Treatment of Mice with the Suppressor of Cytokine Signaling-1 Mimetic Peptide, Tyrosine Kinase Inhibitor Peptide, Prevents Development of the Acute Form of Experimental Allergic Encephalomyelitis and Induces Stable Remission in the Chronic Relapsing/Remitting Form

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We have previously characterized a novel tyrosine kinase inhibitor peptide (Tkip) that is a mimic of suppressor of cytokine signaling 1 (SOCS-1) and inhibits JAK2 phosphorylation of the transcription factor STAT1α. We show in this study that Tkip protects mice against experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis. Mice are immunized with myelin basic protein (MBP) for induction of disease. Tkip (63 μg) administered every other day suppressed the development of acute EAE in 75% of New Zealand White (NZW) mice. Furthermore, Tkip completely protected SJL/J mice, which where induced to get the relapsing/remitting form of disease. Protection of mice by Tkip was similar to that seen with the type I IFN, IFN-β. Protection of mice correlated with lower MBP Ab titers in Tkip-treated groups as well as suppression of MBP-induced proliferation of splenocytes taken from EAE-afflicted mice. Cessation of Tkip and IFN-γ administration resulted in SJL/J mice relapsing back into disease. Prolonged treatment of mice with Tkip produced no evidence of cellular toxicity or weight loss. Consistent with its JAK2 inhibitory function, Tkip also inhibited the activity of the inflammatory cytokine TNF-α, which uses the STAT1α transcription factor. The data presented in this study show that Tkip, like the type I IFN, IFN-γ, inhibits both the autoreactive cellular and humoral responses in EAE and ameliorates both the acute and chronic relapsing/remitting forms of EAE. The Journal of Immunology, 2005, 175: 5077–5086.

Tyrosine kinases play an important role in both normal and abnormal cell function. Uncontrolled or constitutive tyrosine kinase activity can result in diseases such as cancer and immunological disorders associated with inflammatory Th lymphocyte production of pathological cytokines, growth hormones, and growth factors (1, 2). To keep these inflammatory and other factors in check, a family of proteins called suppressor of cytokine signaling (SOCS) negatively control the tyrosine kinases involved in the signal pathways of these inflammatory factors (3–6). There are currently eight identified SOCS (3, 5). SOCS-1 is a negative regulator of cytokines such as IFN-γ and TNF that use the JAK/STAT signal transduction pathway by binding to the JAK2 autophosphorylation site (7–10). Specifically, SOCS-1 binds to a region on JAK2 that contains a phosphorylated tyrosine (Y1007) (11). This interaction blocks JAK2 tyrosine phosphorylation of substrates such as the transcription factor STAT1α (5, 6).

We have developed a 12-residue peptide, WLVFFVIFYFFR, called tyrosine kinase inhibitor peptide (Tkip), that mimics the negative regulatory activity of SOCS-1 (7, 12). We have shown that Tkip binds to the autophosphorylation site of JAK2, thus preventing JAK2 autophosphorylation and other associated signal transduction pathway events, such as phosphorylation of receptor chain IFN-γR-1 and STAT1α (7). Tkip has been shown to block the activity of IFN-γ, an inflammatory cytokine that uses the JAK/STAT signal transduction pathway, such as by inhibition of both IFN-γ up-regulation of MHC class I molecules and its induction of antiviral activity against viruses (7). Recently, we have shown that IL-6 activity is similarly suppressed by Tkip via inhibition of JAK2 phosphorylation of STAT3 (12). Tkip thus represents a novel approach to specific control of tyrosine kinases and their associated cytokines. Because of the potential of Tkip for regulation of inflammatory conditions where tyrosine kinases play a role in the resultant pathology, we have tested Tkip in a mouse model of multiple sclerosis (MS) called experimental allergic encephalomyelitis (EAE).

In the EAE model, immunization of mice with CNS Ags such as myelin basic protein (MBP), proteolysis protein, and myelin oligodendrocyte glycoprotein results in tail and limb paralysis due to lymphocytic infiltration and demyelination in the CNS (13, 14). Inflammatory cytokines that use the JAK/STAT pathway of signal transduction, such as IFN-γ, TNF-α, and IL-2, have been implicated in the pathogenesis of both EAE and MS (15–19). Furthermore, Th2
cytokines, such as IL-4, IL-5, and IL-6, may contribute to the pathogenesis of EAE (and MS) by the induction of a specific humoral immune response against CNS Ags such as MBP (13, 20, 21).

In this study we have evaluated the therapeutic effects of Tkip on the acute and chronic forms of EAE and have compared them to the effects of type I IFN, IFN-γ, which has previously been shown to ameliorate EAE (13, 14, 22). IFN-β, also a type I IFN, is the only Federal Drug Administration-approved cytokine therapy for the treatment of MS (13, 14, 22). Similar to IFN-γ, treatment of SJL/J mice with Tkip after induction of chronic EAE induced stable remission. Furthermore, Tkip prevented the development of the acute form of EAE in New Zealand White (NZW) mice. The protection correlated well with the suppression of MBP-specific cellular and humoral immune responses in EAE mice. Thus, these findings serve as the basis for determining and understanding the therapeutic potential of this novel small molecule SOCS-1 mimetic in autoimmune diseases such as MS.

