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Targeting kallikrein 6-proteolysis attenuates CNS inflammatory disease

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ABSTRACT

Kallikrein 6 (K6, MSP) is a newly identified member of the Kallikrein family of serine proteases that is preferentially expressed in the adult central nervous system (CNS). We have previously demonstrated that K6 is abundantly expressed by inflammatory cells at sites of CNS inflammation and demyelination in animal models of multiple sclerosis (MS) and in human MS lesions. To test the hypothesis that this novel enzyme is a mediator of pathogenesis in CNS inflammatory disease, we have evaluated whether autonomously generated K6 antibodies alter the clinicopathological course of disease in murine proteolipid protein139-151-induced experimental autoimmune encephalomyelitis (PLP139-151 EAE). We demonstrate that immunization of mice with recombinant K6 generates antibodies that block K6 enzymatic activity in vitro, including the breakdown of myelin basic protein (MBP), and that K6-immunized mice exhibit significantly delayed onset and severity of clinical deficits. Reduced clinical deficits were reflected in significantly less spinal cord pathology and meningeal inflammation and in reduced Th1 cellular responses in vivo and in vitro. These data demonstrate for the first time that K6 participates in enzymatic cascades mediating CNS inflammatory disease and that this unique enzyme may represent a novel therapeutic target for the treatment of progressive inflammatory disorders, including MS.

Key words: experimental autoimmune encephalomyelitis • myelin • inflammation • injury • enzyme

The family of Kallikrein enzymes are serine protease for which a growing body of evidence suggests contribute to a wide range of disease processes, including certain types of cancer and neurodegenerative disorders. Although only three such genes were believed to exist in humans, that is K1 (tissue kallikrein), K2 (glandular kallikrein,) and K3 (prostate-specific antigen), 12 additional, unique members have recently been identified, all aligned in tandem on chromosome 19q13.4 (1-3).

Kallikrein 6 (K6) was identified in our laboratory using a degenerate primer PCR cloning strategy based on regions of homology between known serine proteases (4). With the use of rat and human central nervous system (CNS) cDNA, a novel serine protease was amplified that we termed myelencephalon specific protease (MSP). We showed that MSP was abundantly expressed in the normal CNS with more limited expression in nonneural tissues (5, 6). This gene was cloned in near parallel by other research teams and referred to as zyme, protease M, and neurosin in the human (7-9) and brain and skin serine protease or brain serine protease in the mouse (10, 11). We have recently described in detail the enzymatic properties of rat MSP and of human K6 (hK6), in addition to the crystal structure of the human recombinant enzyme (12). These studies established that MSP is the rodent homologue of human K6 and will hereafter be referred to as K6 in the rodent and hK6 in human.

Importantly, altered levels of hK6 in sera, cerebrospinal fluid, or brain have been associated with ovarian and breast cancers (13), as well as certain neurological disorders, such as Alzheimer's (8, 14, 15) and Parkinson's disease (16), in addition to MS (17). Cumulatively, these data point to important and potentially pleiotropic biological activities for K6-mediated proteolysis in human disease, including neurodegenerative and demyelinating conditions. This is an attractive model of disease pathophysiology, since enzymatic activity is amenable to regulation by pharmacological or molecular interventions.

In MS, and its prototypical animal model experimental autoimmune encephalomyelitis (EAE), CNS demyelination is the end result of a slowly progressive process characterized by the accumulation of T cell infiltrates and activated macrophages. Although the cause of MS remains uncertain, it is believed to be a polygenic immune-mediated disease, governed by environmental and genetic factors. The hypothesis that K6 proteolytic cascades participate in inflammatory CNS demyelination stemmed from findings of its abundant expression by inflammatory cell subsets within CNS perivascular cuffs and at sites of CNS demyelination not only in animal models of MS but also in the human disease (17, 18). These findings pointed to a novel role for K6 outside the nervous system in immune function. Moreover, we have shown that substrates relevant to MS pathogenesis, including components of the blood-brain barrier, such as laminin, fibronectin, and heat-denatured collagen as well as myelin proteins, are rapidly and effectively cleaved by K6. Indeed, we have characterized K6 as a "trypsin-like" enzyme with rather broad substrate specificity for cleavage C-terminal to arginine residues, which is tightly regulated at the posttranslational level (12, 18). Our interest in evaluating the functional activity of K6 in immune-mediated CNS pathogenesis has been with an eye to the identification of effector molecules, which may be targeted to limit or reverse disease progression, including inflammation, demyelination, and axonal loss, which characterize MS lesions.

