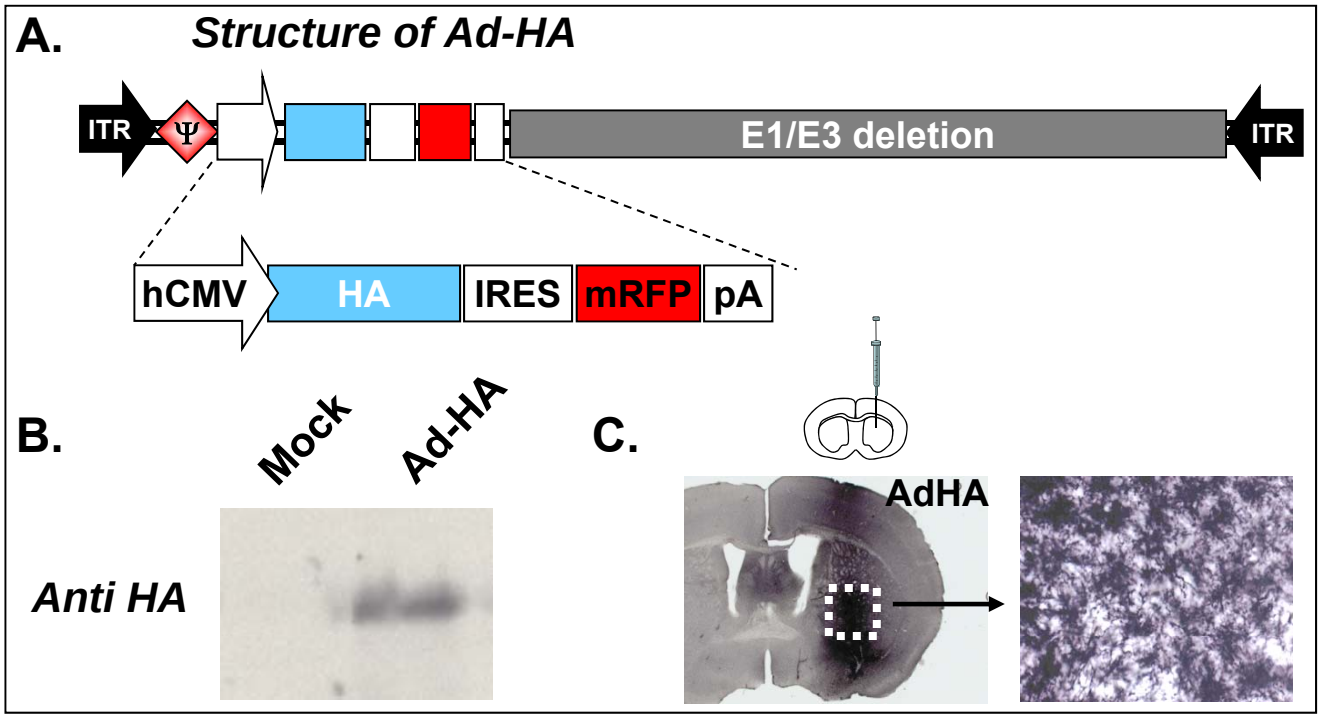
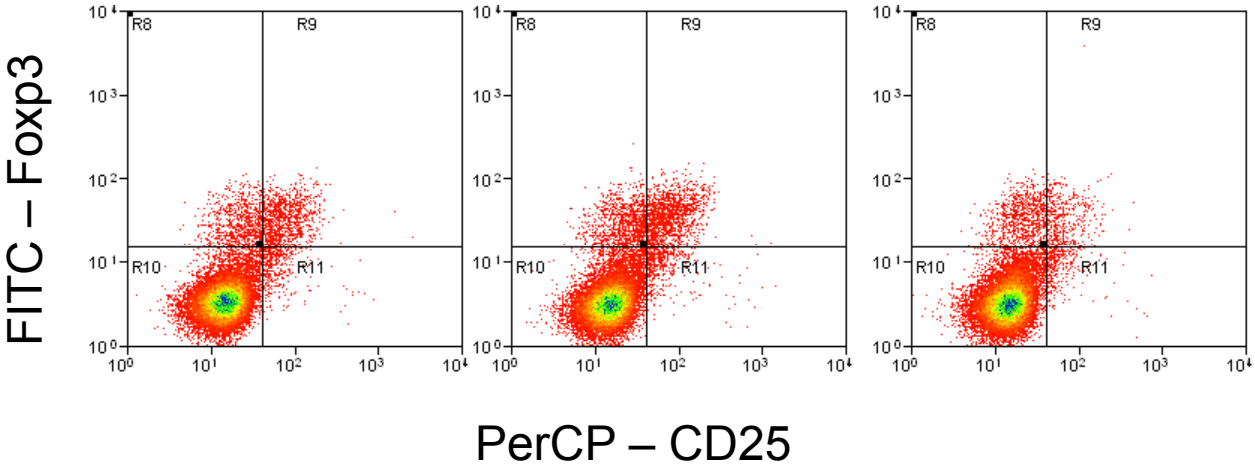
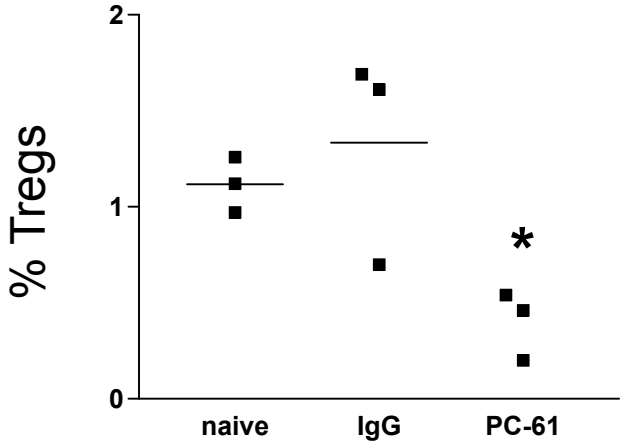
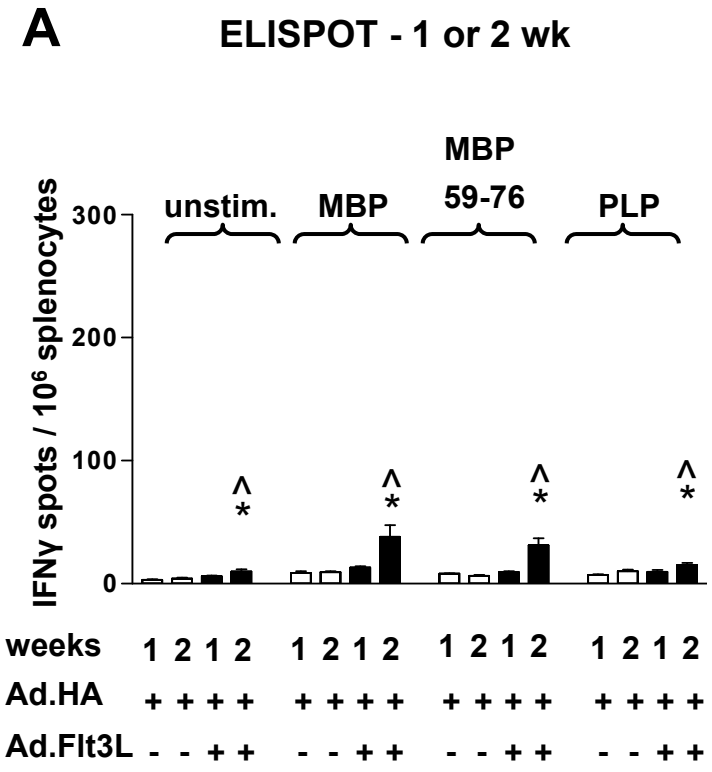