Materials and Methods

Peptides and cytokines

Tkip and IFN-γ<sub>95–125</sub> peptides were synthesized in our laboratory on an Applied Biosystems 9050 automated peptide synthesizer using conventional fluorenylmethoxycarbonyl chemistry as described previously (23). The addition of a lipophilic group (palmitoyl-lysine) to the N terminus of a synthetic peptide was performed as the last step, using a semiautomated protocol. Peptides were characterized by mass spectrometry and were purified by HPLC. Tkip and control peptides were dissolved in DMSO (Sigma-Aldrich).

The ovine IFN-γ gene was expressed in Pichia pastoris using a synthetic gene construct that was provided by Dr. G. Van Heeke (Ciba Pharmaceutical, London, U.K.). IFN-γ was secreted into the medium and was purified by successive DEAE-cellulose and hydroxyapatite chromatography to electrophoretic homogeneity, as determined by SDS-PAGE and silver staining analysis. The purified protein had a specific activity of 2.9–4.4 × 10<sup>7</sup> U/mg protein, as measured by antiviral activity using a standard viral microplate reduction assay on MDBK cells (24). TNF-α was purchased from Sigma-Aldrich and was diluted with tissue culture medium as required.

Induction of EAE in mice

For induction of acute EAE, 300 μg of bovine MBP was emulsified in CFA containing 8 mg/ml H37Ra (Mycobacterium tuberculosis; Difco) and injected s.c. into two sites at the base of the tail of NZW mice. On the day of immunization and 48 h later, 400 ng of pertussis toxin (List Biologicals) was injected i.p. For induction of chronic relapsing/remitting EAE, 300 μg of MBP was emulsified in a Ribi adjuvant (Corixa) consisting of monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and cell wall skeleton and was injected into two sites at the base of the tail of SJL/J mice (The Jackson Laboratory). On the day of immunization and 48 h later, 400 ng of pertussis toxin (List Biologicals) was also injected. SJL/J mice were immunized again 7 days after the initial immunization. Mice were clinically examined daily for signs of EAE, and severity of disease was graded using the following scale: 1, loss of tail tone; 2, hind limb weakness, 3, paraparesis, 4, paraplegia; and 5, moribund/death. These studies were approved by the institutional animal care and use committee of University of Florida.

Administration of lipopeptides and IFN-γ

Mice were administered lipo-Tkip, lipo-IFN-γ<sub>95–125</sub>, and PBS starting at the time of MBP immunization for NZW mice and SJL/J mice that were used to study prophylactic treatment or 68 days after MBP immunization to study drug effects on chronic relapsing/remitting EAE. Mice were treated with the lipopeptides or IFN-γ at a dosage of 63 μg/mouse or 100 μg/mouse, respectively, every other day after the initiation of treatments. PBS was also used as the vehicle of transfer. DMSO concentrations were equalized in all injection mixtures.

Proliferation assay

NZW mice were immunized with MBP as described above, and spleens were extracted and homogenized into a single-cell suspension. Splenocytes (3 × 10<sup>6</sup> cell/well) were incubated with medium, MBP (50 μg/ml), lipo-Tkip, and/or IFN-γ<sub>95–125</sub> for 96 h at 37°C in 5% CO<sub>2</sub>. The cultures were then pulsed with <sup>3</sup>H]thymidine (1.0 μCi/well; Amer sham Biosciences) 18 h before harvesting onto filter paper discs using a cell harvester. Cell-associated radioactivity was quantified using a beta scintillation counter, and data were reported as cpm.

ELISA for MBP-specific Abs

Bovine MBP was resuspended in binding buffer (0.1 M carbonate/bicarbonate; pH 9.6), adsorbed to the flat bottoms of plastic 96-well tissue culture wells overnight at 4°C at a concentration of 1.2 μg/well, and subsequently evaporated to dryness. The plates were treated with blocking buffer (5% powdered milk (Carnation) in PBS) for 2 h to block nonspecific binding and then washed three times with PBS containing 0.05% Tween 20. Various dilutions of sera from NZW mice that were untreated or treated with lipo-Tkip, IFN-γ, lipo-IFN-γ<sub>95–125</sub>, or PBS by i.p. injection were added to the wells and incubated for 3 h at room temperature. Binding was assessed with the secondary Ab, goat anti-mouse Ig, to which alkaline phosphatase had been coupled. Color development was monitored at 405 nm in an ELISA plate reader (Bio-Rad) after the substrate solution p-nitrophenyl phosphate (5 mg/ml) was added, and the reaction was terminated with 3 N NaOH.

Toxicity studies

Naive NZW mice were treated for 1 wk with PBS or various doses of Tkip (100–200 μg/mouse). Mice were weighed before initiation of treatment and after completion of treatment. Blood smears were also prepared on slides for quantification of white blood cell count. Blood smear slides were stained with the Leukostat staining kit (Fisher Scientific) for determination of the differential white blood cell count. A total of 150 white blood cells were evaluated per slide. During the course of EAE treatment with the lipopeptides or IFN-γ, SJL/J mice were weighed daily, and blood smears were prepared on slides for quantification of white blood cell count as described above.