To test the hypothesis that K6 is a mediator of inflammatory demyelination, we have, in the absence of available K6-specific small molecule inhibitors, used an active immunization approach with recombinant K6. We describe herein that high K6 sera antibody titers are associated with reduced inflammation, demyelination, and behavioral deficits, as well as Th1 responses, in a murine autoimmune model of MS.

METHODS

EAE induction and K6-immunization protocol

K6-coimmunization

EAE was induced in 12-wk-old female SJL (H-2^S) mice (Jackson Laboratories, Bar Harbor, MA) by immunization with 100 μg of the highly encephalitogenic peptide PLP139-151 (HSLGKWLGHPDKF) dissolved in PBS and emulsified with Incomplete Freund's adjuvant (IFA), containing 400 μg of *Mycobacterium Tuberculosis*, strain H37Ra (Difco, Detroit MI). All peptides were synthesized by the protein core facility at the Mayo Clinic, with amino acid composition and purity (>98%) verified by mass spectroscopy. In the K6-coimmunization experiments, mice were immunized with recombinant rat K6 at the time of EAE induction by addition of 75 μg per mouse of K6 in PBS, or PBS alone, at the time of emulsification. Rat K6 was expressed in the baculovirus system, purified, and activated as described in detail elsewhere (18). A BLAST search of the Swiss Protein data bank indicated that rat and mouse K6 are 88% identical at the amino acid level. K6 is specific for cleavage of substrates with arginine in the P1 position (12, 18). Since no such residues are present in the PLP139-151 peptide, we were able to induce EAE and immunize with K6 in a parallel fashion. Groups of 14 mice were injected subcutaneously with 0.2 ml of the peptide emulsions and on the same day, and 48 h later, injected intraperitoneally with 400 ng of *Bordetella Pertussis* toxin in 0.1 ml of PBS. Each experiment was repeated twice. This high-dose PLP139-151 priming protocol produced severe disease with substantial mortality before the 21-day endpoint, precluding meaningful analysis of pathology. All studies were performed according to the guidelines of the Institutional Animal Care and Use Committee. Unless otherwise indicated all reagents were obtained from Sigma (St. Louis, MO).

K6-preimmunization

To induce consistent clinical disease, but in which mortality was reduced, the effects of K6-preimmunization were examined in a less severe form of EAE. Milder EAE was induced in SJL mice by administering 50 μg of PLP139-151 in IFA containing 200 μg of *M. Tuberculosis*. Mice were given 100 ng of *B. Pertussis* toxin on the day of immunization and 48 h later. High levels of K6 antibody titers were established in these mice before the induction of EAE by immunizing mice with 75 μg of K6 in 100 μl of PBS, mixed with 100 μl of PBS resuspended Ribi adjuvant (Corixa, Hamilton MT), ~4 wk before PLP139-151 priming. Ribi adjuvant was used to avoid repeated CFA administration. Two-hundred microliters of the Ribi-K6 suspension, or Ribi adjuvant alone, were injected subcutaneously at two sites on the flanks of mice following the instructions of the manufacturer. As a second negative control, other groups of mice received no immunization before the induction of EAE. K6-immunized mice received an additional 20 μg of K6 emulsified with CFA at the time of PLP139-151 priming to boost K6 antibody production. Experiments included at least six mice per group and were repeated three times.

Administration of K6-specific antibodies

To address whether the effects of K6-immunization were primarily due to antibody or T cell responses, we generated K6-specific monoclonal antibodies and have evaluated whether in vivo

administration attenuates clinical disease in myelin oligodendrocyte glycoprotein-35-55 (MOG35-55) induced EAE. The monoclonal antibodies mK6-2 and mK6-3 were generated against recombinant hK6 in Balb/c mice (Jackson Laboratories) following standard methods with the assistance of the Mayo Antibody Core Facility (5). The monoclonals were isotyped as $IgG1_{kappa}$, grown in serum free media and the IgG fraction isolated on a protein G-Sepharose column (Amersham Pharmacia Biotech., Upsala, Sweden).

MOG35-55 EAE was induced in 12-wk-old female C57BL/6J mice (Jackson Laboratories) by immunization with 100 μg of MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in IFA containing 200 μg of *M. Tuberculosis*. Mice were administered 100 ng of *B. Pertussis* toxin on the day of immunization and 48 h later. The day before MOG35-55 immunization and every 48 h thereafter, mice were given 75 μg of a cocktail of the monoclonal antibodies mK6-2 and mK6-3 in PBS by intraperitoneal injection. Animals received a total of 11 antibody treatments over the 21 day period examined for a total of 825 μg. An immunoglobulin control antibody, mouse monoclonal $IgG1_k$ specific for human β2-microglobulin (mAb L368) (American Type Tissue Collection) was administered to separate groups of mice in parallel fashion. Other groups of mice were administered only PBS. Experiments included 12 mice in each experimental group and were repeated twice.

