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(54) Title: USE OF IL-6 IN THE DIAGNOSIS AND TREATMENT OF NEUROINFLAMMATORY CONDITIONS

(57) Abstract: The invention features compositions and methods that are useful for the diagnosis and treatment of neuroinflammatory disorders (e.g., transverse myelitis, multiple sclerosis, optic neuritis, neuromyelitis optica).



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USE OF IL-6 IN THE DIAGNOSIS AND TREATMENT OF NEUROINFLAMMATORY CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of the following U.S. Provisional Application No.:60/835,116, filed on August 2, 2006, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Neuroinflammatory conditions, including transverse myelitis (TM), multiple sclerosis, and neuromyelitis optica, are characterized by abrupt neurologic deficits associated with inflammation, demyelination, and axonal damage. In each of these disorders, neuroinflammation damages the myelin sheath that insulates nerve cell fibers in the brain and spinal cord, ultimately causing extensive and often permanent damage to the underlying nerves. Patients suffering from a neuroinflammatory condition experience dramatic and sometimes permanent losses in sensory and motor function. Neuroinflammatory disorders are notoriously difficult to diagnose and treat. Inaccurate diagnoses result in uncertainty for patients and their families. Quick and accurate methods of diagnosing neuroinflammatory conditions are important to ensure that appropriate methods of treatment are implemented to ameliorate neuroinflammatory symptoms and preserve neurological function. Patients who have experienced a single neuroinflammatory attack may be at risk for a recurrence. Such recurrences are often associated with further losses in neurological function. Current diagnostic methods are incapable of identifying patients at risk of relapse. Improved methods of diagnosing the presence or recurrence of a neuroinflammatory condition are urgently required, as are more effective methods of preventing or treating such conditions.

SUMMARY OF THE INVENTION

The invention features compositions and methods that are useful for the diagnosis and treatment of neuroinflammatory disorders (e.g., transverse myelitis, multiple sclerosis, optic neuritis, neuromyelitis optica).

In various aspects, the invention generally provides methods for diagnosing, treating, or preventing a neuroinflammatory disorder (e.g., transverse myelitis, multiple sclerosis, optic neuritis and neuromyelitis optica); as well as methods for determining a patient

prognosis and selecting an appropriate therapy (i.e., an aggressive therapy for a patient having a poor prognosis, and a less aggressive therapy for a patient having a good prognosis).

In one aspect, the invention provides a method of identifying a subject as having or having a propensity to develop a neuroinflammatory disorder (e.g., transverse myelitis, multiple sclerosis, optic neuritis and neuromyelitis optica), the method involving detecting an increase in the level of IL-6 in a biological sample of the subject relative to a reference level, where the increase in IL-6 indicates that the subject has or has a propensity to develop a neuroinflammatory disorder.

In another aspect, the invention provides a method of identifying a subject as having or having a propensity to develop a neuroinflammatory disorder, the method involving detecting an increase in the level of nitric oxide (NO) in a biological sample of the subject relative to a reference level, where the increase in NO indicates that the subject has or has a propensity to develop a neuroinflammatory disorder.

In another aspect, the invention provides a method of selecting a therapy for a subject identified as having a neuroinflammatory disorder, the method involving detecting an increase in the level of IL-6 in a biological sample of a subject relative to a reference level, where the level of IL-6 indicates an appropriate therapy.

In yet another aspect, the invention provides a method of selecting a therapy for a subject identified as having a neuroinflammatory disorder, the method involving detecting an increase in the level of NO in a biological sample of a subject relative to a reference level, where the level of NO indicates an appropriate therapy.

In yet another aspect, the invention provides a method of identifying a subject as having a propensity to develop a neuroinflammatory relapse, the method involving detecting a decrease in levels of NO or IL-6 in response to therapy, where a failure to observe a reduction in NO or IL-6 levels identifies a patient as having a propensity to relapse. In one embodiment, levels greater than about 15-20 μ M total nitrate indicates and nitrite levels are indicative of poor prognosis. In yet another embodiment, the method indicates that an aggressive therapy should be selected.

In yet another aspect, the invention features a method of monitoring therapy for a subject identified as having a neuroinflammatory disorder, the method involving detecting an alteration in the level of IL-6 in a biological sample of the subject relative to a reference level, where a reduction in the level of IL-6 indicates therapeutic efficacy.

In yet another aspect, the invention features a method of determining the prognosis of a subject identified as having a neuroinflammatory disorder, the method involving detecting

an increase in the level of IL-6 or NO in a biological sample of a subject relative to a reference level, where the level of IL-6 or NO is indicative of a clinical outcome. In one embodiment, an amount of IL-6 between about 3.5 pg and 50 pg/ml CSF or NO levels less than about 15 μ M indicates a good prognosis. For patients having a good prognosis (i.e., unlikely to experience long term disability or relapse) less aggressive (i.e., steroid therapy) is appropriate. In another embodiment, an amount of IL-6 between about 3.5 pg and 50 pg/ml or NO levels less than about 10-15 μ M in CSF indicates that the subject has a good prognosis. In another embodiment, the good prognosis identifies the subject as unlikely to experience severe neurological disability or relapse. In another embodiment, a level of IL-6 in the biological sample of greater than about 50 pg and 5000 pg or NO levels of about 15-20 μ M indicates that the subject has a poor prognosis. In another embodiment, the poor prognosis identifies the subject as likely to experience severe neurological disability or relapse. For patients having a poor prognosis, aggressive therapy is indicated.

In yet another aspect, the invention features a pharmaceutical composition for the treatment of neuroinflammation, containing an effective amount of an agent selected from the group consisting of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, or analogs thereof. In one embodiment, the agent is any one or more of 4-amino-1, 8-naphthalimide, the iNOS inhibitor 1400W, GPI-5693, 15427, 16539, 16072, and GPI-21016. In one embodiment, where the agent is GPI 15427 or GPI 21016, then the effective amount is 10 mg/kg, 20 mg/kg, preferably 30 mg/kg or 100 mg/kg. In yet another embodiment, where the agent is GPI 5693, the effective amount is selected from the group consisting of 10 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 75 mg/kg, and 100 mg/kg.

In another aspect, the invention features a method of treating a neuroinflammatory disorder, the method involving administering to a subject an effective amount of an agent selected from the group consisting of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, or analogs thereof.

In still another aspect, the invention features a method of preventing a neuroinflammatory relapse in a subject at risk thereof, the method involving administering to a subject an effective amount of an agent selected from the group consisting of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, a soluble IL-6 receptor or analogs thereof.

In another aspect, the invention features a kit for the treatment of a neuroinflammatory disorder, the kit containing a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, a soluble IL-6 receptor or analogs thereof. In

one embodiment, the agent is any one or more of GPI-5693, 15427, 16539, 16072, or GPI-21016. In yet another embodiment, the kit is labeled for the treatment of a neuroinflammatory disorder.

In yet another aspect, the invention features a kit for the diagnosis of a neuroinflammatory disorder, the kit containing an IL-6 detecting agent. In one embodiment, the IL-6 detecting agent is an IL-6 antibody.

In various embodiments of the previous aspects, the kit further contains directions for the use of the kit in diagnosing or treating a neuroinflammatory condition or a neuroinflammatory relapse. In yet another embodiment, the kit further comprises an agent for detecting NO.

In various embodiments of any of the previous aspects, the neuroinflammatory disorder is any one or more of transverse myelitis (TM), multiple sclerosis (MS), optic neuritis and neuromyelitis optica (NMO). In still other aspects, the method involves measuring the amount of IL-6 or NO present in a biological fluid selected from the group consisting of: cerebrospinal fluid, serum, urine, and saliva. In still other embodiments of the previous aspects, the reference level is the amount of IL-6 or NO present in a biological sample derived from a control subject. In still other embodiments of the previous aspects, the reference level of IL-6 is between about undetectable 0.5 and 3 pg/ml in cerebrospinal fluid. In still other embodiments of the previous aspects, an increase in IL-6 levels of at least about 2-, 3-, or -4 fold relative to a reference identifies the subject as having a neuroinflammatory disorder. In still other embodiments of the previous aspects, an increase in IL-6 levels between about 5-5,000 (e.g., 5, 10, 20, 50, 100, 200, 300, 500, 750, 1000, 2000, 3000, 5000, 10,000) fold identifies the subject as having a neuroinflammatory condition. In still other embodiments of the previous aspects, an amount of IL-6 between about 3.5 pg and 50 pg indicates that the subject has or has a propensity to develop muscular sclerosis. In still other embodiments of the previous aspects, a level of IL-6 in the biological sample of between about 50 pg and 5000 pg indicates that the subject has or has a propensity to develop transverse myelitis. In still other embodiments of the previous aspects, the method further involves conducting a neurological examination or a diagnostic test. In still other embodiments of the previous aspects, NO is measured indirectly by detecting nitrates, nitrites, or combinations thereof. In still other embodiments of the previous aspects, the level of NO present in a reference is virtually undetectable. In still other embodiments of the previous aspects, the level of NO present in a reference is about 0.5-2 μ M. In still other embodiments of the previous aspects, the level of NO present in a reference is about 0.1 μ M.

In still other embodiments of the previous aspects, a level of NO greater than about 5 μ M identifies the subject as having a neuroinflammation. In still other embodiments of the previous aspects, the reference level is the amount of IL-6 or NO present in a biological sample derived from a control subject. In still other embodiments of the previous aspects, the biological sample is cerebrospinal fluid, serum, saliva, or urine. In still other embodiments of the previous aspects, the reference level of IL-6 is between about 0.5 and 3 pg/ml in cerebrospinal fluid. In still other embodiments of the previous aspects, an increase in IL-6 or NO level of at least about 2-15 (e.g., 2, 3, 4, 5, 10, 15, 20) fold relative to a reference indicates that steroid therapy is appropriate. In still other embodiments of the previous aspects, an amount of IL-6 between about 3.5 pg and 50 pg indicates that steroid therapy is appropriate. In still other embodiments of the previous aspects, a level of IL-6 in the biological sample of greater than about 50 -5000 pg/ml CSF indicates that the subject has or has a propensity to develop transverse myelitis. In still other embodiments of the previous aspects, a level of IL-6 in the biological sample of greater than about 50-5000 pg/ml CSF indicates that an aggressive In still other embodiments of the previous aspects, the method further involves measuring the amount of NO in the biological sample. In still other embodiments of the previous aspects, total levels of nitrate are detected as a method of measuring NO. In still other embodiments of the previous aspects, an effective amount of any one or more of the following agents are used or are contained in a composition as therapeutics: the agent is 4-amino-1, 8-naphthalimide (e.g., administered at about 1 μ M, 5 μ M, 10 μ M, or 20 μ M) an iNOS inhibitor 1400W (e.g., administered at about 50 μ M, 100 μ M, 200 μ M, or 300 μ M), GPI-5693, 15427, 16539, 16072, GPI 15427, or GPI-21016, where an effective amount of GPI 15427 or GPI 21016 is 10 mg/kg, 20 mg/kg, preferably 30 mg/kg or 100 mg/kg; and for GPI 5693, an effective amount is 10 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 75 mg/kg, or 100 mg/kg. In still other embodiments of the previous aspects, the method further involves administering a corticosteroid, plasma pheresis, or other conventional therapy.

The invention provides diagnostic compositions and methods for measuring IL-6 or NO in a biological sample, which are useful for the identification of a neuroinflammatory condition, as well as improved methods for treating or preventing such a condition. The invention further provides methods for determining a patient prognosis and selecting an appropriate therapy. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

By "neuroinflammatory condition" is meant a disease associated with inflammation, demyelination, or axonal damage. Exemplary neuroinflammatory conditions include transverse myelitis (TM), neuritis optica, multiple sclerosis, and neuromyelitis optica.

By "neuroinflammatory relapse" is meant the recurrence of a neuroinflammatory condition or symptom thereof.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

By "alteration" is meant a change (increase or decrease) in the levels or activity of a gene, polypeptide, or other marker as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% change in levels, preferably a 25% change, more preferably a 40% change, and most preferably a 50% or greater change in levels. "

By "antibody" is meant any immunoglobulin polypeptide, or fragment thereof, having immunogen binding ability.

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include bacterial invasion or colonization of a host cell.

By "reference" is meant a standard or control condition.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

As used herein, the terms “prevent,” “preventing,” “prevention,” “prophylactic treatment” and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

By “marker” is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

“Microarray” means a collection of nucleic acid molecules or polypeptides from one or more organisms arranged on a solid support (for example, a chip, plate, or bead).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A – 1F show that IL-6 is selectively upregulated in the cerebrospinal fluid (CSF) of transverse myelitis (TM) patients and correlates with long-term disability. In Figure 1A, a cytokine array was used to profile forty-two inflammatory proteins in the CSF of six TM and eight control patients. The mean value of each cytokine was defined for the control group, and fold induction was calculated for each TM patient. The inset is an immunofluorescent micrograph showing the results of IL-6 immunohistochemistry performed on the cervical spinal cord of a TM patient who died of respiratory failure. In that section, IL-6 expression colocalized with glial fibrillary acidic protein (GFAP)-positive astrocytes. Magnification, $\times 60$. In Figure 1B, quantitative IL-6 levels in the CSF and serum of control (Con) and TM patients were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA). Box plots represent the interquartile range, and the outliers shown are outside the fifth and ninety-fifth percentiles. Mean $\pm$ SEM for each group is indicated above each box. Figure 1C is a graph showing that among TM patients, acute CSF IL-6 levels strongly correlated with sustained disability (as measured by expanded disability status scale (EDSS)). Figure 1D is a graph showing that CSF IL-6 levels strongly correlated with total nitric oxide (NO) metabolites during the acute phase of TM. Figure 1E is a graph showing that total nitric oxide (NO) levels correlated with 14-3-3, a neuronal injury marker in TM patients. Figure 1F is a graph showing that levels of 14-3-3 strongly correlated with sustained disability in TM patients. For panels 1C – F, correlation coefficients and statistical significance are shown. Intensity denotes chemiluminescent signal intensity.

Figures 2A – 2H show that IL-6 is necessary and sufficient to induce injury of oligodendrocytes and axons in spinal cord organotypic cultures by generating nitric oxide. Figure 2A is a graph that quantitates cell death in spinal cord organotypic cultures. CSF (100 μ l) from a TM or control patient was added to the culture media of spinal cord organotypic

cultures, and cellular injury was assessed by ethidium homodimer uptake with Hoechst counterstain (inset). IL-6 was immunodepleted by preincubating TM CSF with an IL-6 antibody and clearing the IL-6 antibody complex with protein A sepharose. Magnification, $\times 20$. Figure 2B shows the results of SDS-PAGE and immunoblot analysis. Tissue lysates from spinal cord organotypics were generated at various times after the administration of IL-6 and subjected to SDS-PAGE followed by immunoblot analysis. Figure 2C is a graph showing a quantification of the data shown in Figure 2B by chemiluminescent signal intensity of three independent experiments. Figure 2D shows RT-PCR analysis of RNA derived from spinal cord organotypic cultures at the indicated times after addition of IL-6 at 2,000 pg/ml. In Figure 2E is a photomicrograph. Dual-color confocal microscopy was carried out with spinal cord organotypic cultures treated with IL-6 for twenty-four hours. Microglia were identified by incubating live cultures with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) -acetylated (Ac)-low density lipoprotein (LDL), which is endocytosed by phagocytosing cells. After fixation, inducible nitric oxide synthase (iNOS) immunohistochemistry was carried out, revealing the expression of iNOS within microglia. Scale bar: 50 μm . Figure 2F shows that nitrotyrosine (NT) and iNOS preferentially accumulated within the exterior white matter of spinal cord organotypic cultures. Scale bar: 200 μm . Figure 2G is a graph showing the effect of adding IL-6 to a final concentration of 2,000 pg/ml to spinal cord organotypic cultures in the presence or absence of the iNOS inhibitor 1400W. $*P < 0.05$. In Figure 2H, wild-type (WT) and iNOS-heterozygous and iNOS knockout (KO) spinal cord organotypic cultures were exposed to IL-6 and assessed for cellular death.

Figures 3A – 3H show cellular participants in IL-6-induced injury. In Figure 3A – 3E, dual-color confocal microscopy was performed using an antibody to nitrotyrosine (NT) as a marker of NO excess, and the cell-specific markers GFAP, RIP, and neurofilament (NF). Figure 3F is a graph showing the results of experiments where dissociated rat spinal neuron cultures were exposed to IL-6 either alone or 2 days after plating 10^4 purified microglial cells in coculture with the dissociated neurons. Figure 3G is a graph showing the results of experiments where purified, cultured microglial cells were exposed to either 500 or 2,000 pg/ml of IL-6. Twenty-four hours later, supernatants were harvested and examined for total nitrates produced by the cultured cells. LPS, lipopolysaccharide. Figure 3H is a graph showing the results of experiments where microglia were plated in a trans well, exposed to IL-6 for 2 hours, and then washed. Microglia were then transferred to 24-well dishes with

plated, dissociated neurons, and the neurons were assessed for cellular death up to 24 hours later. * $P < 0.05$.

Figures 4A – 4G show that IL-6 induced weakness with axonal degeneration and loss of myelin when infused into the spinal subarachnoid space of adult rats. Figure 4A is a graph showing the results of experiments where IL-6 was infused via a subarachnoid spinal catheter into adult rats over a 7-day period. Control animals received saline through the spinal catheter, while another cohort of animals received intrathecal IL-6 and were also given the iNOS inhibitor aminoguanidine (AG) intraperitoneally for the length of the experiment beginning at day 0. * $P < 0.05$. Figure 4B is a micrograph showing that pathologic specimens from IL-6-infused rat spinal cords exhibited reduced myelin staining and white matter vacuolation (asterisks). Scale bar: 20 μm . Figure 4C is a micrograph showing that white matter vacuoles (asterisks) were strongly neurofilament (NF) positive, confirming the presence of axonal degeneration. Scale bar: 20 μm . Figures 4D and 4E show micrographs of plastic sections (1 μM) from IL-6-infused rat spinal cords. These sections showed pathologic features seen principally in superficial white matter regions: panel 4D shows swollen axons with intact myelin (asterisks), consistent with axonal degeneration, and panel 4E shows demyelinated axons (arrows). The term “NT” denotes Scale bar: 10 μm (D and E). Figure 4F is a series of four micrographs showing that these pathologic features could also be seen in autopsy material from a patient with severe TM and high CSF IL-6 levels (1,997 pg/ml). Regions of demyelination (arrows) were seen throughout the cervical spinal cord both with H&E staining and with luxol fast blue (LFB), and there were areas of vacuolation within the white matter (asterisks), consistent with axonal degeneration. Figure 4G shows an immunohistochemical analysis of the autopsy material. This revealed axonal dysfunction as defined by disruption of NF staining and accumulation of APP, a marker of disrupted axonal transport.

Figures 5A – 5F show that PARP activation is necessary for IL-6-induced toxicity. Figure 5A is a graph that shows the results of experiments where PARP activity of IL-6-treated spinal cord (SC) organotypic cultures was assessed in the presence or absence of the PARP inhibitor 4-ANI or the iNOS inhibitor 1400W. Figure 5B is a graph showing the results of experiments where spinal cord organotypic cultures were incubated with IL-6 in the presence or absence of the PARP inhibitor 4-ANI. The amount of nitrotyrosine (NT) accumulation was assessed as a marker of iNOS activity. Figure 5C is a graph showing the results of experiments where spinal cord (SC) organotypic cultures were incubated with IL-6 in the presence or absence of 4-ANI, and cell death was assessed by propidium iodide uptake.

Figure 5D includes two micrographs showing PARP immunoreactivity in the spinal cord of IL-6-infused rats 4 days after initiation of IL-6 infusion. Magnification, $\times 20$. Figure 5E is a graph showing the results of experiments where PARP activity was assessed in spinal cord tissue lysates generated from IL-6-infused rats, control rats, or rats both infused with IL-6 and given the PARP inhibitor 4-ANI intraperitoneally. Lysates were generated 4 days after initiation of IL-6 infusion. Figure 5F is a graph showing the results of experiments where IL-6 was infused into the spinal subarachnoid space of adult rats in the presence or absence of the PARP inhibitors 4-ANI or 3-AB given systemically. Hind limb grip strength was assessed daily for 7 days as described in Methods. $*P < 0.05$.