Cell viability experiments were performed on murine L929 cells (American Type Culture Collection). L929 cells were plated out to confluence, then incubated in 5% CO<sub>2</sub> at 37°C with medium or various concentrations of lipo-Tkip or lipopeptide-IFN-γ<sub>95–125</sub>. After 24 h, supernatants were removed, cells were stained with crystal violet solution, and plates were scanned and analyzed using ImageJ 1.29 software (National Institutes of Health).

Detection of cytokine mRNA expression

Microarray procedures were performed as described by the manufacturer (SuperArray) for detection of IL-2, IL-5, IL-6, IFN-γ, and TNF-α mRNA expression using a microarray kit. Briefly, total RNA was used to prepare cDNA probes. During this process the cDNA was labeled with biotinylated dUTPs. The probes were denatured and allowed to hybridize overnight at 60°C to the nylon microarray membrane, which is spotted with cDNA from common inflammatory cytokines (SuperArray). After a series of washes and additional blocking, the membrane was incubated with alkaline phosphatase-conjugated streptavidin. After incubation with the substrate, the membranes were exposed to film and developed. The exposed film was scanned using an Astra 2100U flatbed computer scanner (UMAX Technologies), and the inverted image was analyzed using ImageJ 1.29 software (National Institutes of Health) for determination of gray values to assess the presence of cytokine bands compared with background. Cytokine regions on the film (bands) that had a higher gray value than the background gray value were marked as positive, denoting the presence of cytokine mRNA, and those cytokine regions on the film that had a gray value equal to or lower than the background gray value were marked as negative, denoting the absence of cytokine mRNA expression.

Histological evaluation

Naive and MBP-immunized NZW mice that were variously treated were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde. The vertebral columns were removed, and spinal cords were dissected out and postfixed for an additional 48 h in 4% paraformaldehyde. Samples were submitted to the molecular pathology and histology core laboratory at University of Florida for tissue processing. Briefly, the tissues were dehydrated and embedded in paraffin. Sections were cut at 7 μm and mounted onto subbed slides. Cresyl violet staining was used to visualize inflammatory infiltrates, detected using a Nikon microscope attached to a Kodak MDS290 zoom digital camera (Eastman Kodak).

TNF activity and production

To determine TNF-α activity in the presence or absence of lipopeptides and medium, murine L929 cells (American Type Culture Collection) were plated to confluence in a 96-well plate, after which cells were incubated...
Control mice received PBS. As shown in Fig. 1, all four of the PBS mice developed EAE, with a mean severity of ~2.0 at its peak. Only one of five mice treated with Tkip developed EAE with a mean severity of <1 for the group. Thus, Tkip protects mice from developing the acute form of EAE.

We next determined the therapeutic effects of Tkip on the relapsing/remitting, or chronic, form of EAE. Unlike NZW mice, SJL/J mice that are immunized with bovine MBP develop the relapsing/remitting form of EAE (13, 14). We have previously shown that IFN-τ was able to induce stable remission in SJL/J mice that had the chronic active EAE disease and protected mice against relapses (13). Accordingly, we administered lipo-Tkip (63 μg/mouse), lipo-IFN-τ (63 μg/mouse), or PBS to SJL/J mice every other day starting the day of immunization with bovine MBP for induction of EAE (Fig. 2). The initial episode of EAE was milder in Tkip-treated mice than in PBS- and control peptide-treated mice. Importantly, none of the Tkip-treated mice (7) experienced a relapse, whereas control peptide mice (four of six) experienced a relapse starting at approximately day 42. The IFN-τ-treated mice were significantly protected against an

FIGURE 2. Tkip completely suppresses disease relapses in mice afflicted with the chronic relapsing/remitting form of EAE, similar to the effect of IFN-τ. SJL/J mice were injected i.p. with PBS, lipo-Tkip (63 μg/mouse), lipo-IFN-τ (63 μg/mouse), or IFN-τ (10^5 U/mouse), or PBS to SJL/J mice every other day starting the day of immunization with MBP for induction of EAE. Mice were followed daily for signs of EAE, and the mean severity of paralysis for each group was graded based on the following scale: 1, loss of tail tone; 2, hind leg weakness; 3, paraparesis; 4, paraplegia; and 5, moribund, death. Control (PBS-treated) mice had a maximum average disease severity of 2.1, whereas Tkip-treated mice had a maximum average disease severity of 0.8. All mice in the control group had disease (disease incidence, four of five).

Western blot analysis
L929 mouse fibroblast cells (3 × 10^5) were preincubated in the presence or the absence of lipophilic Tkip (lipo-Tkip) or lipophilic control peptides, and lipo-murine (Mu) IFN-τ for 20 h in a 5% CO2 atmosphere in six-well plates. After preincubation with peptides, cells were treated with increasing concentrations of TNF-α (0, 1, 10, 100, 300, and 1000 pg/ml; Sigma-Aldrich) for 30 min. To prepare whole cell lysates, L929 cells were washed twice in ice-cold PBS and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM sodium fluoride, 2 mM Na3VO4, 2 mM DTT, 20 mM β-glycerophosphate, 1 mM PMSF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 0.25% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS for 1 h at 4°C (rocking) to ensure complete lysis. After centrifugation, supernatants were resolved by 12% SDS-PAGE gels (Bio-Rad), and proteins were transferred to nitrocellulose membranes (Amersham Biosciences) overnight at low voltage. To reduce nonspecific protein binding, membranes were incubated in blocking buffer (5% nonfat dry milk in PBS) for 1 h at room temperature and incubated with polyclonal Abs specific for phospho-STAT1 (p-STAT1; Cell Signaling Technology) overnight at 4°C. After incubation with primary Ab, membranes were washed in wash buffer (1% nonfat dry milk and 0.1% Tween 20 in PBS) three times for 7 min each time and incubated with HRP-conjugated goat anti-rabbit IgG secondary Abs (Santa Cruz Biotechnology) for 45 min at room temperature. ECL (Amersham Biosciences) detection methods were used after washing steps. Membranes were then exposed to x-ray film to detect p-STAT1 proteins. To control for equal protein loading, membranes were stripped and reprobed with Abs specific for STAT1 (Santa Cruz Biotechnology).