Serological examination and antibody specificity

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate K6-specific antibody responses in immunized mice. Briefly, Maxisorb plates (Nunc) were coated overnight at 4°C with K6 or with other recombinant kallikrein available to us that is hK1, hK2, hK3, and hK13 at 0.1 μg/ml in 0.1 M carbonate buffer. Plates were washed and blocked with PBS containing 1% BSA. Two-hundred microliters of 1:1,000 to 1:64,000 serial serum dilutions were then added to wells in triplicate and incubated at room temperature for 1 h before washing and application of alkaline phosphatase-conjugated anti-mouse immunoglobulin (Jackson Immunoresearch, West Grove, PA). P-nitrophenyl phosphate was used as the substrate and plates read at 405 nm on a microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). Specificity of the monoclonals mK6-2 and mK6-2 was also examined by ELISA in which plates were coated with recombinant rK6 or hK6 protein or with other recombinant kallikrein proteins described above.

Function blocking activity of IgG from K6-immunized mice

To determine the ability of antibodies generated by K6-immunization to block K6 activity, we isolated immunoglobulin from pooled sera of mice immunized with K6 in CFA or with CFA alone, collected before EAE induction, using protein G-Sepharose (Amersham Pharmacia Biotech.). Immunoglobulin isolated from K6-immunized mice was also compared with commercially available normal mouse IgG (ChromPure mouse IgG, Jackson ImmunoResearch), as an alternative negative control.

The function blocking capacity of K6-antibodies was examined in vitro by determining their ability to inhibit K6-mediated cleavage of rat myelin basic protein (MBP) isolated from whole rat spinal cord (18), as well as an arginine-specific chromogenic substrate, Ac-Ala-Thr-Arg-pNa (Bachem, King of Prussia, PA). For analysis of MBP digestion, 10 ng of active K6 was mixed with 2 μg of IgG isolated from mice immunized with K6, or PBS alone, in 50 mM Tris and 100

mM NaCl buffer (pH 8.0). The reaction mix was incubated for 15 min at RT. IgG negative controls were substituted with an equal volume of suspension buffer alone in place of K6. MBP (5 μg), or Tris buffer alone, was then added to each reaction tube, and the reactions were allowed to proceed for 3 h at 37°C. The reaction was stopped by adding SDS-PAGE sample buffer with β-mercaptoethanol and analyzed by 20% Tricine SDS-PAGE.

To examine the ability of IgG isolated from the sera of K6-immunized mice to block hydrolysis of the chromogenic substrate AcATRpNA, 20 ng of active K6 were preincubated with 4 μg of K6-IgG or control IgG (1:35 molar ratio) at RT for 15 min in 50 mM Tris, 1 mM EDTA, pH 8.5. The kinetic conditions of the assay were 1 nM of activated K6 with 400 μM AcATRpNA, incubated at 37°C. Absorbance was read at 405 nm at 15 min intervals over a period of 2 h, with a final reading at the 3 h time point, on a Beckman coulter DU640 spectrophotometer interfaced with temperature controller.

Behavioral analysis

Mice were housed under barrier conditions, and paralyzed mice were afforded easier access to food and water. The primary end point in all experiments was clinical outcome and animals were observed daily and graded without knowledge of treatment groups according to their clinical severity as follows: grade 0, no clinical disease; grade 1, loss of tail tonis; grade 1.5, impairment of righting reflex; grade 2, paresis or paralysis of one hind limb; grade 3, complete paralysis of both lower extremities; grade 4, nonamubulatory and moribund; and grade 5, death.

Histopathology

PLP139-151 primed animals were killed (30 mg/kg sodium pentobarbital) at day 12 or day 21 for histological analysis by perfusion with 4% paraformaldehyde (pH 7.4). Before perfusion in the preimmunization groups, spleens were collected into 4°C RPMI for examination of PLP-specific T cell proliferation and cytokine secretion. Sera were collected from all mice just before perfusion for examination of K6-antibody titers.

At 12 and 21 days post-PLP induced EAE, quantitative morphologic analysis was performed on 1.0 μm erichrome/cresyl violet stained transverse sections through 10-15 different spinal cord segments per mouse (19). A pathological score reflecting the frequency of pathology was assigned to each animal based on meningeal inflammation and white matter parenchymal pathology, which includes parenchymal inflammation and demyelination, in each of the four quadrants of the spinal cord. The score was expressed as a percentage of the total number of spinal cord quadrants positive for each pathological measurement, divided by the total number of spinal cord quadrants examined. A maximum score of 100 reflects the presence of pathology in all four quadrants of every spinal cord segment examined from an individual spinal cord (20).