Figures 6A – 6H show that IL-6 induced regionally specific neural injury in the spinal cord. Figure 6A is a graph showing the results of experiments where IL-6 was administered to cortical, hippocampal, and spinal cord organotypic cultures at increasing doses, and cell death was assessed 36 hours later. Data is plotted as the fold induction of death relative to cultures with no IL-6 addition. Figure 6B is a graph showing the results of experiments where adult rats were infused with IL-6 or saline through an intracerebroventricular (IC) cannula at the same rate (0.5 $\mu\text{l/h}$ for 7 days) and concentration (2,000 pg/ml) as that previously administered via a spinal subarachnoid catheter and assessed for weakness. Figure 6C is a graph showing the results of experiments where cortical organotypic cultures were treated with IL-6 and assessed for PARP activity up to 20 hours later. Figure 6D shows confocal micrographs of cortex and spinal cord organotypic cultures, performed after the administration of IL-6 to the culture. The term “NT” denotes nitrotyrosine; the term “RIP” denotes an oligodendrocyte marker. Scale bars: 50 μm . Figure 6E is a gel showing an RT-PCR analysis of iNOS from cortex or spinal cord organotypic cultures performed at various times after the addition of IL-6. GAPDH serves as a PCR control. Figure 6F is a graph showing results of a quantitative immunoblot of IL-6R expression (inset) present in human autopsy tissue lysates. Spinal cord grey and white matter (SCGM and SCWM, respectively) and cortex grey and white matter (CoWM and CoGM, respectively) lysates were generated, subjected to SDS-PAGE, and probed for IL-6R immunoreactivity. Figure 6G is a graph showing results of a quantitative immunoblot of sIL-6R from the same lysates (shown in chemiluminescent units). Figure 6H is a graph showing the results of experiments where adult rats were infused with either IL-6 or IL-6 plus sIL-6R at a 1:1 molar ratio through a spinal subarachnoid catheter as before. Animals were assessed for hind limb grip strength for the 10-day duration of the experiment. $*P < 0.05$; $**P < 0.04$.

Figure 7 is a graph showing that iNOS knockout mice exhibit an early peak of PARP activity following IL-6 administration in spinal cord organotypic cultures. No late peak was observed. Mouse spinal cord organotypic cultures were generated from wild-type (WT) or iNOS knockout (KO) mice and IL-6 was added at 500 pg/ml. At 4 and 15 hours after IL-6 administration, tissue lysates were generated and PARP activity was assessed. The iNOS KO mice cultures exhibited an early increase in PARP activity that was no different from that observed in wild-type controls. At 15 hours and at other, later time points, iNOS KO mice failed to activate PARP ($P < 0.05$). This indicates that the second peak of PARP activity (as described in Figure 5A) is dependent upon iNOS activation.

Figure 8 is a graph showing that intracerebroventricular infusion of IL-6 (2000 pg/ml) does not induce cognitive/behavioral changes in adult rats. The figure depicts the latencies to eating in the IL-6-treated and control rats. Although the control rats had a trend towards a greater latency to eating, no significant differences between the two groups were seen, $P > 0.05$.

DETAILED DESCRIPTION OF THE INVENTION

The invention features compositions and methods that are useful for the diagnosis and treatment of a neuroinflammatory condition (e.g., transverse myelitis, multiple sclerosis, optic neuritis, neuromyelitis optica). The invention is based, at least in part, on the observation that IL-6 and nitric oxide levels are significantly increased in biological samples derived from subjects suffering from a neuroinflammatory condition, and that levels of IL-6 correlate with the degree of neurological pathology. Accordingly, the invention provides diagnostic and prognostic methods for detecting increases in the level of IL-6 present in a biological sample derived from a patient, where an increase in IL-6 indicates not only that the subject has a neuroinflammatory condition, but can also indicate whether the patient is likely to have a good or a poor prognosis, as well as whether the patient is likely to relapse. Because the treatment regimen selected for each patient is determined by the patient's prognosis and risk of relapse, the invention further provides for the selection of appropriate prophylactic and/or therapeutic regimens.

As reported in more detail below, the invention also provides methods for preventing or treating a neuroinflammatory disorder. Such methods are based, at least in part, on the observation that IL-6 activates the JAK/STAT pathway, resulting in increased iNOS and poly(ADP-ribose) polymerase (PARP) activity, which are associated with neuroinflammatory pathology. Accordingly, the invention further provides compositions and methods for

reducing the activity of the JAK/STAT pathway, iNOS, and PARP. Such compositions and methods were found to be useful for the treatment or prevention of neuroinflammatory disease *in vivo*.

IL-6

IL-6 is a glycoprotein cytokine that mediates signal transduction between immune cells, induces acute-phase protein synthesis, and controls growth and differentiation of cells of the immune and hematopoietic systems (2). IL-6 is likely to be a trophic factor that, under some circumstances, supports neuronal and glial differentiation and survival (2). Introduction of members of the IL-6 superfamily, including IL-6 itself, into some systems has been shown to ameliorate demyelination, perhaps by providing trophic support for oligodendrocytes (3). IL-6 levels in the adult CNS are usually low or undetectable under baseline conditions. Surprisingly, as reported in more detail below, levels of IL-6 were increased dramatically in patients suffering from transverse myelitis, a neuroinflammatory disorder. Similar increases were observed in patients suffering from multiple sclerosis and neuromyelitis optica.

IL-6 within the CNS may stimulate iNOS expression, resulting in the production of NO and leading to free radical-induced tissue injury (10). IL-6 produces its effects by binding to IL-6 receptors (IL-6Rs), which form complexes with gp130. Once formed, the IL-6/IL-6R/gp130 complex stimulates the following two main signal transduction cascades that lead to activation of a number of transcription factors responsible for IL-6-mediated effects: JAK/STAT and Ras/MEK/MAPK (11).

IL-6-induced activation of the JAK2/STAT3 signaling pathway in cardiac myocytes results in activation of iNOS with subsequent NO production and decreased cardiac contractility (10). iNOS is normally expressed either minimally or not at all in the CNS, but in pathological conditions, iNOS levels can increase dramatically in glial cells or influxing macrophages in response to injury or inflammation (12). Compared with constitutive forms of NOS (neuronal and constitutive NOS), iNOS generates significantly greater, sustained amounts of NO (picomolar vs. micromolar levels, respectively) (13). Expression of iNOS causes delayed neurotoxicity in mixed neuronal-glial cortical cultures (14), and NO is directly cytotoxic to oligodendrocytes in culture (15). Much of NO-mediated pathogenicity depends on the formation of secondary intermediates such as the peroxynitrite anion (ONOO^-) (19). ONOO^- triggers DNA single-strand breakage that activates the DNA-repair enzyme poly(ADP-ribose) polymerase (PARP) (20). PARP catalyzes the transfer of ADP-ribose

from its substrate NAD^+ to various proteins, including histones (21, 22). Excessive activation of PARP in response to extensive DNA damage has been associated with the depletion of NAD^+ , which causes cell death through energy depletion (22). It was recently reported that ONOO^- -induced toxicity in spinal cord neurons is associated with DNA strand breakage and prevented by PARP inhibition (23).

Neuroinflammatory disorders

Neuroinflammatory conditions, such as transverse myelitis (TM), neuritis optica, multiple sclerosis, and neuromyelitis optica, are associated with inflammation, demyelination, and axonal damage. TM, for example, can exist as part of a multifocal CNS disease (e.g., multiple sclerosis), a multi-system disease (e.g., systemic lupus erythematosus), or as an isolated idiopathic entity. Neuroinflammation causes extensive damage to nerve fibers of the spinal cord. Subjects suffering from TM typically display any one or more of the following symptoms: weakness of the legs and arms, pain, sensory alterations, and/or bowel and bladder dysfunction. These symptoms may develop over the course of days or weeks. About one-third of patients suffering from TM fail to recover motor function, remaining wheel chair bound or bedridden for the remainder of their lives. In the most severe cases, TM patients lose the ability to breathe on their own, and are dependent on mechanical ventilation. In some patients, the initial TM attack is followed by a recurrence that results in further losses in neurological function.

Multiple sclerosis is another neuroinflammatory condition where inflammation damages the myelin sheath of nerves within the spinal cord and CNS. It is a progressive disease where loss of sensory and motor function occurs over months or even years. Symptoms of MS vary because the location and extent of each attack varies. Symptoms may include pain, tingling, muscle weakness, paralysis loss of vision or hearing, incontinence, vertigo, and spasticity.

Neuromyelitis optica (NMO) is a disease of the central nervous system (CNS) that affects the optic nerves and spinal cord. Individuals with NMO develop transverse myelitis and optic neuritis. Optic neuritis is an inflammation or demyelination of the optic nerve that is typically associated with an acute loss or blurring of vision effecting one eye. Similar to other neuroinflammatory disorders, NMO is associated with spinal cord and CNS demyelination, and patients suffering from NMO often experience unpredictable relapses.

As reported in more detail below, the present inventors found that IL-6 was elevated in the cerebrospinal fluid (CSF) of transverse myelitis patients at the time of their acute

clinical presentation and that levels of IL-6 correlated with the patients' eventual long-term disability. By utilizing organotypic cultures and developing an animal model of TM, IL-6 was shown to mediate the kind of spinal cord injury found in patients with TM. Without wishing to be tied to theory, nitric oxide (NO) production was necessary to achieve this tissue damage. Furthermore, evidence is provided herein showing that the targets of IL-6-mediated injury are oligodendrocytes and axons, which result in demyelination and axonal injury. This work provides the first evidence that a single signaling protein acts as the central mediator of tissue injury in an autoimmune CNS disease. Also described herein is the signaling pathway of IL-6-mediated spinal cord neural injury from JAK/STAT activation to iNOS expression to PARP activation and cell death. The regional specificity of the cytotoxicity of this IL-6 signaling pathway, which involves the spinal cord but not the brain, provides the beginning of an explanation for the selective involvement of different regions of the CNS in autoimmune spectrum diseases.

Accordingly, the invention features compositions and methods that are useful for the treatment or prevention of symptoms associated with any one or more of these neuroinflammatory conditions. The present invention provides methods of treating disease and/or disorders or symptoms thereof which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a therapeutic agent that disrupts or reduces the activity of the JAK/STAT signal transduction pathway or a downstream effector of this pathway, such as PARP or iNOS herein to a subject (e.g., a mammal such as a human). Thus, one embodiment is a method of treating a subject suffering from or susceptible to a neuroinflammatory disease or disorder or symptom thereof. The method includes the step of administering to the mammal a therapeutic amount of a compound herein (e.g., a compound that reduces the expression or activity of a molecular component of the JAK/STAT pathway or reduces the expression or activity of inducible Nitric Oxide Synthase (iNOS) or PARP, such as 1400W or soluble IL-6 receptor) sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

The methods herein include administering to the subject (including a subject identified as in need of such treatment) an effective amount of a compound described herein, or a composition described herein to produce such effect. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method).

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of the compounds herein, such as a compound of the formulae herein to a subject (e.g., animal, human) in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, Marker (as defined herein), family history, and the like). The compounds herein may be also used in the treatment of any other disorders in which neuroinflammation may be implicated.

In one embodiment, the invention provides a method of monitoring treatment progress. The method includes the step of determining a level of diagnostic marker (Marker) (e.g., IL-6, NO, 14-3-3, or any target delineated herein modulated by a compound herein, a protein or indicator thereof, etc.) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to a disorder or symptoms thereof associated with neuroinflammation, in which the subject has been administered a therapeutic amount of a compound herein sufficient to treat the disease or symptoms thereof. The level of Marker determined in the method can be compared to known levels of Marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In preferred embodiments, a second level of Marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain preferred embodiments, a pre-treatment level of Marker in the subject is determined prior to beginning treatment according to this invention; this pre-treatment level of Marker can then be compared to the level of Marker in the subject after the treatment commences, to determine the efficacy of the treatment.

Diagnostics

The present invention provides methods for diagnosing a neuroinflammatory condition by assaying the IL-6 and/or NO in a biological sample. In one embodiment, the method involves detecting an increase in the level of IL-6 in a sample of cerebrospinal fluid relative to the level present in a sample obtained from a control subject. In one embodiment, the method employs an immunoassay, such as a cytokine antibody assay. Patients having a significant increase (e.g., about 3-5 fold, 10-100 fold, 100-300 fold, 300-500 fold, 500-1000

fold, or 1000-5000 fold or more) in IL-6 protein level are identified as having a neuroinflammatory disorder. Preferably, an increase of at least about 250, 300, or 350 fold identifies the patient as having transverse myelitis. Other approaches for measuring IL-6 are known in the art and are within the scope of the present invention. For example, methods for measuring IL-6 protein levels include, but are not limited to ELISA, ELIspot assays, chemiluminescent enzyme immunoassay (Shikano et al., Nephron. 2000 May;85(1):81-5); radioimmunoassay, and Western blot. Methods of measuring NO include measuring total nitrate levels (e.g., using the Griess method). Other assays for measuring NO are commercially available, for example, from R & D Systems (Minneapolis, MN). Methods of measuring IL-6 or iNOS gene expression are also known in the art and include but are not limited to quantitative PCR, RT-PCR, and Northern blot. Kits for measuring IL-6 are commercially available, for example, from R & D Systems (Minneapolis, MN).

Optionally, the diagnostic methods of the invention may be combined with conventional methods for diagnosing neuroinflammatory disorders. Such methods involve taking a medical history, carrying out a neurological examination, including characterizing focal neurologic deficits, which may include decreased or abnormal sensation, decreased ability to move a part of the body, speech or vision changes, or other loss of neurologic functions, head MRI scan, spine MRI scan, lumbar puncture (spinal tap), myelography, CSF oligoclonal banding, and/or CSF IgG index.

Therapeutics

The invention provides compositions and methods for the treatment or prevention of a neuroinflammatory disorder (e.g., transverse myelitis (TM), neuritis optica, multiple sclerosis, and neuromyelitis optica) or neuroinflammatory relapse. In particular, the invention provides therapeutic and prophylactic compositions that inhibit the expression or activity of a component of the JAK/STAT pathway or a downstream effector of that pathway, such as PARP or iNOS, or that disrupt IL-6 signalling, such as blocking antibodies or soluble IL-6 receptors (see, for example, Jones et al., 2002, Biochim. Biophys. Acta 1592:251.-263; Kaplanski et al., 2003, Trends Immunol. 24:25.-29; and Jones et al., 2005, J. Interferon Cytokine Res. 25:241.-253). The activity of a specified compound as an inhibitor of a JAK kinase may be assessed *in vitro* or *in vivo*. In some embodiments, the activity of a specified compound can be tested in a cellular assay. Suitable assays include assays that determine inhibition of either the phosphorylation activity or ATPase activity of a JAK kinase. Thus, an agent is said to inhibit the JAK/STAT pathway if it inhibits the activity of a JAK kinase, if it

inhibits phosphorylation of STAT3, for example at Tyr705, or if it inhibits the ATPase activity of a JAK kinase. In one preferred embodiment, the invention provides therapeutic and prophylactic compositions comprising an effective amount of 4-amino-1,8-naphthalimide (4ANI) and/or 1400W. In other preferred embodiments, the PARP inhibitors are In one preferred embodiment, 10-(4-methyl-piperazin-1-ylmethyl)-2H-7-oxa-1,2-diaza-benzo[de]anthracen-3-one (GPI 15427) and 2-(4-methyl-piperazin-1-yl)-5H-benzo[c][1,5]naphthyridin-6-one (GPI 16539) (Di Paola et al., European Journal of Pharmacology 527 (2005) 163–171; Tentori et al., Clinical Cancer Research Vol. 9, 5370–5379, November 1, 2003). In other preferred embodiments, the PARP inhibitor is GPI-5693, 15427, 16539, 16072, or GPI-21016.

GPI 5693 bulk drug should be kept at 4°C. To prepare GPI 5693 and GPI 16072 for efficacy studies 50 mM HEPES buffered saline (e.g., to prepare 400 ml of 50 mM Hepes buffered saline solution: dilute 20 ml of 1M Hepes stock solution with 380 ml of normal saline solution. Degas by bubbling Argon or nitrogen through the buffer) to solubilize the compounds for efficacy studies) and solubilize concentrations of up to 100 mg/ml GPI 5693 or 10 mg/ml GPI 16072 in HEPES buffered saline (high concentrations may require some sonication). Solutions are somewhat acidic. Therefore, the pH may be adjusted to 6-7 with NaOH before systemic dosing, (pH should not exceed pH 7.0). For oral dosing pH 4 is fine. Some NaOH addition may be needed to help solubilize higher concentrations. Add this dropwise and vortex between additions. Dosing solutions may be made from dry powder preferably fresh daily or at least every two days. Minimize exposure of the drug to air by capping solutions frequently. Capped solutions should be refrigerated when not in use.

Inhibitors of the JAK/STAT pathway are known in the art and are described, for example, in U.S. Patent Publication Nos. 20050159385, 20060293311, and 20040209799. Other examples of JAK/STAT inhibitors which may be useful in the methods of this invention include, but are not limited to: PIAS proteins, which bind and inhibit at the level of the STAT proteins; members of an SH2 containing family of proteins, which are able to bind to JAKs and/or receptors and block signaling; cytokine-inducible Src homology 2-containing (CIS) protein, an inhibitor of STAT signaling; CIS-related proteins, which can inhibit STAT signaling or directly bind to Janus kinases; Suppressor of Cytokine Signaling-I protein (SOCS-1, also referred to as JAB or SSI-1), which appears to associate with all JAKs to block the downstream activation of STAT3; Tyrphostins, which are derivatives of benzylidene malononitrile, resembling tyrosine and erbstatin moieties; AG-490, a member of the tyrophostin family of tyrosine kinase inhibitors; 4,5-dimethoxy-2-nitrobenzoic acid and

4,5-dimethoxy-2-nitrobenzamide, which specifically inhibit JAK3; 4-(phenyl)-amino-6,7-dimethoxyquinazoline (parent compound WHI-258) and derivatives of this compound which are structurally-derived from dimethoxyquinazoline compounds; compounds containing a 4'-OH group, including 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131), 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P154), and 4-(3',5'-dibromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P97); WHI-P180, another dimethoxyquinazoline compound; and cAMP elevating agents, such as forskolin, a direct activator of adenylate cyclase and dibutyryl cAMP, and 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase. Examples of JAK/STAT inhibitors which may be useful in the methods of this invention include, but are not limited to: PIAS proteins, which bind and inhibit at the level of the STAT proteins; members of an SH2 containing family of proteins, which are able to bind to JAKs and/or receptors and block signaling; cytokine-inducible Src homology 2-containing (CIS) protein, an inhibitor of STAT signaling; CIS-related proteins, which can inhibit STAT signaling or directly bind to Janus kinases; Suppressor of Cytokine Signaling-I protein (SOCS-1, also referred to as JAB or SSI-1), which appears to associate with all JAKs to block the downstream activation of STAT3; Tyrphostins, which are derivatives of benzylidene malononitrile, resembling tyrosine and erbstatin moieties; AG-490, a member of the tyrophostin family of tyrosine kinase inhibitors; 4,5-dimethoxy-2-nitrobenzoic acid and 4,5-dimethoxy-2-nitrobenzamide, which specifically inhibit JAK3; 4-(phenyl)-amino-6,7-dimethoxyquinazoline (parent compound WHI-258) and derivatives of this compound which are structurally-derived from dimethoxyquinazoline compounds; compounds containing a 4'-OH group, including 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131), 4-(3'-bromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P154), and 4-(3',5'-dibromo-4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P97); WHI-P180, another dimethoxyquinazoline compound; cAMP elevating agents, such as forskolin, a direct activator of adenylate cyclase and dibutyryl cAMP, and 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, debromohymenialdisine and hymenialdisine.

In other embodiments, the invention provides therapeutic and prophylactic agents that inhibit the expression or activity of iNOS. Methods for assaying the activity or expression of iNOS are known in the art and described herein. Such methods include assaying nitrosylation of tyrosine residues using an immunoassay, assaying iNOS RNA, for example, using RT-PCR, and assaying iNOS protein levels, for example, using immunohistochemistry.

Agents that reduce the expression or activity of iNOS are known in the art and described herein. INOS inhibitors include, but are not limited to 1400W.