Results
We first determined the effects of Tkip on the acute form of EAE. We have previously used the NZW mouse model of EAE to demonstrate a number of important aspects of the use of the type I IFN, IFN-τ, in the treatment of EAE, including efficacy and mechanism (13, 14). The acute form of EAE was induced in NZW mice with bovine MBP; the mice were treated with Tkip (63 μg/mouse) i.p. starting the day of immunization, with treatment every other day. Control mice received PBS. As shown in Fig. 1, all four of the PBS mice developed EAE, with a mean severity of ~2.0 at its peak. Only one of five mice treated with Tkip developed EAE with a mean severity of <1 for the group. Thus, Tkip protects mice from developing the acute form of EAE.

We next determined the therapeutic effects of Tkip on the relapsing/remitting, or chronic, form of EAE. Unlike NZW mice, SJL/J mice that are immunized with bovine MBP develop the relapsing/remitting form of EAE (13, 14). We have previously shown that IFN-τ was able to induce stable remission in SJL/J mice that had the chronic active EAE disease and protected mice against relapses (13). Accordingly, we administered lipo-Tkip (63 μg/mouse), lipo-IFN-τ (63 μg/mouse), or PBS to SJL/J mice every other day starting the day of immunization with bovine MBP for induction of EAE (Fig. 2). The initial episode of EAE was milder in Tkip-treated mice than in PBS- and control peptide-treated mice. Importantly, none of the Tkip-treated mice (7) experienced a relapse, whereas control peptide mice (four of six) experienced a relapse starting at approximately day 42. The IFN-τ-treated mice were significantly protected against an

FIGURE 1. Tkip protects mice from the acute form of EAE. NZW mice were injected i.p. with PBS or Tkip (63 μg/mouse) every other day starting on the day of immunization with MBP for induction of EAE. Mice were followed daily for signs of EAE, and the mean daily severity of paralysis for each group was graded based on the following scale: 1, loss of tail tone; 2, hind leg weakness; 3, paraparesis; 4, paraplegia; and 5, moribund, death. Control (PBS-treated) mice had a maximum average disease severity of 2.1, whereas Tkip-treated mice had a maximum average disease severity of 0.8. All mice in the control group had disease (disease incidence, four of five).

with medium, lipo-Tkip, and lipo-IFN-τ at various concentrations. After 4 h of incubation, human TNF-α (Sigma-Aldrich) at various concentrations and actinomycin D-mannitol (Sigma-Aldrich), resulting in final concentrations of 2.7 μg/ml, were added to the cells. After overnight incubation at 37°C, cell were stained with crystal violet solution, and plates were scanned and analyzed using Image J 1.29 software (National Institutes of Health) to assess cell viability.

For TNF production, human promonocytic U937 cells (American Type Culture Collection) were treated with medium and/or 33 ng/ml PMA and 5 μg/ml PHA (both from Sigma-Aldrich) for 24 h. Supernatants were then collected and evaluated for TNF activity on murine L929 cells as stated above.

Western blot analysis
L929 mouse fibroblast cells (3 × 10^5) were preincubated in the presence or the absence of lipophilic Tkip (lipo-Tkip) or lipophilic control peptides, and lipo-murine (Mu) IFN-τ for 20 h in a 5% CO2 atmosphere in six-well plates. After preincubation with peptides, cells were treated with increasing concentrations of TNF-α (0, 1, 10, 100, 300, and 1000 pg/ml; Sigma-Aldrich) for 30 min. To prepare whole cell lysates, L929 cells were washed twice in ice-cold PBS and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM sodium fluoride, 2 mM Na3VO4, 2 mM DTT, 20 mM β-glycerophosphate, 1 mM PMSF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 0.25% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS for 1 h at 4°C (rocking) to ensure complete lysis. After centrifugation, supernatants were resolved by 12% SDS-PAGE gels (Bio-Rad), and proteins were transferred to nitrocellulose membranes (Amersham Biosciences) overnight at low voltage. To reduce nonspecific protein binding, membranes were incubated in blocking buffer (5% nonfat dry milk in PBS) for 1 h at room temperature and incubated with polyclonal Abs specific for phospho-STAT1 (p-STAT1; Cell Signaling Technology) overnight at 4°C. After incubation with primary Ab, membranes were washed in wash buffer (1% nonfat dry milk and 0.1% Tween 20 in PBS) three times for 7 min each time and incubated with HRP-conjugated goat anti-rabbit IgG secondary Abs (Santa Cruz Biotechnology) for 45 min at room temperature. ECL (Amersham Biosciences) detection methods were used after washing steps. Membranes were then exposed to x-ray film to detect p-STAT1 proteins. To control for equal protein loading, membranes were stripped and reprobed with Abs specific for STAT1 (Santa Cruz Biotechnology).
initial episode of EAE as well as against a relapse (Fig. 2 and Table 1). Thus, these preliminary results suggest that Tkip can protect SJL/J mice against EAE relapses and that this protection is comparable to that seen with IFN-γ, as shown in this study and in our previous IFN studies (13, 14).