Larocque et al. Supplementary Fig 1

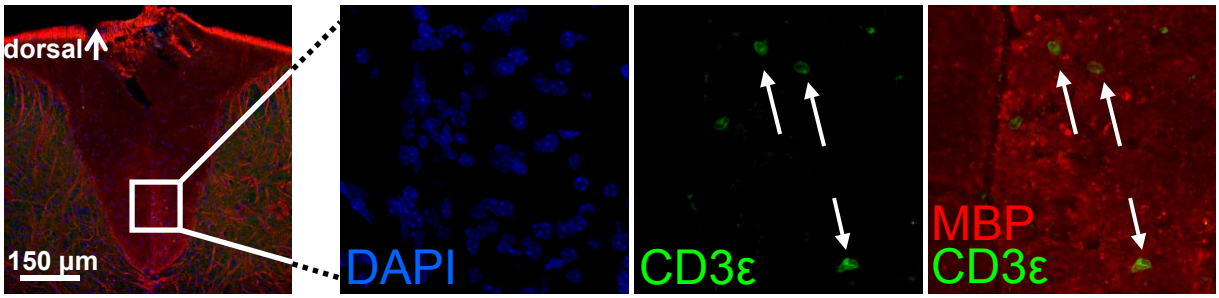




Larocque et al. Supplementary Fig 2



Larocque et al. Supplementary Fig 3



Larocque et al. Supplementary Fig 4

Supplementary Figure Legends

Supplementary Figure 1. Construction of Ad.HA.