In still other embodiments, the invention provides therapeutic and prophylactic agents that inhibit the expression or activity of PARP. Such agents are known in the art and described herein. Agents that inhibit PARP include but are not limited to 4-amino-1,8-naphthalimide (4ANI); nicotinamide, 3 aminobenzamide, 6(5H)-Phenanthridinone, 5-Aminoisoquinolinone (5-AIQ), Hydrochloride, 4-Hydroxyquinazoline, 4-Quinazolinol, 1,5-Isoquinolinediol, 5-Hydroxy-1(2H)-isoquinolinone, 3,4-Dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ); 3-aminobenzamide; 1,5-isoquinolinediol; 6(5H)-phenanthridone; 1,3,4,5,-tetrahydrobenzo(c)(1,6)- and (c)(1,7)-naphthyridin-6-ones; adenosine substituted 2,3-dihydro-1H-isoindol-1-ones; AG14361; 2-(4-chlorophenyl)-5-quinoxalinecarboxamide; 5-chloro-2-[3-(4-phenyl-3,6-dihydro-1(2H)-pyridinyl) propyl]-4(3H)-quinazolinone; isoindolinone derivative INO-1001; 4-hydroxyquinazoline; 2-[3-[4-(4-chlorophenyl)-1-piperazinyl]propyl]-4-3(4)-quinazolinone; DHIQ; 3,4-dihydro-5 [4-(1-piperidinyl)(butoxy)-1(2H)-isoquinolone; CEP-6800; GB-15427; PJ34; and imidazobenzodiazepines. Other PARP inhibitors useful in the methods of the invention are described, for example, in U.S. Patent Publication Nos: 20060079510, 20050288310, 20050288310, 20070142430, and 20070032496.

In one preferred embodiment, 10-(4-methyl-piperazin-1-ylmethyl)-2H-7-oxa-1,2-diaza-benzo[de]anthracen-3-one (GPI 15427) and 2-(4-methyl-piperazin-1-yl)-5H-benzo[c][1,5] naphthyridin-6-one (GPI 16539) (Di Paola et al., European Journal of Pharmacology 527 (2005) 163–171; Tentori et al., Clinical Cancer Research Vol. 9, 5370–5379, November 1, 2003). In other preferred embodiments, the PARP inhibitor is GPI-5693, 15427, 16539, 16072, or GPI-21016.

In one example, the PARP inhibitors, GPI 15427 and GPI 21016 are dissolved in saline and PBS, and the compounds are administered, for example, at 10 mg/kg, 20 mg/kg, preferably 30 mg/kg or 100 mg/kg. For GPI 5693, the compounds are administered at about 30 mg/kg and 50 mg/kg for GPI 5693. For mice, dosing solutions of 3 mg/ml, 5 mg/ml and 10 mg/ml as appropriate.

Pharmaceutical Compositions

The present invention features pharmaceutical preparations for the treatment or prevention of a neuroinflammatory disorder (e.g., transverse myelitis (TM), neuritis optica,

multiple sclerosis, and neuromyelitis optica) or neuroinflammatory relapse comprising an agent that inhibits the expression or activity of a component of the JAK/STAT signal transduction pathway, a PARP inhibitor, an iNOS inhibitor, an agent that blocks IL-6 signalling, such as a blocking antibody or soluble IL-6 receptor, or analogs thereof, together with pharmaceutically acceptable carriers, where the compounds provide for the treatment of virtually any neuroinflammatory condition or neuroinflammatory relapse. In one preferred embodiment, the pharmaceutical composition comprises an effective amount of 4-amino-1,8-naphthalimide (4ANI) and/or 1400W. Pharmaceutical preparations of the invention have both therapeutic and prophylactic applications. In one embodiment, a pharmaceutical composition includes an effective amount of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor. The compositions should be sterile and contain a therapeutically effective amount of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor in a unit of weight or volume suitable for administration to a subject (e.g., a human patient). The compositions and combinations of the invention can be part of a pharmaceutical pack, where the JAK/STAT signal transduction pathway inhibitor, PARP inhibitor, or iNOS inhibitor is present in individual dosage amounts.

Pharmaceutical compositions of the invention to be used for prophylactic or therapeutic administration should be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 μm membranes), by gamma irradiation, or any other suitable means known to those skilled in the art. Therapeutic compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. These compositions ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution.

A JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor may be combined, optionally, with a pharmaceutically acceptable excipient. The term "pharmaceutically-acceptable excipient" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances that are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate administration. The components of the pharmaceutical compositions also are capable of being co-mingled with a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor of

the present invention, and with each other, in a manner such that there is no interaction that would substantially impair the desired pharmaceutical efficacy.

Compounds of the present invention can be contained in a pharmaceutically acceptable excipient. The excipient preferably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetate, lactate, tartrate, and other organic acids or their salts; tris- hydroxymethylaminomethane (TRIS), bicarbonate, carbonate, and other organic bases and their salts; antioxidants, such as ascorbic acid; low molecular weight (for example, less than about ten residues) polypeptides, e.g., polyarginine, polylysine, polyglutamate and polyaspartate; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers, such as polyvinylpyrrolidone (PVP), polypropylene glycols (PPGs), and polyethylene glycols (PEGs); amino acids, such as glycine, glutamic acid, aspartic acid, histidine, lysine, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, sucrose, dextrans or sulfated carbohydrate derivatives, such as heparin, chondroitin sulfate or dextran sulfate; polyvalent metal ions, such as divalent metal ions including calcium ions, magnesium ions and manganese ions; chelating agents, such as ethylenediamine tetraacetic acid (EDTA); sugar alcohols, such as mannitol or sorbitol; counterions, such as sodium or ammonium; and/or nonionic surfactants, such as polysorbates or poloxamers. Other additives may be included, such as stabilizers, anti-microbials, inert gases, fluid and nutrient replenishers (i.e., Ringer's dextrose), electrolyte replenishers, and the like, which can be present in conventional amounts.

The compositions, as described above, can be administered in effective amounts. The effective amount will depend upon the mode of administration, the particular condition being treated and the desired outcome. It may also depend upon the stage of the condition, the age and physical condition of the subject, the nature of concurrent therapy, if any, and like factors well known to the medical practitioner. For therapeutic applications, it is that amount sufficient to achieve a medically desirable result.

With respect to a subject having a neuroinflammatory condition associated with demyelination and/or neurological symptoms, an effective amount is sufficient to prevent, reduce, stabilize, or reverse an alteration associated with neuroinflammation. With respect to a subject having a neuroinflammatory condition or disorder, an effective amount is an amount sufficient to stabilize, slow, or reduce a symptom associated with the neuroinflammatory

condition. Generally, doses of the compounds of the present invention would be from about 0.01 mg/kg per day to about 1000 mg/kg per day. In one embodiment, 25, 50, 75, 100, 125, 150 or 200 mg of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor is administered to a subject. Preferably, 100 mg of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor is administered. Effective doses range from 0.1 nM to 200 nM, where the bottom of the range is any integer between 1 and 199, and the top of the range is any integer between 2 and 200. It is expected that doses ranging from about 5 to about 2000 mg/kg will be suitable – depending on the specific JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, or an iNOS inhibitor used. Lower doses will result from certain forms of administration, such as intravenous, subcutaneous, intramuscular, oral, intranasal administration, intraspinal, or arterial release catheter into brain or spinal cord provides local high dose. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of a composition of the present invention.

A variety of administration routes are available. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. In one preferred embodiment, a composition of the invention is administered orally. Other modes of administration include rectal, topical, intraocular, buccal, intravaginal, intracisternal, intracerebroventricular, intratracheal, nasal, transdermal, within/on implants, or parenteral routes. The term “parenteral” includes subcutaneous, intrathecal, intravenous, intramuscular, intraperitoneal, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Compositions comprising a composition of the invention can be added to a physiological fluid, such as cerebrospinal fluid or blood, for example. Oral administration can be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule.

Pharmaceutical compositions of the invention can comprise one or more pH buffering compounds to maintain the pH of the formulation at a predetermined level that reflects physiological pH, such as in the range of about 5.0 to about 8.0. The pH buffering compound used in the aqueous liquid formulation can be an amino acid or mixture of amino acids, such

as histidine or a mixture of amino acids such as histidine and glycine. Alternatively, the pH buffering compound is preferably an agent which maintains the pH of the formulation at a predetermined level, such as in the range of about 5.0 to about 8.0, and which does not chelate calcium ions. Illustrative examples of such pH buffering compounds include, but are not limited to, imidazole and acetate ions. The pH buffering compound may be present in any amount suitable to maintain the pH of the formulation at a predetermined level.

Pharmaceutical compositions of the invention can also contain one or more osmotic modulating agents, i.e., a compound that modulates the osmotic properties (e.g, tonicity, osmolality and/or osmotic pressure) of the formulation to a level that is acceptable to the blood stream and blood cells of recipient individuals. The osmotic modulating agent can be an agent that does not chelate calcium ions. The osmotic modulating agent can be any compound known or available to those skilled in the art that modulates the osmotic properties of the formulation. One skilled in the art may empirically determine the suitability of a given osmotic modulating agent for use in the inventive formulation. Illustrative examples of suitable types of osmotic modulating agents include, but are not limited to: salts, such as sodium chloride and sodium acetate; sugars, such as sucrose, dextrose, and mannitol; amino acids, such as glycine; and mixtures of one or more of these agents and/or types of agents. The osmotic modulating agent(s) may be present in any concentration sufficient to modulate the osmotic properties of the formulation.

Compositions comprising a compound of the present invention can contain multivalent metal ions, such as calcium ions, magnesium ions and/or manganese ions. Any multivalent metal ion that helps stabilize the composition and that will not adversely affect recipient individuals may be used. The skilled artisan, based on these two criteria, can determine suitable metal ions empirically and suitable sources of such metal ions are known, and include inorganic and organic salts.

Pharmaceutical compositions of the invention can also be a non-aqueous liquid formulation. Any suitable non-aqueous liquid may be employed, provided that it provides stability to the active agents (s) contained therein. Preferably, the non-aqueous liquid is a hydrophilic liquid. Illustrative examples of suitable non-aqueous liquids include: glycerol; dimethyl sulfoxide (DMSO); polydimethylsiloxane (PMS); ethylene glycols, such as ethylene glycol, diethylene glycol, triethylene glycol, polyethylene glycol ("PEG") 200, PEG 300, and PEG 400; and propylene glycols, such as dipropylene glycol, tripropylene glycol, polypropylene glycol ("PPG") 425, PPG 725, PPG 1000, PPG 2000, PPG 3000 and PPG 4000.

Pharmaceutical compositions of the invention can also be a mixed aqueous/non-aqueous liquid formulation. Any suitable non-aqueous liquid formulation, such as those described above, can be employed along with any aqueous liquid formulation, such as those described above, provided that the mixed aqueous/non-aqueous liquid formulation provides stability to the compound contained therein. Preferably, the non-aqueous liquid in such a formulation is a hydrophilic liquid. Illustrative examples of suitable non-aqueous liquids include: glycerol; DMSO; PMS; ethylene glycols, such as PEG 200, PEG 300, and PEG 400; and propylene glycols, such as PPG 425, PPG 725, PPG 1000, PPG 2000, PPG 3000 and PPG 4000.

Suitable stable formulations can permit storage of the active agents in a frozen or an unfrozen liquid state. Stable liquid formulations can be stored at a temperature of at least -70°C, but can also be stored at higher temperatures of at least 0°C, or between about 0.1°C and about 42°C, depending on the properties of the composition. It is generally known to the skilled artisan that proteins and polypeptides are sensitive to changes in pH, temperature, and a multiplicity of other factors that may affect therapeutic efficacy.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of compositions of the invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as polylactides (U.S. Pat. No. 3,773,919; European Patent No. 58,481), poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acids, such as poly-D-(-)-3-hydroxybutyric acid (European Patent No. 133, 988), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman, K.R. et al., *Biopolymers* 22: 547-556), poly (2-hydroxyethyl methacrylate) or ethylene vinyl acetate (Langer, R. et al., *J. Biomed. Mater. Res.* 15:267-277; Langer, R. *Chem. Tech.* 12:98-105), and polyanhydrides.

Other examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems such as biologically-derived bioresorbable hydrogel (i.e., chitin hydrogels or chitosan hydrogels); systatic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like.

Specific examples include, but are not limited to: (a) erosional systems in which the agent is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,667,014, 4,748,034 and 5,239,660 and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,832,253, and 3,854,480.

Another type of delivery system that can be used with the methods and compositions of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. Liposomes are artificial membrane vessels, which are useful as a delivery vector *in vivo* or *in vitro*. Large unilamellar vessels (LUV), which range in size from 0.2 - 4.0 μm , can encapsulate large macromolecules within the aqueous interior and be delivered to cells in a biologically active form (Fraley, R., and Papahadjopoulos, D., Trends Biochem. Sci. 6: 77-80).

Liposomes can be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications, for example, in DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. (USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88, 046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Liposomes also have been reviewed by Gregoriadis, G., Trends Biotechnol., 3: 235-241).

Another type of vehicle is a biocompatible microparticle or implant that is suitable for implantation into a mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System"). PCT/US/0307 describes biocompatible, preferably biodegradable polymeric matrices for containing an exogenous gene under the control of an appropriate promoter. The polymeric matrices can be used to achieve sustained release of the exogenous gene or gene product in the subject.

The polymeric matrix preferably is in the form of a microparticle such as a microsphere (where an agent is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein an agent is stored in the core of a polymeric shell). Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Other forms of the polymeric matrix for containing an agent include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix is introduced. The size of the polymeric matrix further is selected according to the method of delivery that is to be used.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the compositions of the invention to the subject. Such polymers may be natural or synthetic polymers. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multivalent ions or other polymers.

Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, poly-vinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), polyvinyl acetate, poly vinyl chloride, polystyrene, polyvinylpyrrolidone, and polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose,

collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

Selection of a Treatment Method

After a subject is diagnosed as having a neuroinflammatory condition, a method of treatment is selected. The level of IL-6 present in a biological sample, such as CSF, is used in selecting a treatment method. In one embodiment, less aggressive neuroinflammatory conditions have lower IL-6 levels (e.g., less than 50 pg/ml CSF) than neuroinflammatory conditions requiring more aggressive therapies. The level of IL-6 present in a biological sample is correlated with a clinical outcome. Levels of IL-6 greater than 50 pg/ml typically correlate with poor clinical outcomes, and are indicative of a risk for relapse. Accordingly, more aggressive therapeutic interventions are warranted. Aggressive therapeutic interventions include, but are not limited to, combinations of one or more of the following: intravenous steroids (e.g., corticosteroids, methylprednisone, dexamethasone) intravenous IgG therapy, plasmapheresis, cyclophosphamide, thalidomide, cellsept, minocycline, and IL-6 antibody or other cytokine therapy. Aggressive therapies are associated with a variety of adverse side-effects. Thus, where less aggressive therapies may be used, such therapies are preferred (e.g., for patients having a good prognosis and low risk of relapse). Levels of IL-6 below 50 pg/ml typically correlate with good clinical outcomes. Accordingly, less aggressive therapeutic interventions are appropriate because such patients are less likely to sustain severe losses in neurological function or to experience relapse. Less aggressive therapeutic interventions include treatment with intravenous steroids alone. To identify a patient as likely to experience a recurrence, a clinician will look at whether or not levels of NO or IL-6 are reduced in response to therapy. If no reduction in levels is seen in response to therapy (e.g., aggressive or less aggressive therapy) then the patient is likely to experience a relapse. For example, a patient whose levels of IL-6 fail to drop below about 10 pg/ml CSF and/or whose levels of NO fail to drop below about 5-10 μ M is identified as at risk for a relapse. More aggressive therapy is indicated for patients identified as at risk of neuroinflammatory relapse.

In other embodiments, the level of NO is measured. Indirect measures for NO include measuring total nitrate and nitrite species, for example, using a commercially available kit from R & D (Minneapolis, MN). The level of NO present in a reference is virtually undetectable in a standard assay (e.g., Griess assay), for example, less than about 1 μ M. Levels of NO greater than about 5 μ M are indicative of neuroinflammation; levels greater than about 15-20 μ M total nitrate are indicative of poor prognosis; and indicate that an aggressive therapy should be selected.

Patient monitoring

The diagnostic methods of the invention are also useful for monitoring the course of a neuroinflammatory condition in a patient, for assessing the efficacy of a therapeutic regimen, or for assessing the risk of relapse. In one embodiment, the diagnostic methods of the invention are used periodically to monitor the IL-6 or NO levels present in a biological sample of a patient. In one example, the neuroinflammatory condition is characterized using a diagnostic assay of the invention prior to administering therapy. This assay provides a baseline that describes the level of IL-6 present in a biological sample of the subject prior to treatment. Additional diagnostic assays are administered during the course of therapy to monitor the efficacy of a selected therapeutic regimen or the likelihood of a neuroinflammatory relapse. A therapy is identified as efficacious when a diagnostic assay of the invention detects a decrease in IL-6 levels, particularly where levels are reduced to less than 35-50 pg/ml, less than 20-35 pg/ml, less than 5-20 pg/ml, and most preferably to about 1-5 pg/ml, or even to 1-2 pg/ml. A therapy that reduces levels of NO to less than about 15 μ M, 10 μ M, or 5 μ M is efficacious. More preferably, levels of NO are reduced to less than about 1-5 μ M.

Microarray procedure

The methods of the invention may be used for microarray-based assays that provide for the high-throughput analysis of IL-6 and other cytokine levels. Useful substrate materials include membranes, composed of paper, nylon or other materials, filters, chips, glass slides, and other solid supports. The ordered arrangement of the array elements allows hybridization patterns and intensities to be interpreted as methylation levels of particular genes. For each analyzed cytokine, an antibody is fixed to a substrate to allow detection of a bound cytokine

antigen. IL-6 and other cytokines are detected in a biological sample using any method known in the art.

Kits

The invention provides kits for the treatment or prevention of a neuroinflammatory condition, such as transverse myelitis, neuritis optica, multiple sclerosis, and neuromyelitis optica. In one embodiment, the kit includes a pharmaceutical pack comprising an effective amount of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, and/or an iNOS inhibitor. Preferably, the compositions are present in unit dosage form. In some embodiments, the kit comprises a sterile container which contains a therapeutic or prophylactic composition; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

If desired compositions of the invention or combinations thereof are provided together with instructions for administering them to a subject having or at risk of developing a neuroinflammatory condition (e.g., transverse myelitis, neuritis optica, multiple sclerosis, and neuromyelitis optica) or a neuroinflammatory relapse. The instructions will generally include information about the use of the compounds for the treatment or prevention of a neuroinflammatory condition or a neuroinflammatory relapse. In other embodiments, the instructions include at least one of the following: description of the compound or combination of compounds; dosage schedule and administration for treatment of a neuroinflammatory condition or symptoms thereof; precautions; warnings; indications; counter-indications; overdosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

The invention also provides kits for the monitoring of a level of IL-6 present in a biological sample obtained from a subject. In various embodiments, the kit includes at least one antibody or other detection agent whose detection of IL-6 determines the level of IL-6 present in a biological sample, together with instructions for using the antibody to identify a neuroinflammatory condition. In another embodiment, the kit further comprises a detection agent suitable for measuring the level of NO by measuring nitrates and nitrites (e.g., Griess

reaction) in a biological sample. In yet other embodiments, the kit comprises a sterile container which contains the primer or probe; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container form known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding nucleic acids. The instructions will generally include information about the use of an IL-6 antibody or other detection agent and their use in diagnosing a neuroinflammatory condition. Preferably, the kit further comprises any one or more of the reagents described in the diagnostic assays described herein. In other embodiments, the instructions include at least one of the following: description of the antibody; methods for using the enclosed materials for the diagnosis of a neuroinflammatory condition; precautions; warnings; indications; clinical or research studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

Combination Therapies

If desired, therapeutic compositions of the invention for the treatment of a neuroinflammatory condition or neuroinflammatory relapse are administered in combination with any one or more of the following: azathioprine (Imuran), methotrexate, Rituximab, prednisone, or mycophenolate mofetil (CellCept), immune modulators (e.g., interferon, such as Avonex, Betaseron, or Rebif) or glatiramer acetate (Copaxone); steroids, such as corticosteroid medications (e.g., methylprednisone or dexamethasone, methylprednisolone), lioresal (Baclofen), tizanidine (Zanaflex), or a benzodiazepine to reduce muscle spasticity, cholinergic medications for incontinence, antidepressants for mood or behavior symptoms, amantadine for fatigue. In addition, plasma exchange (plasmapheresis) may be used to separate antibodies out of the blood stream, particularly in subjects that are unresponsive to corticosteroid therapy.