We next determined whether protection against relapses required continuous Tkip treatment. As shown in Fig. 3, when Tkip administration was ceased, on day 54 after MBP immunization, SJL/J mice relapsed into disease, and the mean severity of disease was not significantly different from that in the PBS-treated group. Similar relapsing was observed after cessation of IFN-γ treatment. Thus, continuous treatment with Tkip and IFN-γ is required for protection against EAE relapses in mice.

The previous treatments of mice with Tkip were started on the day of MBP immunization for induction of EAE. This could be seen as a prophylactic treatment. We also tested the therapeutic activity of Tkip and the control peptide on active ongoing EAE in SJL/J mice. The chronic relapsing/remitting form of EAE was induced in these mice with MBP, and mice were treated i.p. starting at the time of paralysis (day 68 after MBP immunization) and at 48-h intervals thereafter. Treatment of EAE mice with Tkip reduced the mean disease severity (to <0.5) and protected mice against future relapses, as shown in Fig. 4. PBS- and IFN-γ-treated control groups had mean peak disease severities of ~1.5 and ~2.0, respectively. No additional relapses were seen for Tkip-treated groups with continued administration of Tkip. Thus, i.p. injection of Tkip blocked additional relapses into paralysis in mice afflicted with chronic relapsing/remitting EAE.

Because Abs against CNS proteins may contribute to the exacerbation of EAE and MS (13, 20, 25, 26), we analyzed sera for MBP-specific Abs in Tkip-treated and control mice. In the SJL/J studies of chronic relapsing/remitting EAE, presented above in Fig. 2, during the course of Tkip treatment the mice were tested for MBP-specific Abs by ELISA. Mice that were treated with Tkip had lower MBP-specific Ab titers. Specifically, as shown in Fig. 5A, Tkip-treated mice had >50% lower MBP-specific Ab titers on days 22, 30, and 37 than PBS- or control peptide-treated groups. Similarly, IFN-γ also suppressed Ab production. By comparison, the lipo-IFN-γ50−125 control was relatively much less effective. Titration of sera collected at 22 days showed detectable levels of MBP-specific Abs at dilutions of 1/10,000 in the PBS group, whereas little or no MBP-specific Abs were seen in IFN-γ- or Tkip-treated groups, as shown in Fig. 5B. Thus, Tkip suppressed the humoral arm of the EAE MBP response. If the humoral arm of the immune response plays a role in MS and EAE, as has been suggested (13, 20, 25, 26), then it is subject to regulation by the SOCS-1 mimetic peptide, Tkip.

MBP-specific autoreactive T cells play a central role in the inflammatory reactions seen in EAE and MS (27–29). Previously, we had shown that IFN-γ ameliorated EAE in part by inhibiting the autoreactive MBP-specific T cell responses (14, 30). IFN-γ has also been shown to inhibit the proliferation of MBP-specific TCR-transgenic T cells (31). We therefore determined the in vivo and in vitro effects of Tkip on the proliferation of splenocytes from PBS- and Tkip-treated EAE mice. For in vivo studies, splenocytes (5 × 105 cells/well) from naive (unimmunized), PBS-treated, and Tkip-treated EAE mice were incubated in the presence or the absence of MBP for 96 h. Cultures were then titrated with [3H]thymidine for 18 h before harvesting. The radioactivity of washed cells was counted on a liquid scintillation counter. As shown in Fig. 6, splenocytes from Tkip-treated EAE mice showed significantly lower stimulation by MBP than PBS-treated control splenocytes. For in vitro proliferation studies, MBP-immunized mouse splenocytes (3 × 105 cells/well) were incubated with medium, MBP (50 μg/ml), lipo-Tkip, or lipo-IFN-γ95−125 control peptide for 96 h. Cultures were then titrated with [3H]thymidine for 18 h before harvesting. The radioactivity of washed cells was counted on a liquid scintillation counter. As shown in Fig. 7, Tkip at 3.7 and 11.1 μM significantly inhibited MBP-induced proliferation of MBP-sensitized splenocytes, whereas control lipo-IFN-γ95−125 peptide had no effect. Thus, consistent with the inhibition of EAE in NZW mice, in vivo and in vitro Tkip

![FIGURE 3.](http://www.jimmunol.org)
treatments also inhibited MBP-specific splenocyte proliferation in these mice.

