Supplementary Methods

Materials. The cyanine dyes 1-(5-caboxypentyl)-1-propylindocarbocyanine halide (Cy2), N-hydroxy-succinimidyl ester (Cy3), 1-(5-carboxypentyl)-1-methylindodicarbocyanine halide (Cy5); Immobiline DryStrips pH 3-10 (24cm pH gradient strips), Pharmalyte, 2D Clean-Up kit, 2D Quant kit were purchased from GE Healthcare (Piscataway, NJ). Urea, thiourea, 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonic acid (CHAPS), protease-inhibitor, bromophenol blue, acetonitrile (ACN), trifluoracetic acid (TFA) and ammonioum bicarbonate were obtained from Sigma (St. Louis, MO). Dithiothreitol (DTT) and iodacetamide (IAA) was purchased from Fisher (Pittsburgh, PA). Gels for Ettan Dalt Six System (GE Healthcare) were purchased from Jule, Inc. (Milford, CT). C18 ZipTips were purchased from Millipore (Billerica, MA). The Deep Purple staining kit was from GE Healthcare.

Cell isolation and culture. For isolation of primary murine microglia Brains were removed, placed in Hanks' balance salt solution (HBSS) at 4°C, dissociated using a 10-ml plastic pipette and incubated in 0.25% trypsin at 37°C for 30 min. Trypsinization was stopped by adding cold, heat-inactivated fetal bovine serum (FBS) and the dissociated tissue washed several times with cold HBSS. The washed tissue was triturated by pipeting through a series of sterile Pasteur pipettes with reduced bores and filtered through a 40µm filter. Brain cells were cultured in Dulbecco's Modified Eagles' Media (DMEM) containing 10% FBS, and 2 µg/ml of macrophage colony stimulating factor (MCSF, a generous gift from Wyeth Pharmaceuticals, Cambridge, MA), hereafter referred to as complete media. To obtain highly purified microglia, culture flasks were gently shaken after 7 days of static culture and the supernatants containing floating microglia were transferred to new flasks. The flasks were incubated for 30 min to allow the microglia to adhere, and non-adherent cells were removed by washing with DMEM. Adherent microglia were cultured in DMEM complete media for 7-14 days and then re-plated for experiments.

For isolation of CD4+ T cell subsets, CD4+ T cells were incubated with biotinylated antibody cocktail

and enriched by negative selection on CD4+ T cell columns (R & D Systems). Enriched CD4+ T cells were incubated with PE labeled anti-CD25 antibody and anti-PE microbeads (Miltenyi Biotec, Auburn, CA) and subjected to magnetic separation with AutoMACS (Miltenyi Biotec). The negatively selected fraction contained enriched CD4+CD25- T cells (Teff), while positively selected fraction contained enriched CD4+CD25+ T cells. T cells were cultured in stimulation media [RPMI medium 1640 (GIBCO, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 1 x nonessential amino acids, 55 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin (Mediatech, Herndon, VA) for 4 days in the presence of mouse rIL-2 (100 U/ml) [R & D Systems] and 0.5 μg/ml anti-CD3 (145-2C11; BD Pharmingen, San Diego, CA). Irradiated (3300 rads) splenocytes were used as antigen presenting cells (APCs) at a ratio of 3 APCs:1 T cell. Following activation, T cells were washed in HBSS, resuspended in DMEM (supplemented with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, nonessential amino acids, 55 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin).

Cells from the MES 23.5 dopaminergic cell line kindly provided by Dr. Stanley Appel, were cultured in 75-cm^2 flasks in DMEM/F12 with 15 mM HEPES (Invitrogen) containing N2 supplement (Invitrogen), 100 U/ml of penicillin, 100 μg/ml streptomycin, and 5 % FBS. Cells were grown to 80% confluence then co-cultured in serum free DMEM/F12 at a density of 1 x 10^5 cells (1:1 with microglia) on sterile glass coverslips.

Flow cell analysis. Samples from cell fractions were labeled with fluorescently labeled antibodies to CD4, CD8, CD25, CD11b and intracellular FoxP3 [eBiosciences, San Diego, CA] and analyzed with a FACSCalibur flow cytometer [BD Biosciences, San Jose, CA].