The following examples are offered by way of illustration, not by way of limitation. While specific examples have been provided, the above description is illustrative and not restrictive. Any one or more of the features of the previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1: IL-6 levels are selectively and dramatically elevated in the CSF of transverse myelitis patients.

A cytokine antibody array was used to simultaneously profile 42 inflammatory proteins in the CSF of six transverse myelitis (TM) patients who had not been started on immunomodulatory therapy prior to CSF sampling, and 8 control patients (hydrocephalus, aseptic meningitis, spinal cord infarct, and spinal cord tumors; $n = 2$ per group). Relative to controls, arrays from the TM patients showed an approximately 300-fold mean induction of IL-6 ($P < 0.05$), while all other cytokines were altered less than 10-fold ($P = \text{NS}$; Figure 1A). Two TM patients died of respiratory failure, and immunohistochemical staining of the spinal cord was performed at autopsy in order to define the source(s) of IL-6. In both cases, it was shown that the predominant source of IL-6 secretion was from astrocytes in and around the area of inflammation within the spinal cord (Figure 1A, inset), while microglial cells and influxing immune cells exhibited less robust IL-6 staining (data not shown). To ascertain the absolute levels of IL-6 in the serum and CSF of TM patients, quantitative ELISA in a larger series of TM ($n = 25$) and control patients ($n = 16$; Figure 1B) was performed. Mean CSF IL-

6 levels in patients with acute TM were 262-fold higher than in control patients (TM, 654.3 ± 247.9 pg/ml, range 0–4209 pg/ml; control, 2.5 ± 0.72 pg/ml).

Mean serum IL-6 levels in TM and control patients were not statistically significantly different and were markedly lower than those seen in the CSF, suggesting that the IL-6 measured in the CSF was generated within the CNS. Further details on demographic and clinical characteristics of idiopathic transverse myelitis (TM) and control patients used in the study are provided in Table 1, shown below.

Table 1

Characteristic	Transverse Myelitis	Control
Age (in years) (mean $\pm$ SEM)	48.7 ± 3.5 (n = 21)	49.9 ± 3.8 (n = 16)
Gender (% women)	58.0 % (n = 25)	62.5 % (n = 16)
Time to follow up (months)	6.5 ± 0.79 (n = 22)	18.5 ± 3.8 (n = 16)
(mean $\pm$ SEM)		
Percent with gadolinium enhancing lesions	78% (n = 14)	12.5 % (n = 16)
WBC in CSF (mean $\pm$ SEM)	10.8 ± 4.5 (n = 20)	2.1 ± 1.2 (n = 11)
Protein in CSF (mg/dl) (mean $\pm$ SEM)	58.7 ± 11.4 (n = 16)	39.2 ± 11.5 (n = 10)
Protein in serum (g/dl) (mean $\pm$ SEM)	6.5 ± 0.4 (n = 8)	7.0 ± 0.5 (n = 4)
Acute EDSS (mean $\pm$ SEM)	6.4 ± 0.5 (n = 22)	3.8 ± 0.7 (n = 16)

The hypothesis examined in these experiments was that cytokines play an important role in the pathogenesis of TM, and thus the diffusible derangements within the CSF of a group of TM patients were examined with a cytokine antibody array. A virtually identical pattern in the CSF from all the TM patients examined in this manner was found, that is, dramatic elevations in IL-6 levels. This uniformity in the pattern was surprising in that TM has widely been considered to be a heterogeneous disorder, and one may have expected the cytokine derangements to reflect this heterogeneity. However, it should be noted that recent nosologic strategies have attempted to categorize TM patients into various classifications, including monophasic vs. recurrent and idiopathic vs. those associated with systemic disease (28). For this study, the analysis has been limited to patients with idiopathic TM and has excluded those with identified systemic inflammatory disease. Therefore, this classification scheme may have resulted in a more uniform patient population with relatively homogenous immune system derangements.

Though CSF IL-6 levels reported here are among the highest reported in any human disease (up to 4,209 pg/ml), related disorders have also been found to have elevated IL-6 within the CNS. Acute disseminated encephalomyelitis, like TM, is a monophasic, inflammatory disorder of the CNS that is often post-infectious (29). Similarly, several reports have suggested that IL-6 is involved in the pathogenesis of MS, since patients have elevated IL-6 in the CSF (30) and within plaques (31, 32) and elevated numbers of IL-6-expressing monocytes within the blood and CSF (30).

Example 2: IL-6 levels correlate with total NO production and tissue injury in the spinal cord.

A strong correlation was shown between CSF IL-6 obtained at the time of acute clinical evaluation and long-term disability, as assessed by an expanded disability status scale [EDSS] at 6-month follow-up, as shown in Figure 1C. A significant correlation was also shown between acute CSF IL-6 levels and acute CSF total NO levels (Figure 1D) in TM patients, and also that total CSF NO correlates with 14-3-3, a previously reported marker of acute neuronal injury in TM patients (24) (Figure 1E). Further, it was shown that CSF 14-3-3 levels correlated with long-term disability (Figure 1F). These data are consistent with a direct role of NO in mediating spinal cord injury.

Example 3: IL-6 is necessary and sufficient to cause spinal cord cellular injury by activating the JAK/STAT pathway, resulting in increased iNOS activity.

To test whether IL-6 is simply correlated with or is causative of cellular injury in the spinal cord, studies were carried out using rat organotypic culture spinal cord sections. CSF was added from a TM patient (with IL-6 of 1,997 pg/ml) or a control patient onto spinal cord organotypic culture sections and cell death was evaluated by determining the percentage of cells that took up the red, vital dye propidium iodide. It was found that CSF from the TM patient induced death of spinal cord cells ($33\% \pm 12\%$), while CSF from a control patient with hydrocephalus did not ($4\% \pm 2.4\%$, $P < 0.01$; Figure 2A, black bars and photomicrograph inset). Immunodepletion of IL-6 from the CSF prior to application (decrease in IL-6 to 2 pg/ml; data not shown) abrogated the ability of the CSF to induce death, suggesting that IL-6 was necessary for this death ($P < 0.01$; Figure 2A). Immunodepletion of IgG or a control protein (14-3-3) did not abrogate the killing activity of TM CSF. IL-6 at a dose found in the CSF of TM patients (2,000 pg/ml) triggered a dramatic, transient phosphorylation of STAT3 at Tyr705, peaking 2 hours after the application to the culture medium of spinal cord organotypic cultures (total STAT levels were unchanged; Figure 2B and 2C). There was also a peak of iNOS expression at 4 hours after IL-6 administration. Nitrosylation of tyrosine residues (nitrotyrosine [NT]), a marker of NO excess (25), continued to increase at 7 hours after IL-6 administration. Actin and loading controls did not show any change over the examined period. Quantitative determination of phosphorylated STAT3, iNOS, and nitrotyrosine intensity of several representative immunoblots revealed a nearly 4-fold increase of phosphorylated STAT3 at 2–3 hours, a nearly 4.5-fold increase of iNOS at 4 hours, and a progressive increase in nitrotyrosine immunoreactivity (greater than 8-fold) at 7 hours after IL-6 administration (Figure 2C). RT-PCR analysis of spinal cord organotypic cultures confirmed a marked upregulation of iNOS RNA at 4 hours after IL-6 administration (Figure 2D), demonstrating that the upregulation of iNOS occurs at the level of transcription. Immunofluorescent examination of spinal cord organotypic cultures revealed a dramatic upregulation of iNOS within microglial cells (defined by the ability to phagocytose fluorescent acetylated low-density lipoprotein [DiI-Ac-LDL]; Figure 2E) and the accumulation of both iNOS and nitrotyrosine immunoreactivity, especially within the peripheral white matter of the spinal cord (Figure 2F), in response to IL-6 administration. Next, the consequences of iNOS induction in spinal cord organotypic cultures were investigated and it was found that iNOS is critical for IL-6-induced death (Figure 2G). IL-6 was administered to the culture medium and it was found that the

progressive cellular injury that occurs is completely blocked by 1400W (Figure 2G). Similarly, mouse organotypic spinal cord cultures from either iNOS-heterozygous or -KO mice were resistant to IL-6-induced death (Figure 2H). This suggests that IL-6 induces activation of STAT3, resulting in the upregulation of iNOS and cellular injury within the spinal cord.

Example 4: Targets of IL-6-mediated spinal neural injury.

Next, the target cells injured by IL-6 were identified by examining the colocalization of NT, a commonly utilized marker of cellular injury due to excess NO (26), with other cellular markers. It was found that nitrotyrosine immunoreactivity was diffusely increased within IL-6-treated tissue (Figure 3, A, C, and E). Additionally, while astrocytes (defined by glial fibrillary acidic protein [GFAP] immunoreactivity) did not accumulate nitrotyrosine in IL-6-treated cultures (Figure 3A), oligodendrocytes (defined by receptor interacting protein [RIP] immunoreactivity; Figure 3, B and C) and axons (defined by neurofilament immunoreactivity; Figure 3, D and 3E) did, and were therefore preferentially susceptible to IL-6-induced injury. When dissociated cultures of pure spinal neurons were generated, it was found that IL-6 did not induce cellular death at any of the studied time points or doses (Figure 3F). When microglia were added to the mixed neural culture either in cis (Figure 3F) or in trans (Figure 3H), the ability of IL-6 to induce neural injury was restored, confirming that microglial cells are critical in this pathway. Mixed cultures of neurons and astrocytes were also refractory to IL-6-induced cytotoxicity (data not shown). Indeed, purified microglia were induced to secrete high levels of NO in response to IL-6 at 500 and 2,000 pg/ml (Figure 3G). These results suggest that IL-6 activates microglia to produce NO, resulting in neural injury to spinal cord cultures.

Example 5: Spinal cord from IL-6-infused rats and TM patients exhibit demyelination and axonal degeneration.

To determine whether elevation of spinal IL-6 in adult rats is sufficient to induce weakness and cellular injury, IL-6 or vehicle was infused through a spinal subarachnoid catheter. The catheter was attached to a subcutaneous osmotic minipump that infused IL-6 or vehicle at 0.5 μ l/h for 7 days (24 ng IL-6 infused per day). While saline-infused animals showed no change in hind limb grip strength over the 8-day observation period, IL-6-infused rats became progressively weak (n = 10 per group; Figure 4A). By the completion of the study, IL-6-infused rats had lost nearly 50% of their baseline hind limb strength. IL-6-

infused rats treated with the iNOS inhibitor aminoguanidine did not exhibit weakness, suggesting that iNOS is required for spinal neural injury. Histologic analysis of the spinal cords from IL-6–infused animals revealed white matter disruption (Figure 4B, asterisks) and the accumulation of neurofilamentous material within white matter vacuoles (Figure 4C). Grey matter was largely spared (data not shown). The analysis of plastic-embedded 1- μ m histologic sections from IL-6–infused rats revealed both axonal degeneration, characterized by swollen, empty, myelin-encased chambers (Figure 4D, asterisks), and demyelination, characterized by intact axons with little or no surrounding myelin (Figure 4E, arrows). This pathology in IL-6–infused rats was similar to the axonal degeneration and demyelination seen in the spinal cord of a patient with severe, fatal TM (the same patient with IL-6 levels of 1,997 pg/ml). Histologic analysis of the cervical spinal cord revealed focal areas of demyelination (Figure 4F, arrows) and axonal disruption (Figure 4F, asterisks). Pathologic analysis of this patient's spinal cord also revealed amyloid precursor protein (APP) accumulation within axons, a marker of axonal dysfunction, and neurofilament disruption (Figure 4G, right), whereas in a control patient, no APP accumulation was observed within axons (Figure 4G, left).

There has been recent awareness about the dual role of IL-6 as both protective and injurious (2). In contrast to the view that IL-6 may be purely injurious to the nervous system, several studies have shown that members of the IL-6 superfamily, including IL-6 itself, may be neuroprotective (3). These disparate effects may be explained by differential involvement of the gp130 receptor among different members of the family (3, 33), by distinct inflammatory pathways activated in different injury models (7, 34), or by a dose effect wherein low doses of IL-6 or a family member are protective (35) while higher doses are injurious. In pathological spinal cord specimens from patients with TM, it was found that astrocytes were the predominant source of IL-6 production. Astrocytes have been shown to produce IL-6 in response to direct stimulation by proinflammatory cytokines (e.g., TNF- α and IL-1 β), viral and bacterial pathogens, and neurotransmitters. What triggers the initial biosynthesis of IL-6 in astrocytes is currently being investigated, but potential candidates include an immune response following vaccination or an antecedent infection that could involve mechanisms such as molecular mimicry or superantigen-mediated inflammation (36). Why some individuals mount a dramatic elevation of their IL-6 levels that results in the pathophysiological injury seen in TM is still unknown, but the potential contribution of genetic differences to CNS IL-6 production has been previously described (37, 38). Primary targets of IL-6–mediated cytotoxicity include oligodendrocytes and axons. The finding of

nitrotyrosine accumulation in axons argues for a direct neuropathic effect of IL-6 in mediating neural injury, as opposed to axonal degeneration that is solely the result of demyelination.

The finding that APP axonal transport was blocked in the spinal cords of TM patients is consistent with a recent report that microglial iNOS activation produces a breakdown in axonal transport (39). Both demyelination and axonal degeneration are hallmarks of CNS autoimmune demyelinating conditions, such as TM and MS, and these pathological hallmarks were reproduced in adult rats in which IL-6 was infused into their spinal cords.

Example 6: Activation of PARP is necessary for cell death.

It was next examined whether PARP activation contributes to IL-6-induced neural injury by using spinal cord organotypic cultures and it was found that there was a robust increase in PARP activity in a biphasic manner: at 2 hours and again at 8–15 hours after IL-6 administration (Figure 5A). Since it has previously been shown that the excess production of NO leads to DNA strand breaks and PARP activation, it stands to reason that the second peak of PARP activity may be a consequence of iNOS activity, which is initiated 2–3 hours following IL-6 induction. To test this hypothesis, organotypic cultures were incubated with IL-6 and the iNOS inhibitor 1400W, and PARP activity was assessed. The second peak of PARP activity was prevented when organotypic cultures were incubated with 1400W, confirming that iNOS activity is required. However, the first peak of PARP activity was not prevented by incubation with 1400W, indicating that this early peak is independent of iNOS activity. This data was confirmed by examining the activation of PARP in wild-type and iNOS KO animals (Figure 7).

In order to define whether iNOS induction is dependent upon PARP activity, the converse experiment was carried out, in which the extent of nitrotyrosine reactivity (a marker of iNOS activity) was examined by ELISA in the presence and absence of PARP inhibition (Figure 5B). Here, the IL-6-induced increase in nitrotyrosine immunoreactivity was not blocked by the addition of PARP inhibitor 4-amino-1, 8-naphthalimide (4-ANI), confirming that iNOS activation is not dependent upon PARP activation and that iNOS induction occurs upstream of PARP activation. The importance of PARP in IL-6-induced spinal cellular injury was examined by incubating spinal cord organotypic cultures with IL-6 and either the PARP inhibitor 4-ANI or vehicle. It was found that the PARP inhibitor completely prevented cellular death (Figure 5C). The function of PARP in adult rats infused with IL-6 through a subarachnoid catheter draining into the low thoracic spinal cord was examined. It was found

that IL-6-infused rats demonstrated a dramatic increase in both the amount of nuclear PARP within the white matter (Figure 5D) and the activity of PARP (Figure 5E), suggesting that, as seen *in vitro*, PARP activation occurs within the spinal cord of IL-6-infused rats. Further, inhibition of PARP activity by concurrent treatment of adult rats with IL-6 and either of 2 PARP inhibitors (4-ANI or 3-aminobenzamide [3-AB]) completely prevented IL-6-induced hind limb weakness (Figure 5F). These data suggest that PARP is activated in response to IL-6 by at least 2 distinct pathways and that inhibition of PARP activity is protective against IL-6-induced spinal injury both *in vitro* and *in vivo*.

Excessive NO production can lead to cellular injury by several mechanisms, including loss of membrane integrity through direct lipid peroxidation, altered protein function through nitrotyrosine formation, or energy depletion through PARP activation (40). Studies with PARP-1 KO mice and chemical PARP inhibitors have consistently shown that lack of PARP activation following severe oxidative injury with NO, hydrogen peroxide, and ONOO⁻ results in significantly reduced neuronal death (22). It was recently shown that ONOO⁻ exerts toxic effects on spinal cord neurons at least in part through a PARP-dependent pathway, as demonstrated by the ability of PARP inhibitors to preserve neuronal viability in the presence of ONOO⁻ (20). Here, using spinal cord organotypic cultures, a biphasic activation of PARP was observed: the first peak, occurring 2 hours after addition of IL-6, was not dependent upon iNOS, while the later peak was. These results suggest that NO production was necessary for the second peak of PARP activity, presumably by initiating DNA damage that resulted in increased PARP activation. The observation that PARP inhibition prevented IL-6-mediated spinal cord cell death both in organotypic cultures and in the adult rat spinal cord supports the importance of PARP as a final effector in the pathway of IL-6-mediated cellular injury *in vivo*. The first peak of PARP activity after IL-6 administration coincides with STAT phosphorylation and not NO production. Since IL-6 is capable of mediating JAK/STAT signaling pathways other than JAK2/STAT3 and Ras/MEK/MAPK (11), a speculation is that PARP has a role in signal transduction. Recent evidence from PARP KO mice suggests a role for PARP in enhancing p38/MAPK signaling in response to inflammatory stimulation through lipopolysaccharide (LPS) (41). Perhaps the early-phase IL-6-mediated PARP activation described here plays a role in the augmentation of p38/MAPK signaling. Spinal cord iNOS activation, however, is not due to early PARP activation, because it is unaffected by PARP inhibition.

Example 7: Regional vulnerability of the spinal cord relative to the brain.

Next, to determine whether IL-6 at concentrations seen in TM patients is universally injurious to the nervous system, or selectively injurious to the spinal cord, cortical and hippocampal organotypic culture sections were incubated with increasing concentrations of IL-6 and the fold induction of death relative to sections with no IL-6 administration was plotted (Figure 6A). Within hippocampal and cortical organotypic cultures, low levels of IL-6 were protective against baseline cellular injury that occurs in these cultures, while higher doses were only slightly injurious. This contrasts with spinal cord organotypic cultures, which showed an injurious response to even 50 pg/ml IL-6 and greater than 10-fold increase in cell death at all greater doses tested. In adult rats, infusion of IL-6 (at the same dose previously infused into the spinal subarachnoid space) into the cerebral ventricles did not result in weakness (Figure 6B) or behavioral changes (Figure 8), further supporting a regionally specific nervous system response to IL-6. A series of studies previously performed in the spinal cord were repeated in cortical tissues in order to determine what limits IL-6-mediated injury in cortex organotypic cultures. A small early peak in PARP activity within IL-6-treated cortical cultures was found, but the second peak seen in spinal cord cultures was not observed (Figure 6C). Working further upstream, there was no accumulation of nitrotyrosine within cortical organotypic cultures treated with IL-6, while IL-6-treated spinal cord organotypic cultures did accumulate nitrotyrosine (Figure 6D), and iNOS mRNA induction was not observed (Figure 6E). JAK2 and STAT3 phosphorylation could not be detected in IL-6-treated cortical organotypic cultures (data not shown), suggesting that the regional specificity to IL-6 is even upstream of JAK and STAT. To determine the discrepancy between brain and spinal cord, the expression of the membrane-bound IL-6R and the soluble IL-6R (sIL-6R) was examined within human autopsy material. It was found that IL-6R is similarly expressed within the brain and spinal cord, indicating that IL-6R density does not account for relative susceptibility of spinal cord tissue. There is markedly increased density of IL-6R within white matter compared to grey matter (Figure 6F), suggesting that the relative susceptibility of axons and oligodendrocytes may be mediated by this gradient. Next, the expression of sIL-6R was examined, and it was found to have greater expression in cortical tissue lysates than in spinal cord tissue lysates (Figure 6G). If sIL-6R deficiency in the spinal cord mediates the enhanced susceptibility of the spinal cord to IL-6, then co-infusion of IL-6 and sIL-6R should blunt the effects of IL-6 in spinal cord cellular injury. This was found to be true, since adult rats infused with both IL-6 and sIL-6R did not get weak, while IL-6-infused animals did ($P < 0.04$ at days 2–10; Figure 6H).