We next determined the presence of mRNA of inflammatory cytokines involved in the pathogenesis of EAE in Tkip- and control peptide-treated EAE mice in the lymphoid and CNS tissues. As shown in Table II, cytokines expressed by MBP-specific splenocytes that contained MBP-specific T cells from Tkip- and PBS-treated mice included IL-2, IL-5, TNF-α, and IFN-γ. PBS-treated mouse splenocytes also expressed IL-6, whereas IL-6 was not detected in splenocytes from Tkip-treated mice. CNS tissues of PBS-treated EAE mice had detectable levels of IL-2, IL-5, IL-6, TNF-α, and IFN-γ. With the exception of IL-6, all other cytokines were not expressed in the CNS tissue of Tkip-treated, MBP-immunized mice. Histological evaluation of the CNS showed that mice protected from disease by Tkip had no lymphocytic infiltration in the CNS in contrast to PBS-treated EAE mice that had lymphocytic infiltration in their CNS, as shown in Fig. 8. This is comparable to histological results seen after IFN-γ treatment of EAE mice, which we

FIGURE 4. Tkip protects mice that have ongoing chronic relapsing/remitting EAE. SJL/J mice were immunized with MBP for induction of EAE. Mice were followed daily for signs of EAE, and the mean severity of paralysis for each group was graded based on the scale described in Fig. 1. Mice that had ongoing EAE were treated with PBS, lipo-Tkip (63 μg/mouse), or lipo-IFN-γ95–125 (63 μg/mouse) every other day starting on day 68 after MBP immunization. The dashed line signifies the start of Tkip and other treatments. Statistical analysis using paired two-tailed Student’s t test showed that the mean disease severity of the Tkip-treated group was significantly different from that of the PBS-treated group (p < 0.001), whereas the mean severity of the control lipo-IFN-γ95–125-treated group was not significantly different from that of the PBS-treated group (p = 0.11).

FIGURE 5. Tkip- and IFN-γ-treated mice have lower MBP-specific Ab levels. SJL/J mice were injected i.p. with PBS, lipo-Tkip (63 μg/mouse), lipo-IFN-γ95–125 (63 μg/mouse), or IFN-γ (105 U/mouse) every other day starting on the day of immunization with MBP for induction of EAE. On days 22, 30, and 37 after MBP immunization, mice were bled, and sera were evaluated for MBP-specific Abs by ELISA. A, Sera were diluted 1/900 before incubation on the ELISA plate. Data are representative of three experiments, each performed in duplicate. MBP-specific Ab levels in the sera of Tkip- and IFN-γ-treated groups were significantly lower (p < 0.05) at all three time points than those in the PBS- or IFN-γ95–125-treated groups, as determined by Student’s unpaired t test. B, Twenty-two days after immunization, mice were bled, and sera were evaluated for MBP-specific Abs at various dilutions. MBP-specific Ab levels in the sera of Tkip- and IFN-γ-treated groups at a dilution factor of 1000 were significantly lower (p < 0.05) than those in the PBS- and IFN-γ95–125-treated groups, as determined by Student’s unpaired t test.
have shown previously (13, 14). Thus, Tkip-treated mice do not express inflammatory cytokines in the CNS due to the inhibition of MBP-specific lymphocyte infiltrates.

The continuous treatment of SJL/J mice with Tkip for the above-described EAE studies did not result in any cellular toxicity or animal weight loss. The mean weights for all treatment groups were not statistically different, as shown in Table III. Furthermore, differential white blood cell counts for Tkip-treated mice 60 days after MBP immunization were also not significantly different from those of naive, PBS, or control IFN-γ95–125 groups, as shown in Table III, although eosinophil levels were, in general, higher in all MBP-immunized groups compared with the naive group (unimmunized). Monocyte, neutrophil, basophil, and lymphocyte levels were not statistically different among the different treatment groups, including the naive mice. Similar to the differential white blood count, there was neither a change in the percentage of cell types in the spleen nor any sign of splenomegaly (data not shown).

In vitro treatment of murine L929 cells with various concentrations of Tkip (3.7–100 μM) showed no significant reduction of cell viability, as assessed in a cytopathic assay presented in Table IV, although Tkip at 100 μM had a reduced cell viability (81.3%), but was not statistically different from that of the control peptide IFN-γ95–125 at 100 μM. Thus, Tkip had no associated toxicity at the concentrations used for EAE studies in vitro and in vivo.

Table II. Cytokine expression in the spleen and CNS of control and Tkip-treated micea

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a NZW mice MBP immunized for induction of EAE were administered PBS or Tkip (63 μg/mouse) similar to the Fig. 1 legend. After 2 mo, spleen and CNS tissue were collected and mRNA extracted for detection of specific cytokine RNA using microarray procedures as outlined in Materials and Methods section. Presented above are cytokines that were either expressed (+) or not expressed (−), as assessed by using Image J 1.32 software, in the CNS and spleen tissues (see Materials and Methods).

We have shown previously that Tkip blocks the activities of IL-6 and IFN-γ, cytokines that use the JAK/STAT signal transduction pathway, via inhibition of JAK2 phosphorylation (7, 12). IL-6 and inflammatory cytokines, such as IFN-γ and TNF-α, may contribute to the pathogenesis of immunological diseases, such as EAE and MS (15–20, 32). In this study we also tested Tkip for its ability to inhibit TNF-α-induced cytoxicity of murine L929 cells. Mouse L cells plated to confluence in 24-well plates were incubated with 33 μM lipo-Tkip, control peptide lipo-IFN-γ95–125, or medium. Four hours after pretreatment, the cells were incubated with various concentrations of human TNF-α and actinomycin D (2.7 μg/ml). After overnight incubation at 37°C, the cells were stained, and cell viability was assessed. As shown in Fig. 9, Tkip (33 μM) produced protection similar to that in cells alone at TNF-α concentrations of up to 12.3 pg/ml. Decreasing protection was seen thereafter with increasing concentrations of TNF-α up to 1000 pg/ml. Similar suppression of TNF-α activity was seen with 11 μM Tkip (data not shown), although this protection was lower than that at 33 μM Tkip. Significantly, protection was observed at these higher TNF-α concentrations compared with peptide control and medium protection, where essentially complete cell death was seen at 37 pg/ml and higher concentrations of TNF-α. Thus, similar to SOCS-1, Tkip inhibits TNF-α functions in a dose-dependent manner (10).