Sample preparation and 2D DIGE. Cell lysates of microglia were prepared with 5 mM Tris-HCl, pH 8.0, 1% CHAPS and a cocktail of protease inhibitors (Sigma-Aldrich). Factors known to interfere

with isoelectric focusing were removed from cell lysates and culture supernatants using the 2D Cleanup Kit (GE Healthcare) according to manufacturer's protocol. Protein concentration was determined using 2D Quant (GE Healthcare). Fifty micrograms of protein from N-α-syn stimulated microglia or N-α-syn stimulated microglia treated with Treg were labeled with 400 pmol of Cy3 or Cy5 dyes (CyDye Minimum Labeling kit, GE Healthcare) respectively, according to the manufacturer's protocol. Twenty-five micrograms of proteins from N-α-syn stimulated microglia and 25 µg from N-α-syn stimulated microglia treated with Treg were mixed and labeled with Cy2. Cy2 labeled samples were utilized as an internal standard to provide a point of normalization for each spot and most importantly the ability to compare a protein spot found in each gel using DeCyder Software. The resulting pools of proteins (Cy2-, Cy3-, and Cy5-labeled) were mixed with rehydration buffer [7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 1% Pharmalyte (pH 3–10 NL)]. First dimension separation by isoelectric focusing was carried out in IPGphor II apparatus (GE Healthcare) as described by the manufacturer using gel strips with immobilized pH gradient (24 cm; pH 3–10 NL), followed by the second dimension in an Ettan DALTsix electrophoresis apparatus. Gels were scanned using a Typhoon 9400 Variable Mode Imager set at 100 pixels with excitation-emission filters at 492/510 nm for Cy2 dyes, 540/590 nm for Cy3 dyes and 620/680 nm for Cy5 dyes (GE Healthcare) and images produced using ImageQuant software. Preparative 2D gel electrophoresis of pooled protein samples (450 µg total) was performed and the gel was post-fixed overnight in 10% ethanol/7.5% acetic acid solution overnight then post-stained with Deep Purple Staining solution (GE Healthcare) in 100 mM sodium borate solution, pH 10.5. A total of three separate preparative gels were performed to facilitate adequate acquisition of protein spots for all experimental paradigms.

In gel trypsin digestion. In brief, gel pieces were picked using Ettan™ (GE Healthcare) automated spot picker of 2 mm fragments and destained using 100µL of 50% ACN/50mM NH_4HCO_3. Gel pieces were dried and incubated with trypsin in 10mM NH_4HCO_3 (Promega, Madison, WI) overnight at 37°C.

Peptides were extracted by washing gel pieces twice with 0.1% TFA and 60% ACN. Dried samples were resuspended in 12µL of water containing 0.1% formic acid for automated injection. All samples were purified using ZipTip® (Millipore) prior to mass spectrometric analyses.

Database searches. In the TurboSEQUEST Search Parameters, threshold for Dta generation was 10000, precursor mass tolerance for Dta generation was set at 1.4. For Dta search, peptide tolerance was set at 1.5 and fragment ions tolerance at 0.00. Charge state was set on "Auto." Peptide identifications from protein data base met the criteria of > 5 amino acids long with an Xcorr ≥ 1.5, 2.0, and 2.5 for +1, +2, and +3 charged ions, respectively, with delta Cn values of ≥0.1 and an Sp > 500 were used for protein identifications.Database nr.fasta was retrieved from ftp.ncbi.nih.gov. We excluded keratins from our database search based on previous observations that these are contaminants resulting from sample processing.

Immunocytochemistry and Western blot. Immunocytochemistry was performed on primary microglial cells cultured in Labtek chamber slides. Cells were fixed in formaldehyde and permeabilized, followed by incubation in 5% normal serum. Primary antibodies were diluted in 1% BSA and incubated overnight at 4°C, followed by Alexa fluor-conjugated secondary antibodies (Molecular Probes). For Western blot, 10 µg of protein was electrophoresed in 4-12% gradient Bis-Tris NuPAGE Novex gels (Invitrogen) and transferred onto PVDF membranes (BioRad, Hercules, CA). Blots were blocked in 5% milk and probed with primary antibodies followed by HRP-conjugated secondary antibodies, and detected using SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL).

Primary antibodies. For immunocytochemistry, Prx1 (1:500; R & D systems) and Hsp70 (1:50; Cell Signaling), rhodamine phalloidin (Molecular Probes) was used to label actin, and nuclear staining was achieved using DAPI. For Western blot, antibodies to alpha-tubulin (1:1000; Calbiochem), galectin 3 (1:500; eBioscience), gelsolin (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Gapdh (1:5000; Santa Cruz Biotechnology) were used.