A suggestion, therefore, is that IL-6-mediated signaling is decreased in the brain relative to the spinal cord and that this impairment must be upstream of IL-6R. The observation of higher sIL-6R levels in the context of decreased IL-6 responsiveness in cortical lysates suggests that this molecule may act as an antagonist in this setting (27). Therefore, it is likely that sIL-6R is a critical molecule that dampens the tissue response to IL-6. Moreover, spatially restricted responses to cytokines including IL-6 may underlie the restricted inflammation seen in a variety of CNS inflammatory disorders, including regionally limited forms of MS, TM, neuromyelitis optica, and optic neuritis.

Previous studies have implicated IL-6 family members in preventing cell death as well as potentially playing a causative role in neurodegenerative diseases (2). This discrepancy may result from differences in downstream signaling among IL-6 and its family members, and differing effects in distinct experimental paradigms. The protective or destructive actions of IL-6 may also result from selective dose and regional effects. The results here showed that IL-6 causes preferential cytotoxicity in white matter compared to gray matter in the spinal cord. It was also found that low doses of IL-6 prevented cell death in organotypic cultures of sections from the hippocampus or cortex, whereas higher doses had little effect on cell death. In contrast, no IL-6 dose tested in spinal cord sections was found to be protective, and higher doses were extremely cytotoxic. The observation that IL-6 treatment of cortical organotypic cultures did not cause significant PARP activation, iNOS induction, or STAT phosphorylation suggests that the regional selectivity of IL-6 response occurs at the level of IL-6R and not its downstream signaling pathway. The increased abundance of IL-6R in white compared to grey matter, which may be due to increased abundance of IL-6R on oligodendrocytes, may account for the preferential white matter injury seen both in vitro and in vivo following IL-6 application. However, it is the higher concentration of sIL-6R that may explain the increased susceptibility of the spinal cord to IL-6. Alternative explanations for the role of sIL-6R in preventing cell death include the possibilities that IL-6 is bound and isolated by sIL-6R or that the IL-6/sIL-6R complex initiates signaling through alternative pathways in cells without IL-6R (42). Biological markers of regional selectivity within the CNS has been seen previously, such as the selective clearance of alphavirus from spinal cord relative to the cortex (43) and the selective vulnerability of spinal cord neurons to excitotoxic injury when compared to cortical neurons.

The results presented herein demonstrate that a single signaling molecule can be a determinant of patient outcome in TM. The implications of these findings are that therapeutic strategies capable of modulating this pathway may improve outcomes in TM

patients. The data presented herein provides direct evidence for a signaling cascade involving IL-6, iNOS, and PARP proteins that accounts for the clinicopathologic findings in inflammatory spinal neurodegeneration. Since spinal cord dysfunction is a major determinant of disability in several neurologic disorders including TM and MS, the elucidation of this pathway identifies important therapeutic targets for preventing these and other neurologic disorders or disabilities in the future.

Methods

The above results were obtained using the following methods and materials.

Study subjects.

All patients underwent a lumbar puncture at acute onset of disease as part of their clinical care and diagnosis prior to initiating treatment and consented to the withdrawal of additional CSF for research (approved by the Johns Hopkins Medicine Institutional Review Board). Idiopathic TM patients were defined according to previously published criteria (1). Those who did not meet the inflammatory criteria were classified as noninflammatory controls. Spinal fluid was collected, immediately placed on ice, centrifuged at 1,000 g to remove cellular elements, and stored at -80°C .

Clinical assessments.

Clinical and radiological data was collected: the presence of gadolinium enhancement; the location and extent of the lesion on the spinal MRI; and the levels of protein and number of white blood cells, if any, in the CSF during the acute phase (Table 1). Further, functional status (acute and follow-up EDSS scores) were assessed by neurologists who were blinded to the immunologic assay results. The EDSS is a widely used neurological rating scale, ranging from 0 (normal) to 10 (death).

Immunologic assays.

Cytokine antibody arrays were purchased (TranSignal RayBio Human Cytokine Antibody Array 3, catalog no. MA6020; Panomics Inc.) and used according to package inserts. For each blot, 1,000 μl of CSF was used. Signal was analyzed and quantitated by using a Fuji chemiluminescent detection system. Quantitative IL-6 ELISA assay kits and total NO assay kits were purchased from R&D Systems and the LIVE/DEAD Viability/Cytotoxicity Kit was purchased from Invitrogen Corp.; all were used according to the

manufacturers' instructions. All samples were measured in triplicate and average values were determined. Total nitrite concentrations in supernatants collected from in vitro co-culture experiments were determined by using the Total NO Kit (catalog no. DE1600; R&D Systems) as specified by the manufacturer. PARP activity was assayed using a PARP Activity Assay Kit (catalog no. 4667-50-K; Trevigen) according to the manufacturer's instructions. Nitrotyrosine release in supernatants collected from in vitro co-culture experiments was analyzed using the Nitrotyrosine ELISA Test Kit (catalog no. HK501; Cell Sciences) as directed by the manufacturer.

Reagents.

Recombinant rat IL-6 (catalog no. 557008; BD Biosciences — Pharmingen) was made as a 2 µg/ml stock in 1 mg/ml BSA in PBS and used at final concentrations of 500 pg/ml and 2,000 pg/ml. iNOS inhibitor 1400W dihydrochloride (catalog no. ALX-270-073; Alexis Biochemicals) was made at 100 mM stock in water and used at a final concentration of 100 µM. 4-ANI (catalog no. ALX-270-250-M010; Alexis Biochemicals) was dissolved in 10% DMSO and used this for in vitro studies at a final concentration of 5 µM from a 5.8-mM stock. 3-AB was obtained from Sigma-Aldrich (catalog no. A0788). Microglial identity was determined by incubation with DiI-Ac-LDL (Invitrogen Corp.). This marker identifies phagocytosing cells and has been reported to accurately identify microglial cells in the nervous system (44). Antibodies and dilutions used in this study include the following: PhosphoPlus Stat3 (Tyr705) Antibody Kit (1:1,000; Cell Signaling Technology); iNOS/NOS Type II (1:10,000; BD Biosciences); anti-NT, clone 1A6 (1:1,000; Upstate); PARP antibody (1:75; Cell Signaling Technology), biotinylated anti-human IL-6R antibody (1:2,500; R&D Systems), RIP (SMI 91; Sternberger Monoclonals), neurofilament (NF), Heavy Chain (SMI 31/32; Sternberger Monoclonals), and GFAP (1:400, catalog no. MAB360, or 1:2,000, catalog no. AB5804; Chemicon). CSF samples from TM patients were immunodepleted with anti-human IL-6 antibodies (catalog no. ab6672; Novus Biologicals), precipitated with protein A-coated beads, and verified for IL-6 depletion by human IL-6 ELISA assay.

Immunoblots.

Western blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc.) and quantified with a Luminescent Image Analyzer (LAS-1000 plus camera) and Image Gauge software (Fuji). Protein expression in treated tissue was normalized to levels in untreated controls by dividing measured band intensities.

Supernatants were collected at each time point and centrifuged at 13.2 rpm in a tabletop centrifuge for 5 minutes at 4°C. The clarified supernatant was then analyzed for the presence of total nitrites and NT.

Rat spinal cord organotypic cultures.

All animal experiments were approved by the Johns Hopkins Animal Care and Use Committee, and mice were cared for and euthanized according to the guidelines of the committee. Spinal cord organotypic slice cultures were prepared from 8-day-old Sprague-Dawley rats as previously described (45). Growth media was changed 1 day after culturing and twice more before using. Cultures were used 7–10 days after culturing. Cultures were changed to serum-free organotypic culture media and treated with recombinant human IL-6, PBS, or 100 µl human CSF from TM patients.

Microglial cultures.

Microglial cultures were isolated from adult rats as described by Babas et al. (44), with a few modifications. The cells were derived from the cortical regions of 3 adult Lewis rat brains. Processed tissues were passed through an 18-gauge needle on a 10-CC syringe and filtered over 70-µm nylon cell strainers (Falcon, catalog no. 352350; BD Biosciences) twice to ensure a single cell suspension. Cells were plated at a density of 4×10^6 cells/well. The next day, cells were washed and changed to fresh growth medium. Cells were maintained for a week before experimentation. Microglial cell purity (at least 90%) was verified by fluorescent staining of microglia with Iba-1 (1:150, catalog no. 01-1974; Wako) and the absence of staining with GFAP.

Spinal cannulated rats.

Adult Sprague-Dawley rats with spinal cannulas were purchased from Zivic Laboratories Inc. These rats had cannulas placed into the cisterna magna and extended caudally through the subarachnoid space with the cannula tip terminating adjacent to the T8 vertebral body. Cannulated rats were anesthetized with avertin, an incision was made behind the head, and the subarachnoid cannula was connected to an Alzet pump (1007D, 0.5 µl/hr for 7 days). Pumps were filled with 100 µl IL-6 at 2 µg/ml or saline. Animals were coded and housed individually. There were 10 rats per group, and each rat was scored for hind limb grip strength daily by a blinded examiner. Animals were perfused with 4% paraformaldehyde or 2% paraformaldehyde/2% glutaraldehyde 7 days after initiating IL-6

infusion, and spinal cords were harvested for immunohistochemistry or plastic sectioning. Plastic sections (1 μ M) were generated of the lumbar spinal cord after osmium tetroxide embedding. Additional behavioral studies were carried out as described below.

Animal behavioral studies.

Novelty-induced food intake was utilized as a measure of animal anxiety to determine whether IL-6 induced any change in this measure. The testing apparatus consisted of a Plexiglas box (60 x 60 x 50 cm). Forty-eight hours before testing, all food was removed from the home cage. For testing, 3-4 food pellets were placed in the center of the testing box and an animal was placed into a randomly chosen corner of the testing box and a stopwatch was immediately started. The latency to begin eating was measured and was defined as chewing the food, not simply sniffing or playing with a pellet. If a rat had not eaten within 360 seconds, the test was stopped and the animal was assigned a latency score of 360 seconds and the animal was returned to the home cage. The data for novelty-induced food intake inhibition were analyzed using Student t-test.

Statistics.

SPSS software (version 12.0; SPSS Inc.) was used for all statistical analyses. Box plots were used to represent the distribution of the data. The outliers shown are outside the fifth and ninety-fifth percentile. Correlations were assessed by Spearman's rank correlation coefficient due to the ordinal nature of the data. Group differences in EDSS and IL-6 levels and between CSF and serum IL-6 levels in the different patient groups were compared using the Mann-Whitney U test due to the non-Gaussian appearance of the data. A P value less than 0.05 was considered significant. Due to the nonparametric nature of the data (as determined by using tests of normality), nonparametric equivalent tests of ANOVA and repeated-measures ANOVA were used to increase the robustness of the results. The Kruskal- Wallis test was performed to analyze differences among groups at each time point, and Friedman's nonparametric repeated measures comparison was used to analyze differences across time within a group. The Mann-Whitney U test was used for the comparison of 2 independent samples.

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Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method of identifying a subject as having or having a propensity to develop a neuroinflammatory disorder, the method comprising detecting an increase in the level of IL-6 in a biological sample of the subject relative to a reference level, wherein the increase in IL-6 indicates that the subject has or has a propensity to develop a neuroinflammatory disorder.
2. A method of identifying a subject as having or having a propensity to develop a neuroinflammatory disorder, the method comprising detecting an increase in the level of nitric oxide (NO) in a biological sample of the subject relative to a reference level, wherein the increase in NO indicates that the subject has or has a propensity to develop a neuroinflammatory disorder.
3. The method of claim 1 or 2, wherein the neuroinflammatory disorder is selected from the group consisting of transverse myelitis, multiple sclerosis, optic neuritis and neuromyelitis optica.
4. The method of claim 1 or 2, wherein the detecting involves measuring the amount of IL-6 or NO present in a biological fluid selected from the group consisting of: cerebrospinal fluid, serum, urine, and saliva.
5. The method of claim 1 or 2, wherein the reference level is the amount of IL-6 or NO present in a biological sample derived from a control subject.
6. The method of claim 1, wherein the reference level of IL-6 is between about undetectable 0.5 and 3 pg/ml in cerebrospinal fluid
7. The method of claim 1, wherein an increase in IL-6 levels of at least about 2-, 3-, or - 4 fold relative to a reference identifies the subject as having a neuroinflammatory disorder.
8. The method of claim 1, wherein an increase in IL-6 levels between about 5-5,000 fold identifies the subject as having a neuroinflammatory condition.

9. The method of claim 7, wherein detecting an amount of IL-6 between about 3.5 pg and 50 pg indicates that the subject has or has a propensity to develop multiple sclerosis.
10. The method of claim 7, wherein a level of IL-6 in the biological sample of between about 50 pg and 5000 pg indicates that the subject has or has a propensity to develop transverse myelitis.
11. The method of claim 2, wherein the method further comprises conducting a neurological examination or a diagnostic test.
12. The method of claim 2, wherein NO is measured indirectly by detecting nitrates, nitrites, or combinations thereof.
13. The method of claim 2, wherein the level of NO present in a reference is virtually undetectable.
14. The method of claim 11, wherein the level of NO present in a reference is about 0.5-2 μM .
15. The method of claim 12, wherein the level of NO present in a reference is about 0.1 μM .
16. The method of claim 2, wherein a level of NO greater than about 5 μM identifies the subject as having a neuroinflammation.
17. A method of selecting a therapy for a subject identified as having a neuroinflammatory disorder, the method comprising detecting an increase in the level of IL-6 in a biological sample of a subject relative to a reference level, wherein the level of IL-6 indicates an appropriate therapy.
18. A method of selecting a therapy for a subject identified as having a neuroinflammatory disorder, the method comprising detecting an increase in the level of NO in a biological sample of a subject relative to a reference level, wherein the level of NO indicates an appropriate therapy.

19. The method of claim 17 or 18, wherein the reference level is the amount of IL-6 or NO present in a biological sample derived from a control subject.
20. The method of claim 17 or 18, wherein the biological sample is cerebrospinal fluid, serum, saliva, or urine.
21. The method of claim 18, wherein the reference level of IL-6 is between about 0.5 and 3 pg/ml in cerebrospinal fluid.
22. The method of claim 17 or 18, wherein an increase in IL-6 or NO level of at least about 2-15 fold relative to a reference indicates that steroid therapy is appropriate.
23. The method of claim 17, wherein an amount of IL-6 between about 3.5 pg and 50 pg indicates that steroid therapy is appropriate.
24. The method of claim 17, wherein a level of IL-6 in the biological sample of greater than about 50 -5000 pg/ml CSF indicates that the subject has or has a propensity to develop transverse myelitis.
25. The method of claim 17, wherein a level of IL-6 in the biological sample of greater than about 50-5000 pg/ml CSF indicates that an aggressive
26. The method of claim 17 or 18, wherein the neuroinflammatory disorder is selected from the group consisting of transverse myelitis, multiple sclerosis, and neuromyelitis optica.
27. The method of claim 17, wherein the method further comprises measuring the amount of NO in the biological sample.
28. The method of claim 17 or 18, wherein the detecting involves measuring the amount of IL-6 or NO present in a biological fluid selected from the group consisting of: cerebrospinal fluid, serum, urine, and saliva.

29. The method of claim 18, wherein total levels of nitrate are detected as a method of measuring NO.
30. The method of claim 18, wherein levels greater than about 15-20 μM total nitrate indicates and nitrite levels are indicative of poor prognosis.
31. The method of claim 30, wherein the method indicates that an aggressive therapy should be selected.
32. A method of monitoring therapy for a subject identified as having a neuroinflammatory disorder, the method comprising detecting an alteration in the level of IL-6 in a biological sample of the subject relative to a reference level, wherein a reduction in the level of IL-6 indicates therapeutic efficacy.
33. A method of determining the prognosis of a subject identified as having a neuroinflammatory disorder, the method comprising detecting an increase in the level of IL-6 in a biological sample of a subject relative to a reference level, wherein the level of IL-6 is indicative of a clinical outcome.
34. The method of any of claims 1-33, wherein the reference level is the amount of IL-6 or NO present in a biological sample derived from a control subject.
35. The method of any of claims 1-33, wherein the biological sample is cerebrospinal fluid.
36. The method of any of claims 1-33, wherein the reference level of IL-6 is between about 0.5 and 3 pg/ml in cerebrospinal fluid and the reference level of NO is virtually undetectable.
37. The method of claim 36, wherein an amount of IL-6 between about 3.5 pg and 50 pg/ml CSF or NO levels less than about 15 μM indicates that steroid therapy is appropriate.

38. The method of claim 37, wherein an amount of IL-6 between about 3.5 pg and 50 pg/ml or NO levels less than about 10-15 μ M in CSF indicates that the subject has a good prognosis.
39. The method of claim 38, wherein the good prognosis identifies the subject as unlikely to experience severe neurological disability or relapse.
40. The method of claim 37, wherein a level of IL-6 in the biological sample of greater than about 50 pg and 5000 pg or NO levels of about 15-20 μ M indicates that the subject has a poor prognosis.
41. The method of claim 40, wherein the poor prognosis identifies the subject as likely to experience severe neurological disability or relapse
42. The method of claims 1-33, wherein the neuroinflammatory disorder is selected from the group consisting of transverse myelitis, multiple sclerosis, and neuromyelitis optica.
43. A pharmaceutical composition for the treatment of neuroinflammation, comprising an effective amount of an agent selected from the group consisting of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, or analogs thereof.
44. The pharmaceutical composition of claim 43, wherein the agent is 4-amino-1, 8-naphthalimide.
45. The pharmaceutical composition of claim 43, wherein the agent is the iNOS inhibitor 1400W.
46. The pharmaceutical composition of claim 43, wherein the agent is selected from the group consisting of GPI-5693, 15427, 16539, 16072, and GPI-21016.
47. The pharmaceutical composition of claim 43, wherein the agent is GPI 15427 or GPI 21016 and the effective amount is selected from the group consisting of 10 mg/kg, 20 mg/kg, preferably 30 mg/kg or 100 mg/kg.

48. The pharmaceutical composition of claim 43, wherein the agent is GPI 5693, and the effective amount is selected from the group consisting of 10 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 75 mg/kg, and 100 mg/kg.
49. A method of treating a neuroinflammatory disorder, the method comprising administering to a subject an effective amount of an agent selected from the group consisting of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, or analogs thereof.
50. The method of claim 49, wherein the agent is 4-amino-1, 8-naphthalimide.
51. The method of claim 50, wherein the agent is administered at about 1 μ M, 5 μ M, 10 μ M, or 20 μ M.
52. The method of claim 49, wherein the agent is the iNOS inhibitor 1400W.
53. The method of claim 52, wherein the agent is administered at about 50 μ M, 100 μ M, 200 μ M, or 300 μ M.
54. The method of claim 49, wherein the agent is selected from the group consisting of GPI-5693, 15427, 16539, 16072, or GPI-21016.
55. The method of claim 54, wherein the agent is GPI 15427 or GPI 21016 and the effective amount is selected from the group consisting of 10 mg/kg, 20 mg/kg, preferably 30 mg/kg or 100 mg/kg.
56. The method of claim 54, wherein the agent is GPI 5693, and the effective amount is selected from the group consisting of 10 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 75 mg/kg, and 100 mg/kg.
57. The method of claim 54, further comprising administering a corticosteroid, plasma pheresis, or other conventional therapy.

58. A method of preventing a neuroinflammatory relapse in a subject at risk thereof, the method comprising administering to a subject an effective amount of an agent selected from the group consisting of a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, a soluble IL-6 receptor or analogs thereof.
59. The method of claim 58, wherein the agent is 4-amino-1, 8-naphthalimide or the iNOS inhibitor 1400W.
60. The method of claim 58, wherein the agent is selected from the group consisting of GPI-5693, 15427, 16539, 16072, or GPI-21016.
61. A kit for the treatment of a neuroinflammatory disorder, the kit comprising a JAK/STAT signal transduction pathway inhibitor, a PARP inhibitor, an iNOS inhibitor, a soluble IL-6 receptor or analogs thereof.
62. The kit of claim 59, wherein the agent is selected from the group consisting of GPI-5693, 15427, 16539, 16072, or GPI-21016.
63. The kit of claim 59, wherein the kit is labeled for the treatment of a neuroinflammatory disorder.
64. A kit for the diagnosis of a neuroinflammatory disorder, the kit comprising an IL-6 detecting agent.
65. The kit of claim 64, wherein the IL-6 detecting agent is an IL-6 antibody.
66. The kit of claim 61 or 64, wherein the kit further comprises directions for the use of the kit in diagnosing or treating a neuroinflammatory condition or a neuroinflammatory relapse.
67. The kit of claim 64, wherein the kit further comprises an agent for detecting NO.
68. A method of identifying a subject as having a propensity to develop a neuroinflammatory relapse, the method comprising detecting a decrease in levels of NO or

IL-6 in response to therapy, wherein a failure to observe a reduction in NO or IL-6 levels identifies a patient as having a propensity to relapse.