It has been shown recently that TNF-α-induced apoptosis is mediated by STAT1α, because STAT1α-null cells were resistant to apoptosis by TNF (33). Restoration of the STAT1α gene correlated with sensitivity to TNF-induced apoptosis (33). To determine whether TNF-α could stimulate STAT1α activation in L929 murine fibroblast cells, cells were treated with TNF-α at different concentrations of up to 12.3 pg/ml. Decreasing protection was seen thereafter with increasing concentrations of TNF-α up to 1000 pg/ml. Similar suppression of TNF-α activity was seen with 11 μM Tkip (data not shown), although this protection was lower than that at 33 μM Tkip. Significantly, protection was observed at these higher TNF-α concentrations compared with peptide control and medium protection, where essentially complete cell death was seen at 37 pg/ml and higher concentrations of TNF-α. Thus, similar to SOCS-1, Tkip inhibits TNF-α functions in a dose-dependent manner (10).

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optosis by Tkip involves inactivation of STAT1

Table III.  

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Weight (g)</th>
<th>Eosinophil (%)</th>
<th>Basophil (%)</th>
<th>Neutrophil (%)</th>
<th>Monocyte (%)</th>
<th>Lymphocyte (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive (unimmunized)</td>
<td>20.8 ± 0.2</td>
<td>0.8 ± 0.9</td>
<td>2.7 ± 0.3</td>
<td>6.7 ± 3.5</td>
<td>2.2 ± 1.1</td>
<td>87.0 ± 5.4</td>
</tr>
<tr>
<td>PBS</td>
<td>20.2 ± 1.6</td>
<td>2.6 ± 1.6</td>
<td>1.6 ± 0.3</td>
<td>17.8 ± 1.0</td>
<td>5.9 ± 1.7</td>
<td>71.9 ± 0.7</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>22.7 ± 0.9</td>
<td>4.6 ± 1.6</td>
<td>2.2 ± 0.2</td>
<td>10.7 ± 9.9</td>
<td>7.2 ± 1.4</td>
<td>75.1 ± 13.4</td>
</tr>
<tr>
<td>Lipo-IFN-γ (95–125)</td>
<td>20.5 ± 1.2</td>
<td>5.4 ± 0.7</td>
<td>1.9 ± 0.1</td>
<td>10.5 ± 6.4</td>
<td>3.0 ± 0.1</td>
<td>79.1 ± 7.2</td>
</tr>
<tr>
<td>Lipo-Tkip</td>
<td>21.9 ± 1.0</td>
<td>5.5 ± 1.2</td>
<td>2.1 ± 0.1</td>
<td>10.8 ± 0.1</td>
<td>3.8 ± 1.7</td>
<td>77.7 ± 3.0</td>
</tr>
</tbody>
</table>

* SJL/J mice were administered various treatments at the time of MBP immunization every other day for 60 days, after which blood smears were prepared for all groups including an unimmunized mice group. Furthermore, the mean weight for each group was measured and reported in grams. Blood smear slides were stained for the determination of differential white blood cell count, which is presented as percentage cell type. Percentage of cell types were not statistically different (p > 0.05) among the immunized treatment groups as determined by Student’s t test. Weights of mice in all groups were statistically not different as compared to each other as determined by Student’s t test (p > 0.05).
previous studies (13, 14). In the acute form of EAE, Tkip blocked the development of EAE (incidence, one in five), whereas in the relapsing/remitting form, all mice, including the Tkip-treated group, had an initial episode of EAE, although the Tkip group had the lowest average severity compared with control peptide- and PBS-treated groups. Tkip did block additional relapses into disease in these mice (incidence, zero of seven), whereas the control peptide- and PBS-treated groups had continued relapses (incidence, four of six and four of five, respectively). We also show in this study that chronic relapsing/remitting EAE already established in mice can be significantly alleviated by Tkip treatment (mean severity, <0.5) compared with control IFN-γ and PBS (mean severity, ~1.5) groups. Thus, Tkip blocked the development of EAE in the acute model of EAE and prevented future relapses in the chronic form of EAE.

Prolonged use of Tkip in the above studies at 63 μg/mouse every other day did not result in cellular toxicity or observable signs of toxicity, such as weight loss, in the animal. The weights of Tkip-treated mice were not significantly different from those of PBS- or control peptide-treated groups. Differential white blood cell counts for monocytes, lymphocytes, and granulocytes in blood of Tkip-treated mice were similar to those in control groups. Furthermore, treatment of murine L929 cells with various concentrations of Tkip did not show any statistically significant cytopathic...
effect compared with the effect of medium or control peptide. Thus, Tkip was without any toxicity in vivo and in vitro.