(**A**) A schematic illustration of a first generation adenoviral vector expressing HA (Ad-HA). (**B**) Western blot analysis reveals Ad-HA expresses HA protein *in vitro*. (**C**) IHC analysis of a brain section reveals demonstrates Ad-HA expresses HA protein *in vivo.*

Supplementary Figure 2. Confirmation of Treg depletion by administration of PC-61 rat anti-CD25 antibody. A. representative flow cytometry dot plots showing Foxp3 and CD25 immunofluorescence of splenocytes from naïve BALB/c mice (left panel), a control rat IgG (middle panel), or the CD25 depleting antibody PC61 (right panel). Intact cells gated on forward and side-scatter were further gated on CD45 and CD4 expression and of these cells, those expressing both Foxp3 (vertical axis of dot plots) and CD25 (horizontal axis) were used to prepare column scattergraph below. Column scatter graph of percentages of CD45+, CD4+ cells immunopositive for CD25 and Foxp3 in splenocytes from three groups of mice. *=significantly different from control. Individuals with mean values were chosen for dot plots above.

Supplementary Figure 3. Immune Response against brain self-antigens at one and two weeks

The frequency of myelin and PLP-specific IFNγ secreting T lymphocyte precursors was quantified using an IFNγ ELISPOT assay. Seven or fourteen days after Adv injection, splenocytes were stimulated with either MBP pure protein, the MBP 59-67 peptide, or the PLP 139 peptide, or as controls, unstimulated before quantification of IFNγ production. Data were analyzed by two-way ANOVA followed by Tukey-Kramer Multiple Comparison test. *$p < 0.05$ vs. Day 7 of the same vector treatment and stimulation group; ^$p < 0.05$ vs. splenocytes from Ad-HA injected animals at the same timepoint and stimulation group; n=5.

Supplementary Figure 4. Coronal vibratome section of cervical spinal cord from a C57 mouse from the Flt3L-treated, Treg depleted, two month group, immunofluorescently labeled for MBP (red) and CD3ε (green). White arrows show CD3ε immunopositive T cells in dorsal white matter in region of corticospinal tract.

Supplemental Methods:

Recombinant adenoviruses

The HA cDNA from the Influenza PR8 strain was provided by A. Caton (Wistar Instute, Philadelphia) **(42)** . The HA coding sequence was PCR amplified using the primers (5'C CAA CGC GTG CCA CCA TGA AGG CAA ACC TAC TGGTCCTG3' and 5' CC CAA CGC GTC AGA TGC ATA TTC TGC ACT GCA AAG 3'. The HA coding region was cloned into the unique *Mlu*I site of the pIRES plasmid (Clontech laboratories, Palo Alto, CA) to create the plasmid pHA-IRES. The monomeric red fluorescent protein (mRFP) cDNA was obtained from Dr. Roger Tsien (University of California in San Diego) and was PCR amplified using the primers: 5'CC GTC GAC CGC CAC CAT GGCC TCC TCC GAG GAC GTC ATC3' and 5' G GGC GGC CGC TCA GGC GCC GGT GGA GTG GCG GCC CTC3' and cloned into the *Sal*I and *Not*I sites of the plasmid pHA-IRES. The resulting plasmid pHA-IRES-mRFP was excised using *Bgl*II and *Cla*1 and cloned into the adenoviral vector shuttle plasmid pΔE1sp1a (Microbix, Toronto). The first generation adenoviral vector Ad.HA-IRES-mRFP (Ad.HA, **Suppl. Fig 1A**)was rescued by co-transfection with pJM17 and amplified and purified using methodologies described by us previously (39). The first generation adenoviral vectors expressing either human soluble fms-like tyrosine kinase 3 ligand (Ad.Flt3L) or Herpes Simplex Type 1 thymidine kinase (Ad-TK) under the control of the hCMV promoter have been described by us previously (27, 28, 41, 43-46). All adenoviral vectors are human serotype 5 with deletions in the E1 and E3 regions (39). All viral preparations used were free from replication-competent adenovirus and LPS contamination. To confirm HA expression, HEK293 cells were infected with Ad.HA-IRES-mRFP, cells were lysed and protein was harvested for Western blot analysis using a previously described anti-HA antibody (purified from the H36-37 hybridoma cell line kindly provided by A. Caton, Wistar Instute, Philadelphia) (47) followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase

(HRP) (Amersham) and visualized with ECL chemiluminesence (Amersham) (**Suppl. Fig 1B**).

in the brain by immunohistochemistry following intrastriatal administration of BALB/c mice (**Suppl. Fig. 1C**). Immunohistochemistry analysis of brain sections with an antibody specific for HA revealed widespread expression of HA throughout the brain parenchyma at seven days post-administration.