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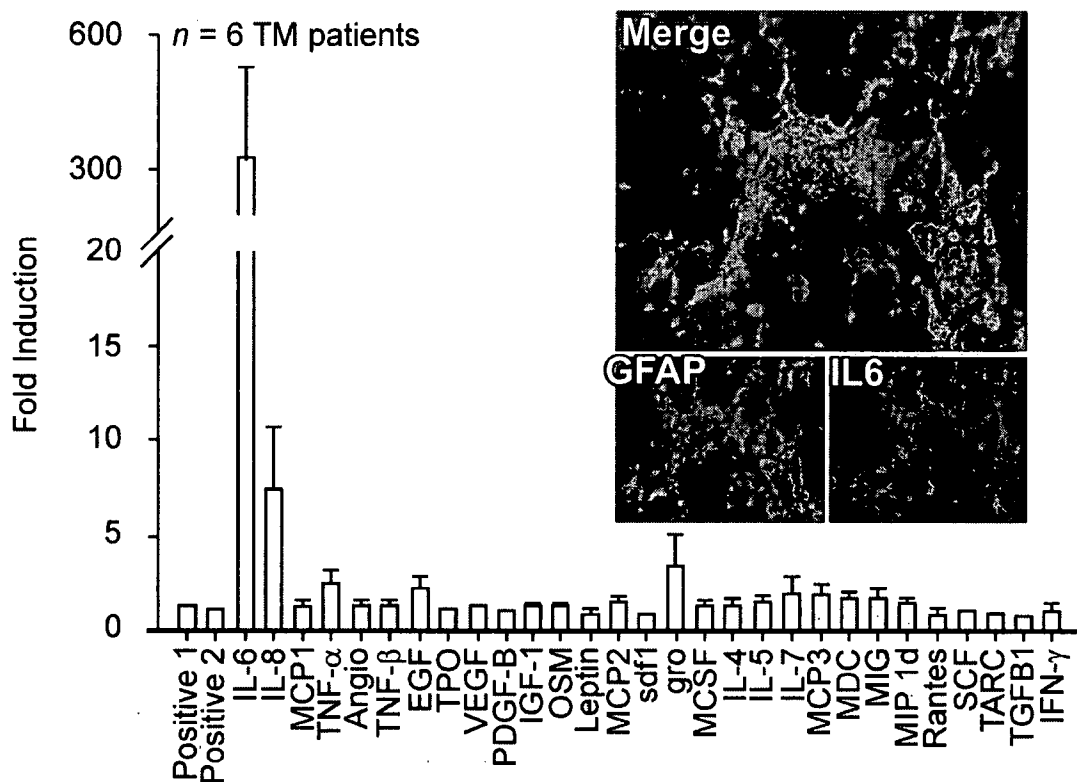


FIG. 1A

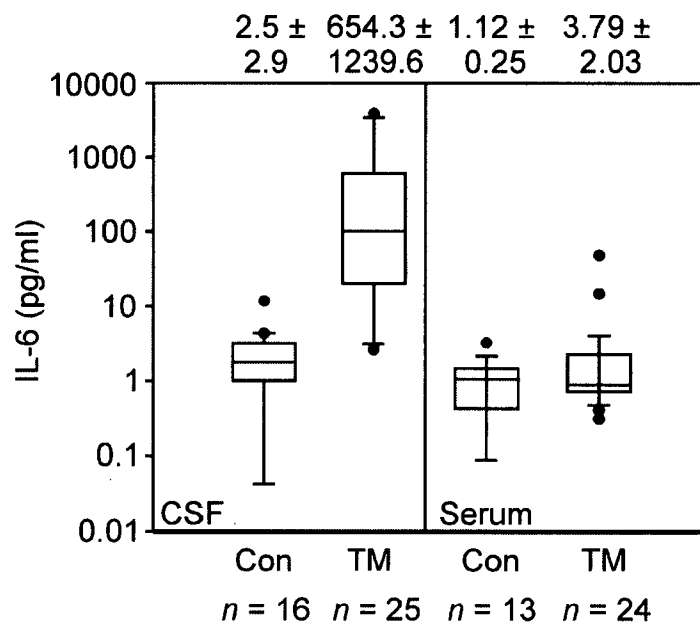


FIG. 1B

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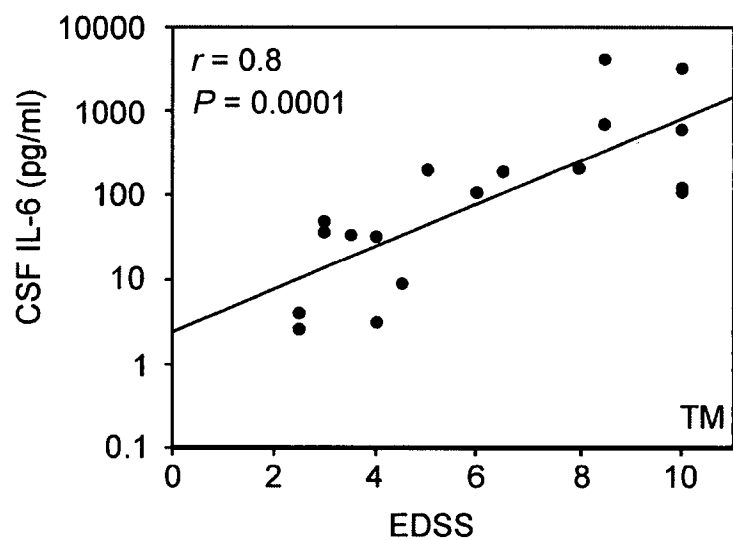


FIG. 1C

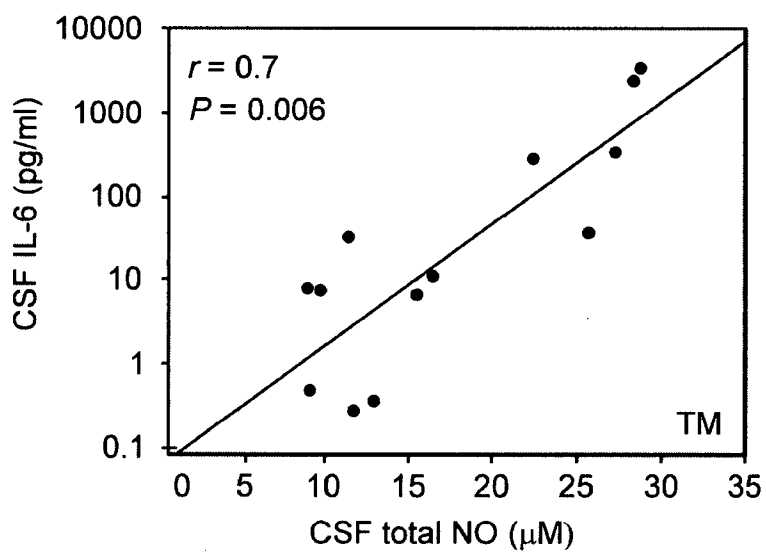


FIG. 1D

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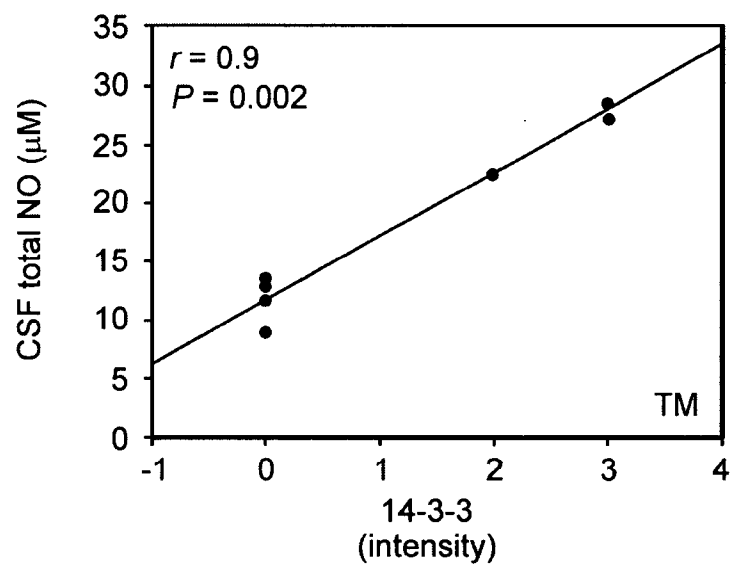


FIG. 1E

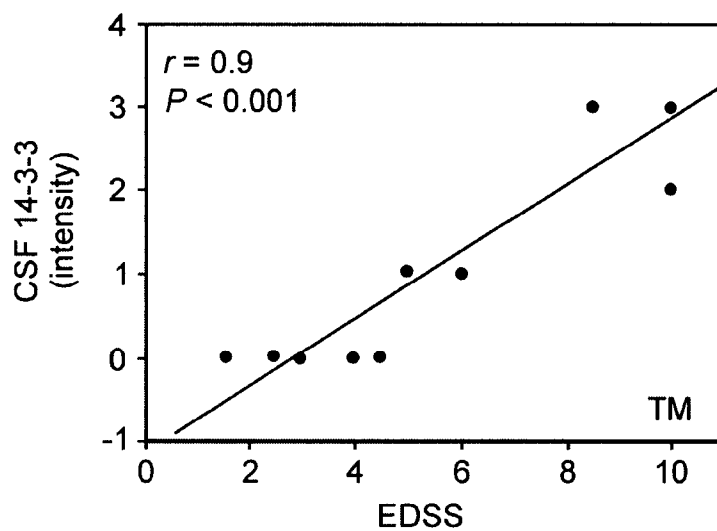


FIG. 1F

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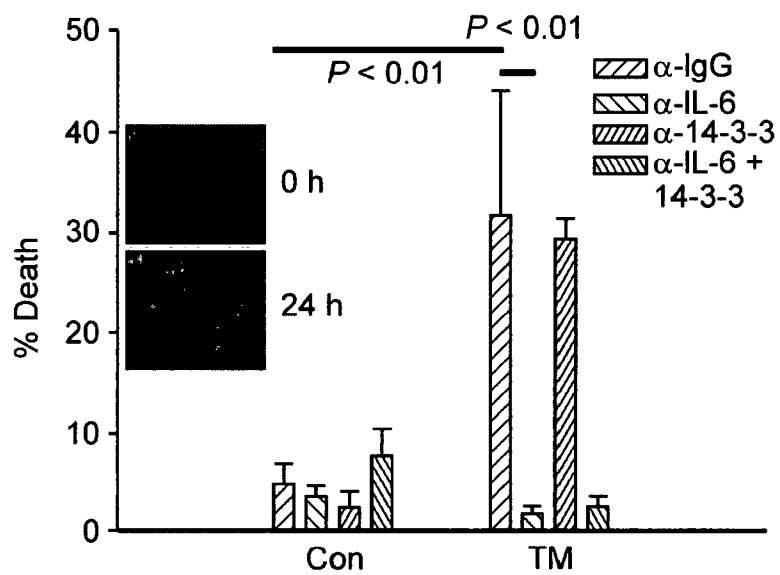


FIG. 2A

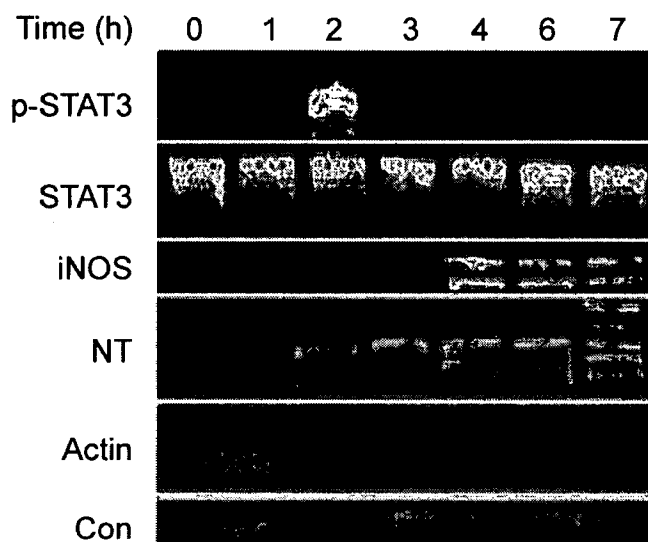


FIG. 2B

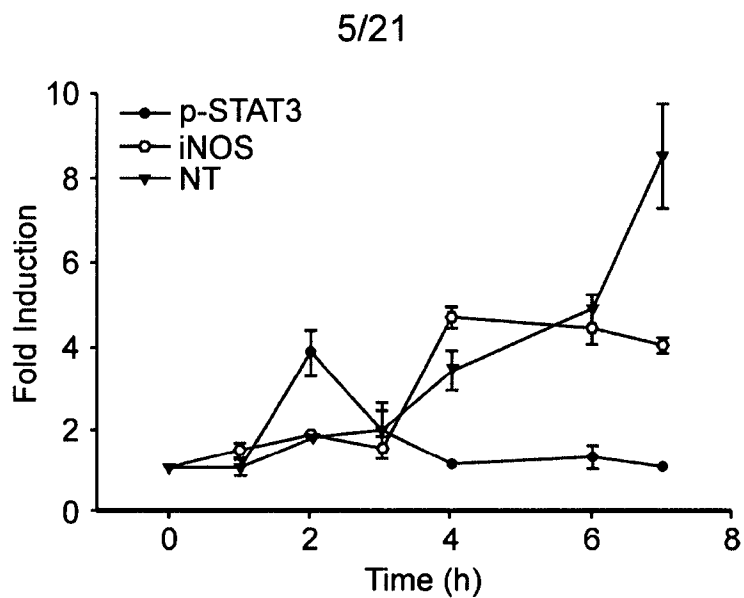


FIG. 2C

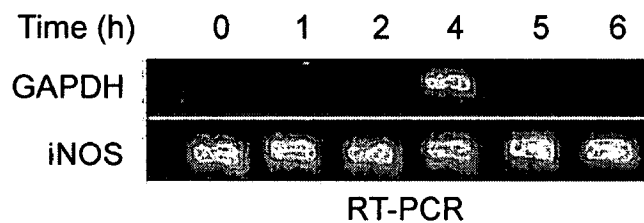


FIG. 2D

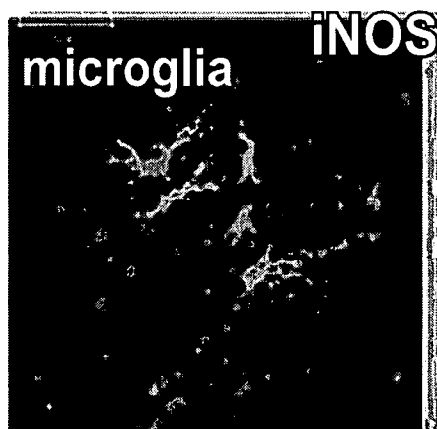


FIG. 2E

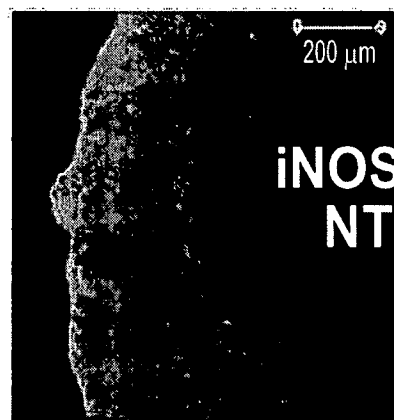


FIG. 2F

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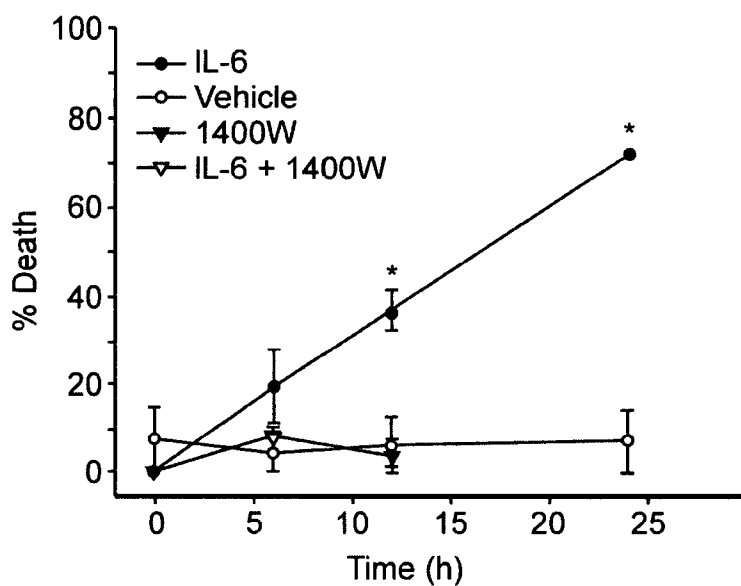


FIG. 2G

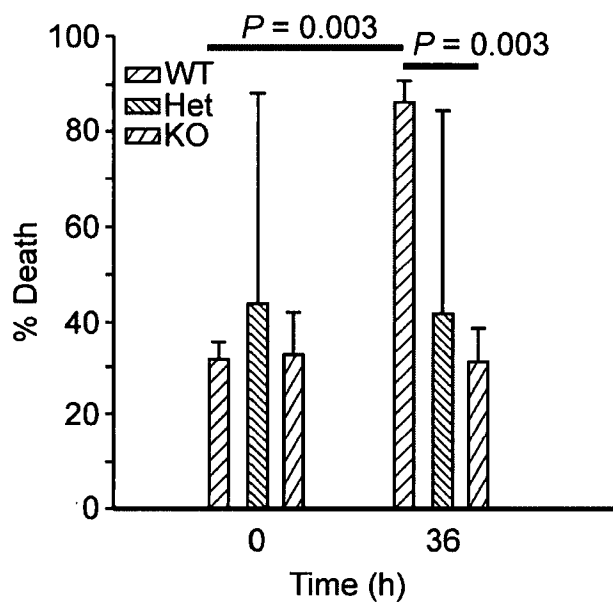


FIG. 2H

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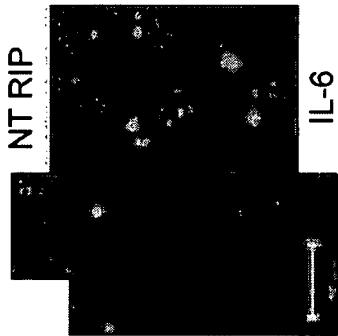