The mechanisms involved in protection from EAE include inhibition of both autoreactive humoral and cellular responses by Tkip. Mice treated with Tkip had lower MBP-specific Ab titers in sera collected over a span of 3 wk starting at 22 days after MBP immunization. This result was comparable to those obtained with IFN-γ treatment, as shown in this and previous studies (13). MBP-specific titers for PBS and control peptide groups were significantly higher than those for Tkip- and IFN-γ-treated groups. Tkip inhibited, in a dose-dependent manner, MBP-specific splenocyte proliferation in the presence of MBP, whereas the control peptide IFN-γ-125/125 was without effect. Furthermore, splenocytes from Tkip-treated mice had lower levels of stimulation than splenocytes from PBS-treated control mice. Inhibition of the MBP-specific cellular responses by Tkip correlated with the inhibition of inflammatory/pathogenic cytokines, such as IL-2, IL-5, TNF-α, and IFN-γ, in the CNS and lymphoid tissues. The presence of IL-6 mRNA in the CNS tissue of Tkip-treated mice suggests the presence of Th2 cells in the CNS. We and other have previously shown that suppressive and regulatory cytokines produced by Th2 cells inhibit EAE inflammatory Th1 cells (13, 30, 31). Thus, the protection conferred on mice by Tkip is due in part to inhibition of the inflammatory cellular and humoral responses in EAE.

The molecular mechanism involved in Tkip inhibition of EAE in mice could be due in part to the suppression of inflammatory cytokine activity via blocking of the JAK/STAT signal transduction pathway in immune cells. The expressions of STAT1 and SOCS-1 in both the CNS and lymphoid cells during active EAE have been previously described (35). We have previously shown that Tkip suppresses the activities of IFN-γ and IL-6 by binding to the autophosphorylation site of JAK2 with inhibition of STAT1 activation (7, 12). In this study we have also shown that TNF-α activity is similarly suppressed by Tkip via inhibition of STAT1 phosphorylation. Thus, the inhibition of autophosphorylation of JAK2 results in the inhibition of STAT1 activation and further downstream signal transduction events, thus inhibiting the activity of JAK2-dependent cytokines (5–7, 12). Other studies have also shown SOCS-1 attenuation of STAT1 activation (36, 37). SOCS-1, similar to Tkip, also binds the autophosphorylation site of JAK2 and inhibits phosphorylation of STAT1α (11, 38, 39). As previously mentioned, the SOCS family (SOCS-1 to -7) members play an essential physiological role in maintenance of homeostasis (3, 5, 40–42). They are negative regulators of inflammatory cytokines such as IFN-γ (7–10). The fact that Tkip did not inhibit the production of TNF in U937 cells activated by PMA/PHA suggests that although Tkip specifically suppresses this cytokine’s function, it does not suppress its induction. It has been shown that PMA/PHA induction of TNF-α protein in U937 cells is mediated through MAPK signaling pathway molecules, which involves serine/threonine kinases (34). Consistent with this result, we have previously shown that Tkip inhibits epidermal growth factor receptor autophosphorylation, but not vascular endothelial growth factor receptor and c-Src kinases (7). The epidermal growth factor receptor is regulated by SOC-1, whereas vascular endothelial growth factor and c-Src kinases are not (7, 42). Thus, at the molecular level Tkip inhibits the cellular immune response via specifically mimicking SOCS-1 function and inhibiting the signal transduction pathway of inflammatory cytokines in part via the JAK tyrosine kinases (7, 12).

It is noteworthy that SOC-1−/− mice, which develop normally through embryogenesis, die within 3 wk due to severe lymphopenia, fatty degeneration, necrosis of liver cells, and extensive macrophage infiltration of internal organs (40, 41). This condition in SOC-1−/− mice occurs primarily as a result of unregulated IFN-γ and erythropoietin signaling, because the administration of IFN-γ-specific Abs to SOC-1−/− mice prevents premature death and produces a normal appearance of internal organs (8). Furthermore, murine embryonic fibroblasts lacking the SOCS-1 gene are more sensitive than their littermate controls to TNF-α-induced cell death (10). Tkip does not inhibit activation of U937 cells by PHA/PPA, which use the MAPK signaling pathway. This suggests that Tkip does not suppress MAPK signaling. Thus, SOCS-1, Tkip, and other SOCS proteins may play key therapeutic roles in a variety of diseases associated with uncontrolled cytokine signaling (39).

We have therefore developed a 12-mer peptide that we have previously shown to mimic SOCS-1 function and that binds to the JAK2 autophosphorylation site, thus inhibiting inflammatory cytokine- and hormone cell-signaling events (7, 12). In this study we show that similar to IFN-γ, continuous Tkip treatment protects mice against the acute form of EAE in NZW mice and suppresses relapses in SJL/J mice afflicted with the chronic relapsing/remitting form of EAE. This protection correlated well with inhibition of the autoreactive cellular and humoral immune responses in EAE without any associated toxicity at the concentrations used in this study. Tkip suppresses cytokines such as IL-6, IFN-γ, and TNF-α via binding specifically to the JAK2 autophosphorylation site, thereby inhibiting the signal transduction pathway of the respective cytokines. We have demonstrated that Tkip is an effective therapy for EAE and as such should have potential for the treatment of MS and other similar inflammatory neurological diseases in humans.

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Disclosures
The authors have no financial conflict of interest.

References