Stereotactic injection of adenoviral vectors

BALB/c (Harlan Laboratories, Indianapolis, Indiana and Simonsen Labs, Gilroy, CA) and C57BL/6J mice (Jackson) were anesthetized with an IP injection of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg), placed in a stereotactic frame modified for mice and a hole was drilled into the skull. Various combinations of adenoviral vectors in 1μl of sterile saline, or saline alone was injected into the right striatum (+0.5 mm AP, +2.2 mm ML, -3.0 mm DV from the bregma) or intra-cerebroventricular space (+0.8 mm AP, +1 mm ML, -2.0 mm DV from the bregma) using a 5uL Hamilton syringe with a 33-gauge needle. Mice were resuscitated using atipamezole (1mg/kg, IP) and administered buprenorphine (0.1mg/kg SQ) as an analgesic. All animal experiments were performed after prior approval by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center and conformed to the policies and procedures of the Comparative Medicine Department. To deplete $CD25^+$ Tregs, mice were treated with a single ip injection of 600 μl ascites fluid (1mg protein) from the PC61 hybridoma as described by us previously (41), or 1 mg of non immune rat IgG in the same volume of saline. According to experiment, some mice were injected saline as a control, rather than IgG. At 7, 14 or 60 days post-injection, animals were perfused with oxygenated Tyrode's solution (132 mM NaCl, 1.8 mM $CaCl_2$, 0.32 mM NaH_2PO_4, 5.56 mM glucose, 11.6 mM $NaHCO_3$, and 2.68 mM KCl). For collection of brain infiltrating immune cells, brains

were dissected out into ice-cold RPMI medium. For Immunohistochemistry, Tyrode's was followed by 4% paraformaldehyde in PBS. Spleens were collected before perfusion for ELISPOT and T cell proliferation assays.

Immunohistochemistry

Forty-micrometer-thick coronal sections of fixed brains were cut through the striatum using a vibratome (Leica VT10005). Free-floating immunohistochemistry was performed using the following primary antibodies: rabbit anti-HA (H36, 1:100) (47) Hamster anti-CD3ε (BD Pharmingen), rat anti-MBP (Chemicon, 1:200) rabbit anti-laminin (Sigma, 1:1000) followed by a biotinylated secondaries for immunoperoxidase labeling or fluorescent secondaries for multi-antigen co-labeling. Biotinylated secondary antibodies were detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and immunoreactive cells were developed with 3,3'-diaminobenzidine (Sigma) and glucose oxidase (Sigma). Sections were mounted on gelatinized glass slides and dehydrated through graded ethanol solutions. A Zeiss Axioplan 2 microscope was used to visualize IHC staining. Immunofluorescently labeled sections were visualized with a Leica DMIRE2 confocal microscope.

Brain-infiltrating immune cells and flow cytometry

A block containing the injected striatum was dissected out into cold RPMI in a Dounce homogenizer and dissociated. Immune cells were isolated by centrifugation in a 30%/70% two step Percoll gradient. To enumerate dendritic cells, fresh cells were labeled with V450 conjugated anti-CD45 and PE-conjugated anti-CD11c. To assess T reg depletion, cells were labeled with anti CD-45, APC-conjugated anti-CD4 and PerCP-conjugated anti-CD25, then fixed and permeabilized and labeled with FITC-conjugated anti-Foxp3. Fluorescence was measured with a CyAn ADP (Beckman Coulter).

His-HA protein purification

The HA coding sequence was PCR amplified using the primers 5′ GGGGATCCAAGGCAAACCTACTGGTCCTGTTA3′ and 5′ CTGGTACCTCAGATGCATATTCTGCACTGCAAAG 3′ and cloned into *Bam*H1 and *Kpn*1 site of the prokaryotic expression plasmid pRSETA containing an N terminal polyhistidine (6xHis) tag (Invitrogen, Carlsbad, CA) to generate the plasmid pRSET-HA. The *E. coli* BL21(DE3)pLysS strain was transformed with the pRSET-HA plasmid vector and His-HA protein was purified by nickel-NTA affinity chromatography as described by the manufacturer (Qiagen, Valencia, CA). The pure protein was desalted on Sephadex G25 column (Amersham, Piscataway, New Jersey) and dialysed in PBS containing 10% glycerol.