FIG. 3C

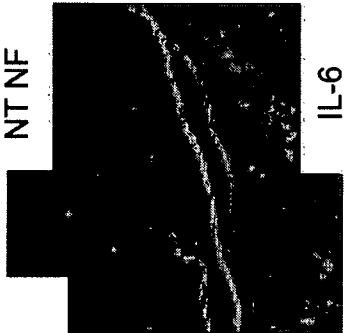


FIG. 3E

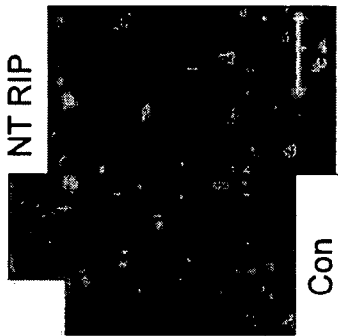


FIG. 3B

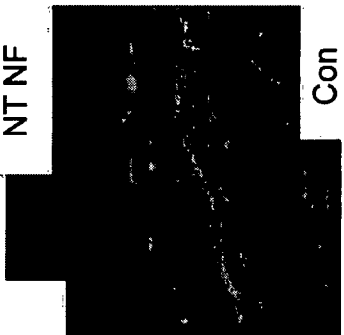


FIG. 3D

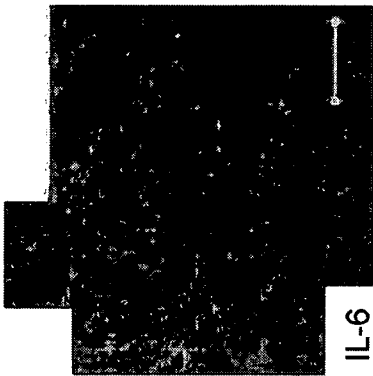


FIG. 3A

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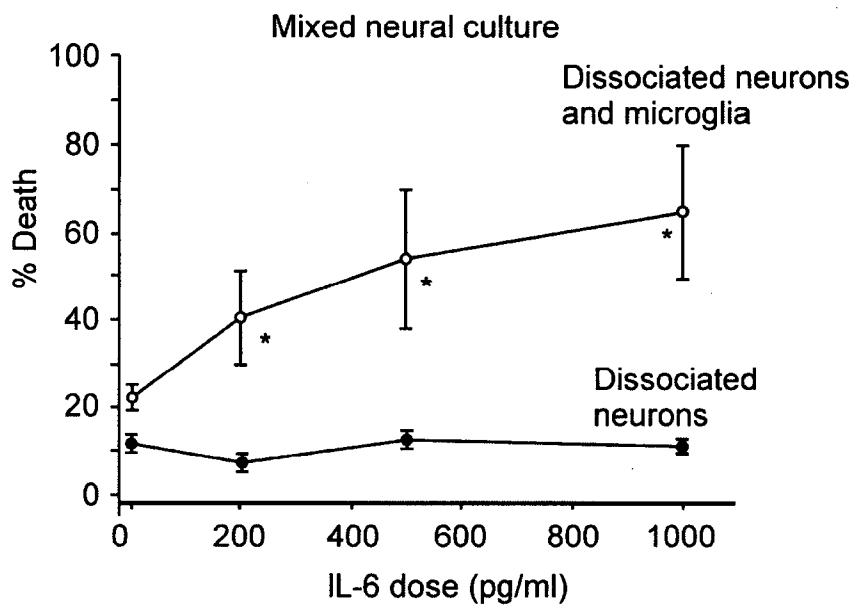


FIG. 3F

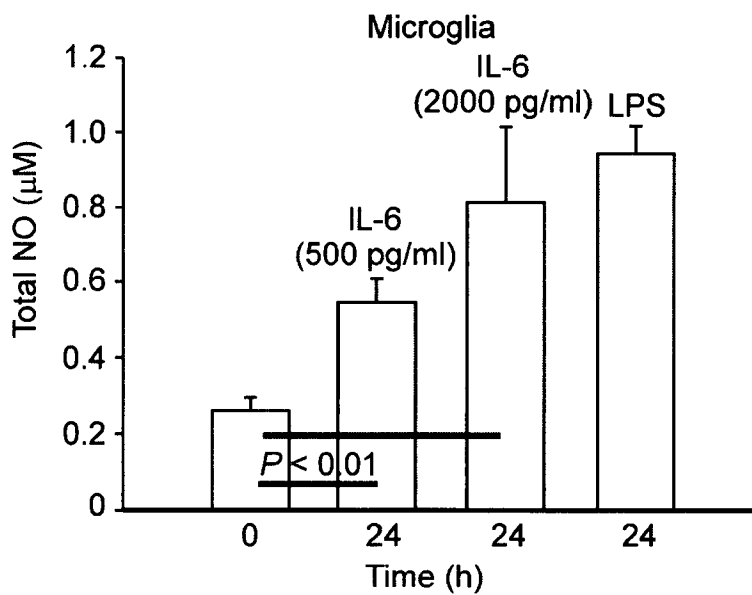


FIG. 3G

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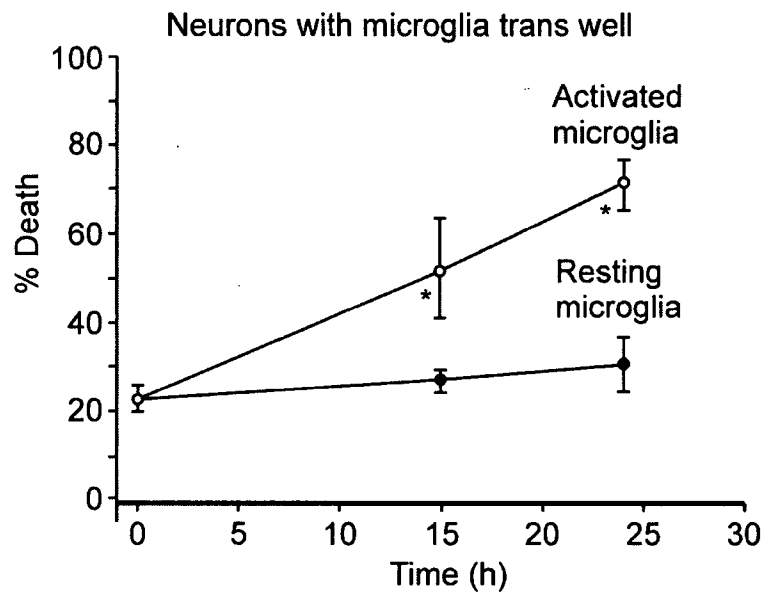


FIG. 3H

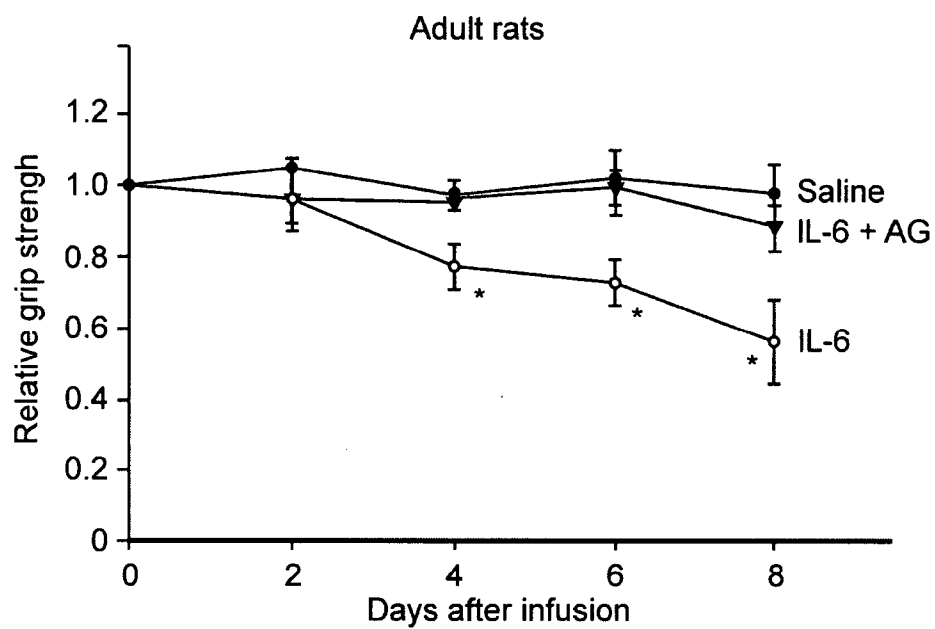


FIG. 4A

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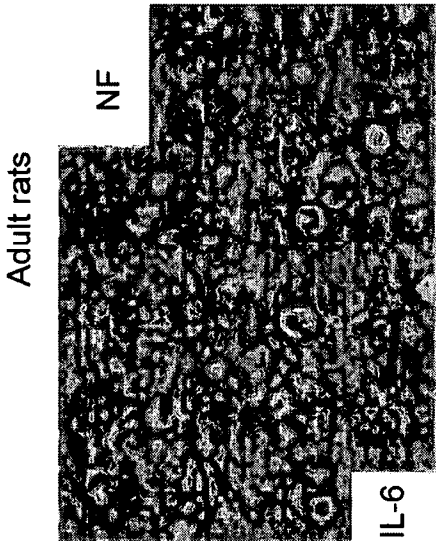


FIG. 4C

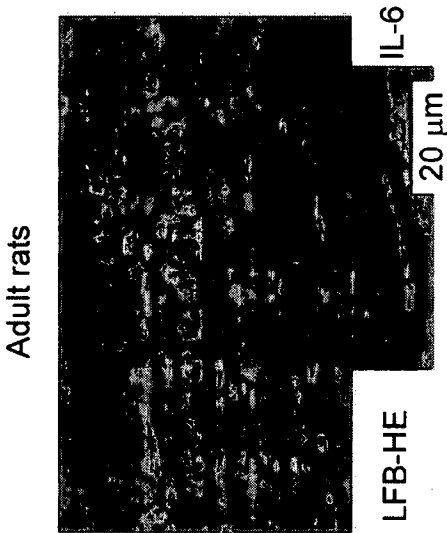


FIG. 4B

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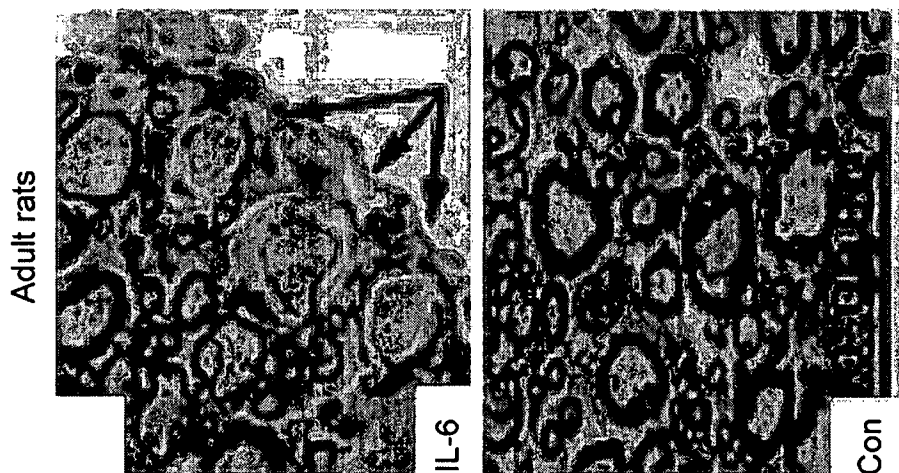


FIG. 4E

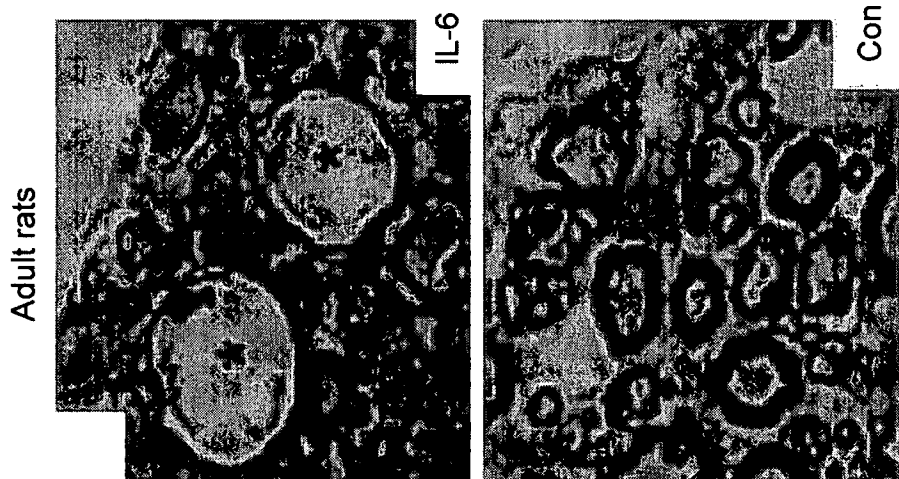


FIG. 4D

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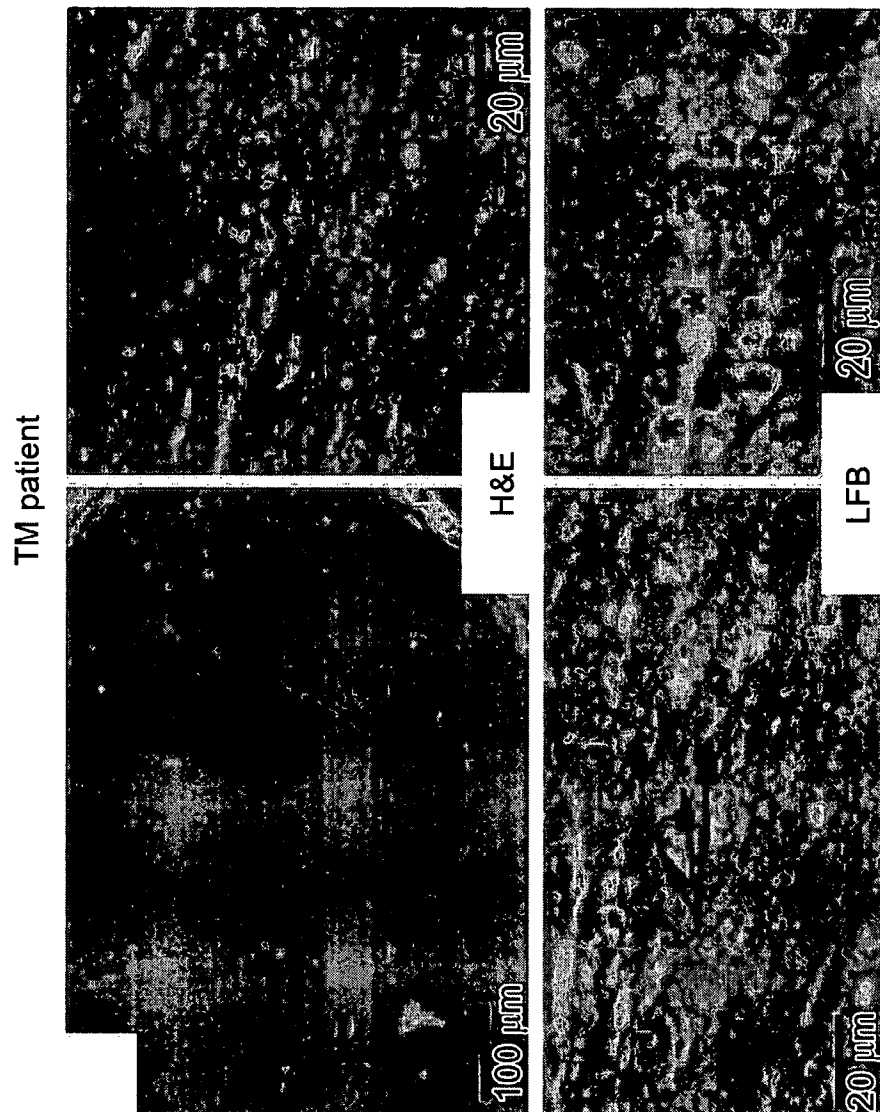


FIG. 4F

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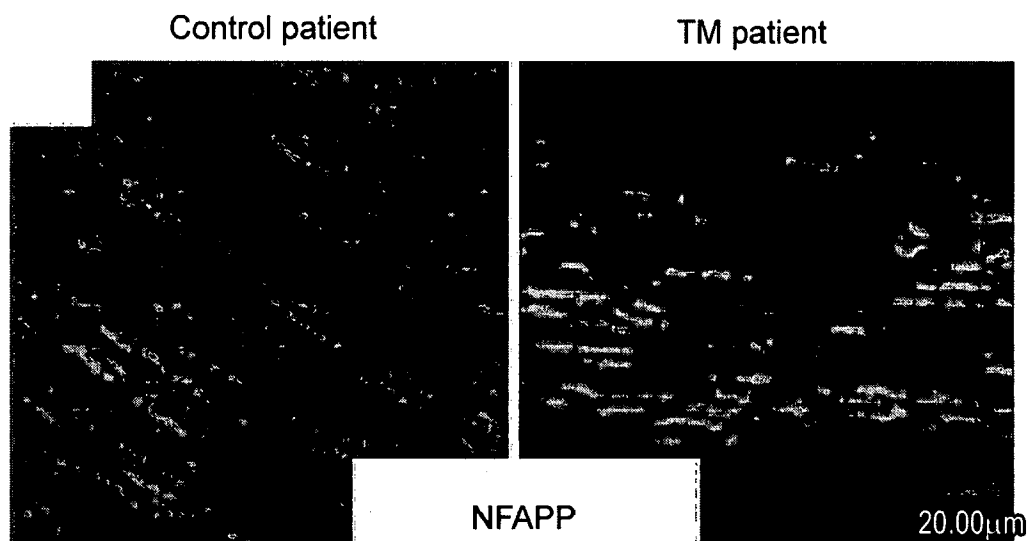


FIG. 4G

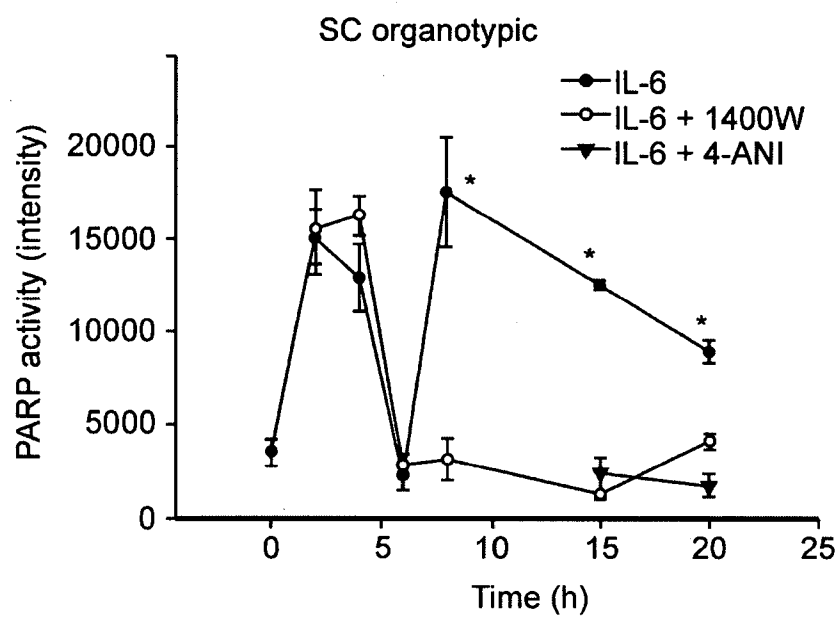


FIG. 5A

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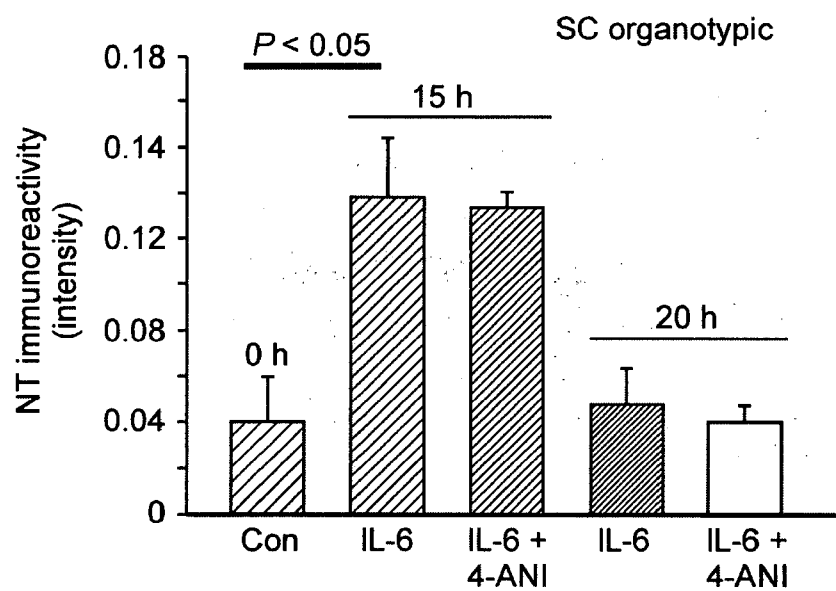


FIG. 5B

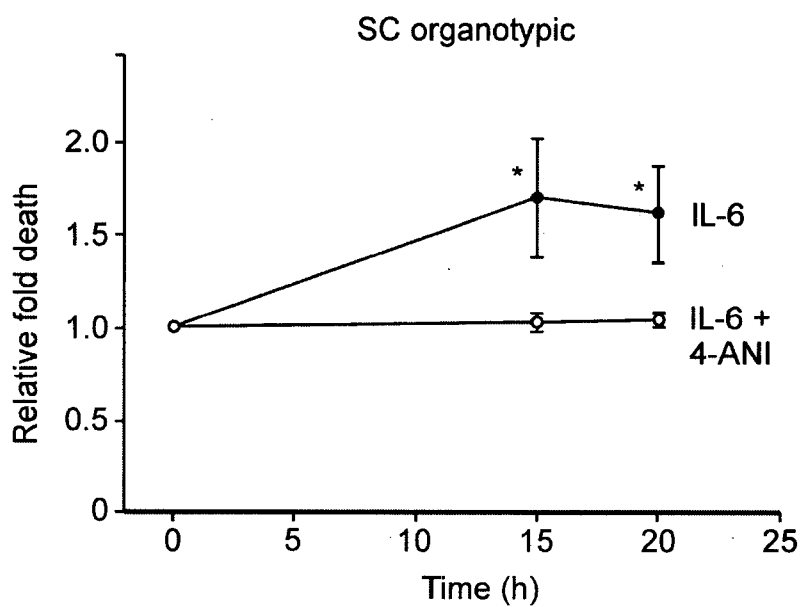


FIG. 5C

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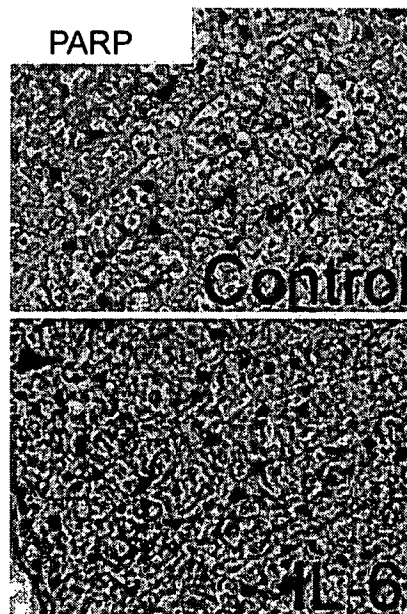


FIG. 5D

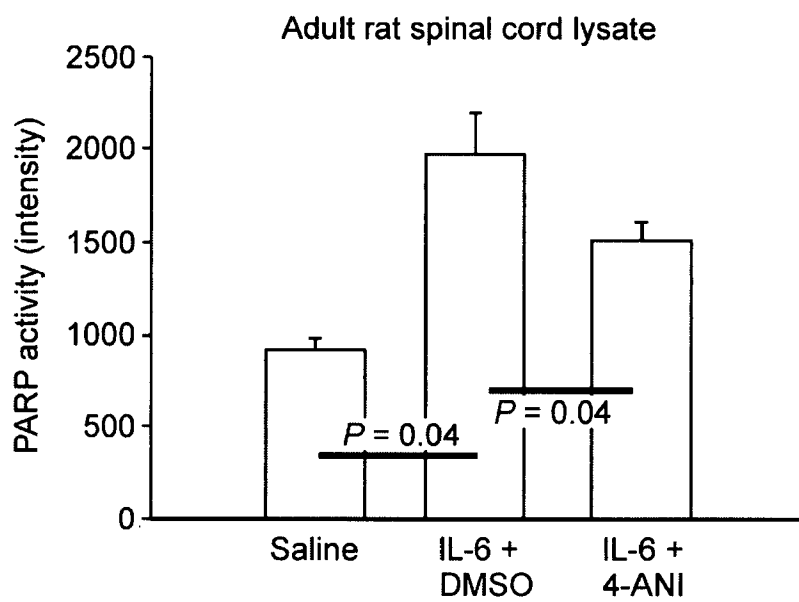


FIG. 5E

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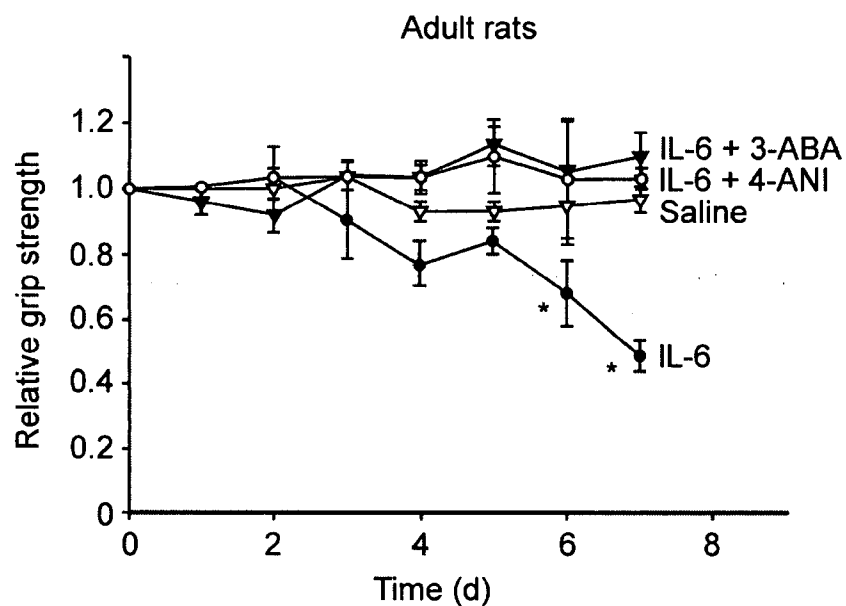


FIG. 5F

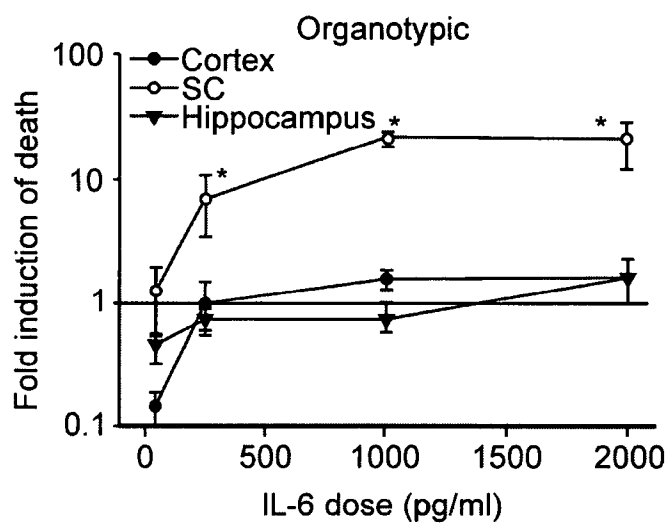


FIG. 6A

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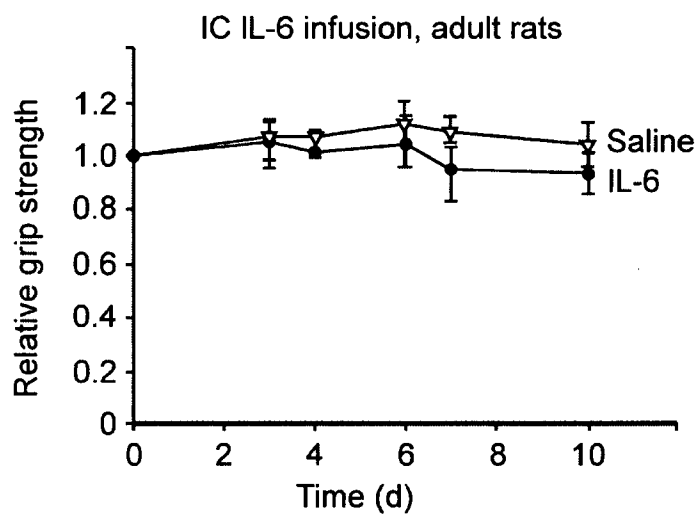


FIG. 6B

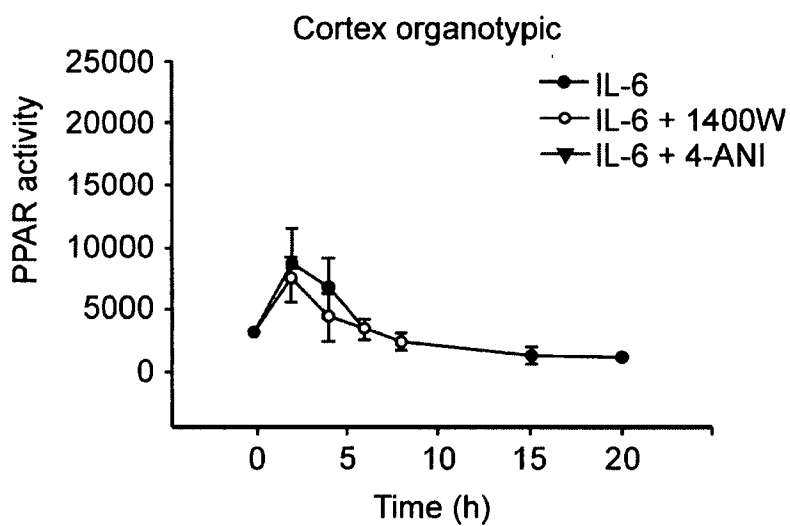


FIG. 6C

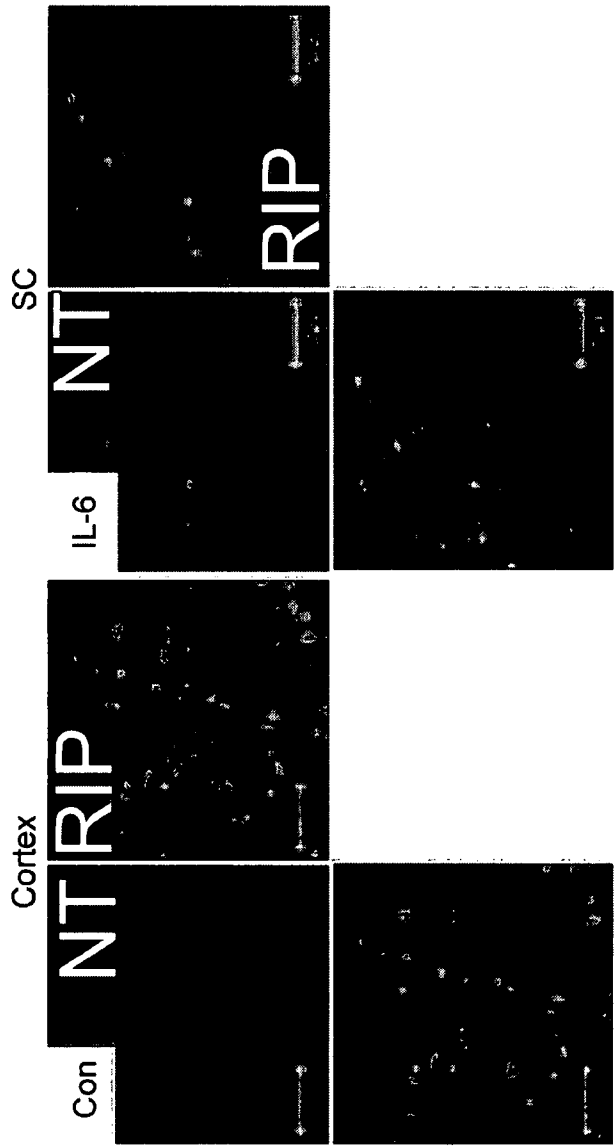


FIG. 6D

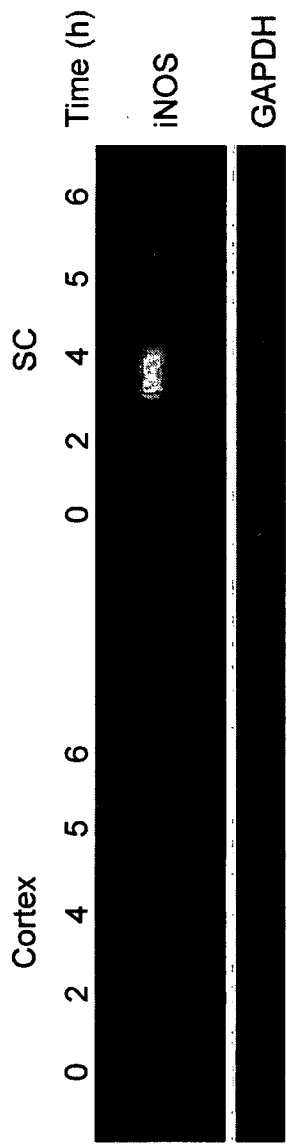


FIG. 6E

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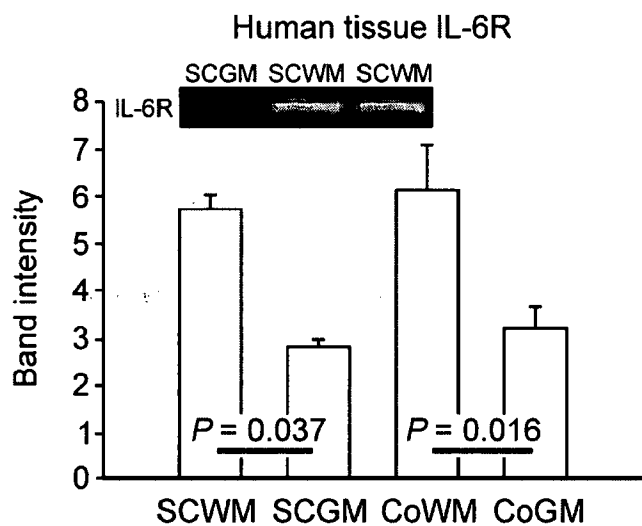


FIG. 6F

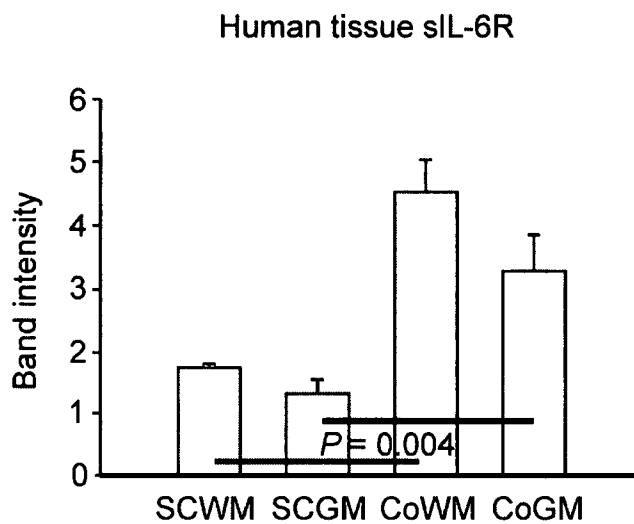


FIG. 6G

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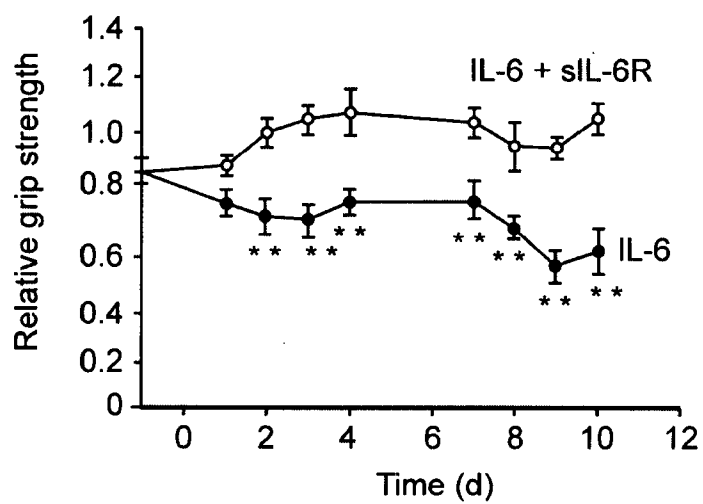


FIG. 6H

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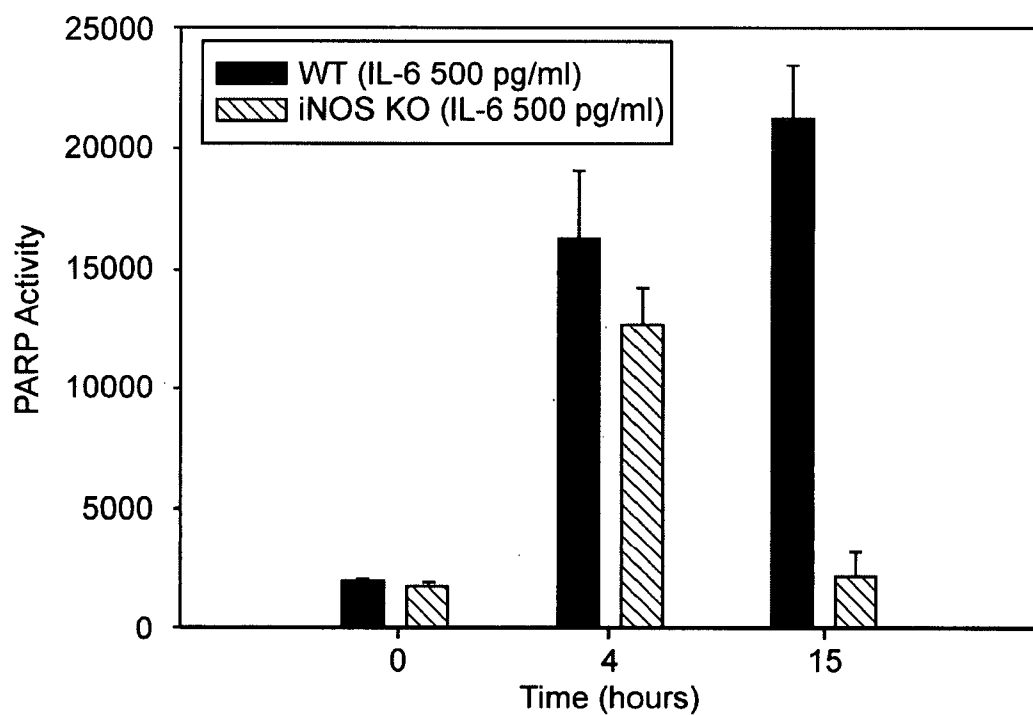


FIG. 7

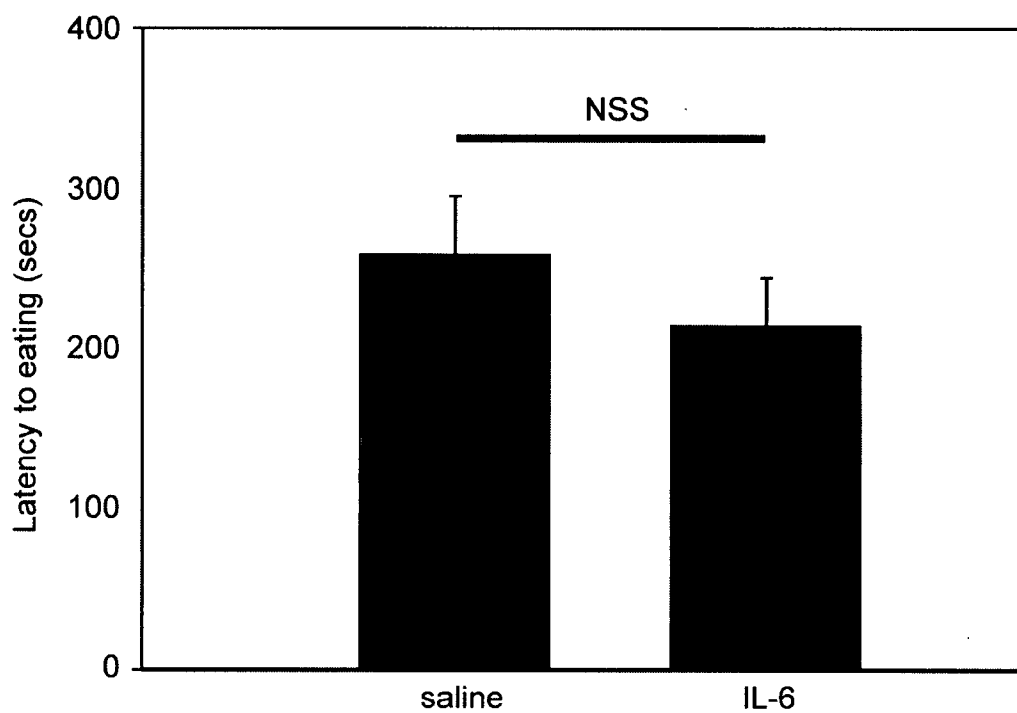


FIG. 8