IFNγ ELISPOT

Splenocytes were purified from mice injected with vector(s) or saline 7 days earlier. The number of IFNγ secreting T lymphocytesin response to various stimuli was assessed using the enzyme-linked immunospot (ELISPOT) kit assay (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. Briefly, splenocytes (1 x 10^6 cells/well) were cultured in Millipore MultiScreen plates (coated with mouse anti-IFNy capture antibody) for 24 hrs in X-Vivo media (Cambrex, Baltimore, MD) containing one of the following stimuli: His-HA protein (5 μg/ml), the HA518 ClassI peptide (IYSTVASSL, 1 μg/μL), the HA ClassII peptide (HNTNGVTAACSHE, 1 μg/μL), the MBP peptide (HTRTTHYGSLPQKSQHGR, 0.1 μg/μL) or the PLP139 peptide (HSLGKWLGHPDKF, 0.1 μg/μL). 24 hours after stimulation, IFNγ spots were developed with an anti-mouse IFNγ development antibody as recommended by the manufacturer's detailed instructions (R&D Systems mouse IFNγ ELISPOT Development Kit). The number of spots per 10^6 splenocytes, which represents the

frequency of IFNy-producing T lymphocyte precursors, was counted with the KS ELISPOT automated image analysis system (Zeiss, Germany).

T cell proliferation assay

Splenocytes were purified from mice injected with vector(s) or saline 7 days earlier. The levels of T cell proliferation in response to various stimuli was measured using a BrdU cell proliferation assay (Exalpha Biologicals, Inc.) according to the manufacturer's instructions. BrdU incorporation into nascent DNA strands was quantified to determine relative proliferation of T lymphocyte precursors. Splenocytes were stimulated with either His-HA protein (5 μg/ml), the HA518 ClassI peptide (IYSTVASSL, 1 μg/μL), the HA ClassII peptide (HNTNGVTAACSHE, 1 μg/μL), the MBP peptide (HTRTTHYGSLPQKSQHGR, 0.1 μg/μL) or the PLP139 peptide (HSLGKWLGHPDKF, 0.1 μg/μL).

Generation of HA specific polyclonal antibody:

We developed a custom polyclonal antibody in New Zealand White rabbits against 15 amino acids of HA (LTEKEGSYPKLKNSC). The peptide was conjugated to KLH using a C-terminal cysteine residue. Rabbits were immunized with the KLH-conjugated peptide in complete Freunds adjuvant and then boosted twice with the KLH-coupled peptide in incomplete Freunds adjuvant 14 d and 28 d after initial immunization. Serum was taken on day 35 and aliquoted and stored at -80 ºC. Serum was used at 1:1000 for immunohistochemistry.

Behavior

Open Field Test This test measures general levels of locomotor activity. Animals were individually placed into a clear Plexiglas box (16" x 16" x 15") surrounded by two bands of photobeams and optical sensors that measure horizontal locomotor and rearing activity.

Movement was detected as breaks within the beam matrices and automatically recorded for 60 minutes.

Mouse Neurological Screen These tests allow basic neurological phenotyping by assessing sensory and neuromuscular function. Screening includes testing the righting response, eye-blink, ear twitch, limb withdrawal, orienting to olfactory and visual stimuli, and startle response to auditory stimuli. A trained experimenter blind to treatment condition gently placed each mouse into a gloved hand and measured the presence or absence of these responses. This screening was administered 2 hours after the open field test and took approximately 5 minutes to complete.

Rotarod The rotarod test measures motor function and coordination of mice placed on an elevated rotating rod (San Diego Instruments, USA). The rod (1.25" dia), elevated 18 inches above a soft surface to lessen the impact of falls, begins accelerating until it reaches a predetermined rotational rate. The latency (amount of time it takes for the mouse to fall off the rod) and the speed at which the fall occurs was recorded. The test was conducted in two phases:

Phase 1: Training

In the first phase, each mouse was placed on the rotating rod for a single 60-second training session. The rotating rod was accelerated from 0 to 20 rpm across the 60-second trial period. The training session was complete once the mouse fell off of the rod.

Phase 2: Test

In the test phase, each mouse was placed on the rotating rod for 3 subsequent trials. Acceleration speed was again set at 0-20 rpm over a 60-second trial period. Latency to fall off the rod was averaged across the 3 trials.

Statistical Analysis

All statistical analyses were performed using one or two-way ANOVA followed by a Tukey-Kramer Multiple Comparison test. When data failed normality or Levene's test for Variance Homogeneity (NCSS), they were square root or log transformed before statistical analysis. These tests were calculated using NCSS 2007 software (NCSS, Kaysville, Utah). Differences between groups were considered significant when p values were below 0.05.