K6 expression in PLP-primed mice and regulation by activation

To confirm the association of K6 with CNS inflammatory cells in the PLP139-151 model of MS, K6 was localized immunohistochemically in 10 μm paraffin sections through the spinal cord of mice at 21 days postpriming. Deparaffinized sections were treated with citrate buffer and stained for K6 using rabbit anti-rat K6 IgG, as described previously (18). Colocalization of K6 within

immune cells in the same or adjacent tissue sections was accomplished using the T cell marker, rat anti-human CD3 (Serotec) or the macrophage marker Isolectin B_4 (Sigma). Immunolabled cells were visualized using species-specific fluorochrome conjugated secondary antibodies or fluorochrome conjugated streptavidin (Jackson Immunoresearch).

To determine whether K6 expression in infiltrating CNS inflammatory cells represents constitutive levels of expression or an up-regulation in activated immune cells, we examined the relative levels of K6 mRNA expression in resting and activated splenocytes, using real-time RT-PCR. Splenocytes were isolated from normal SJL mice and cultured for a period of 72 h in the presence of 10 μg/ml concanavilin A (ConA), 10 μg/ml lipolysaccharide (LPS), or PBS alone, or were grown on tissue culture plastic coated with 10 μg/ml CD3-antibody (CD3-Ab) (PharMingen, San Diego, CA). For each experiment, splenocytes cultures were prepared from combined spleen homogenates obtained from four mice and were grown in complete Click's media at 3×10^6 cells/ml in a total volume of 12 ml. Each culture condition was examined in duplicate, with each experiment being repeated twice. Total RNA was extracted from noncultured and cultured splenocytes using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and quantified spectrophotometrically; 0.5 ug of total RNA were subject to RT-PCR using the Light Cycler-RNA Amplification Kit SYBR Green I in a Roche Light Cycler apparatus following the instructions of the manufacturer (Roche Diagnostics, Mannheim Germany). Primers specific for mouse K6 were (forward, 5′-CCTACCCTGGCAAGATCAC-3′ and reverse, 5′-GGATCCATCTGATATGAGTGC-3′) and for the housekeeping gene glyceraldehyde phosphate 3-dehydrogenase (GAPDH) were (forward, 5′-ACCACCATGGAGAAGGC-3′ and reverse, 5′-GGCATGGACTGTGGTCATGA-3′). Expression levels were quantified relative to K6 and GAPDH nucleic acid templates of known copy number with changes in gene expression reported as percent change relative to PBS-treated controls.

A sensitive capture ELISA was developed to evaluate changes in K6 levels in the sera of PLP139-151 primed mice. Maxisorb plates were coated with rabbit anti-rK6 (17) and nonspecific binding blocked with 1% BSA. The sera from control mice (n=24) or from mice at 12 (n=12) or 21 days (n=12) post-PLP139-151 priming were then applied at fivefold dilutions in 1% BSA containing 0.01% Tween-20. Alternatively, recombinant rK6 was applied to create a standard curve. Bound K6 was detected using the sera of K6-immunized mice at a 1000-fold dilution or the mK6-2 or mK6-3 monoclonals at 20 μg/ml. Binding was visualized with an alkaline phosphatase conjugated mouse IgG specific secondary antibody (Jackson Immunochemicals), as described above.

Effect of IgG isolated from the sera of K6-immunized mice on immune cell invasion

The possibility that direct immunization of mice with K6 generates antibodies that block immune cell invasive capacity was examined by determining the effect of IgG isolated from the sera of K6-immunized mice on the invasion of splenocytes in vitro, using a modified Boyden Chamber assay. Splenocytes were isolated from normal mice and grown in complete Click's media containing 10 μg/ml ConA. After 48 h in culture, cells were harvested and grown for an additional 3 h in serum starvation media (phenol red free RPMI, 1% bovine serum albumin, 1 mM HEPES buffer, 50 U/ml penicillin-streptomycin, and 2 mM glutamine). Cells were labeled with calcein AM according to the recommendations of the manufacturer (Molecular Probes, Eugene, OR); 1.5×10^6 calcein AM labeled cells were applied in 250 μl of serum starvation

media to each of the upper wells of Matrigel coated Fluoroblock 24 well plates (Pharmigen), with or without the addition of 25 μg/ml of IgG from K6-immunized mice. Addition of the same concentration of normal mouse IgG (Jackson Immunoresearch), served as an immunoglobulin control. Seven-hundred and fifty microliters of serum starvation media were added to the lower chamber with SDF-1α (50 ng/ml), which was added as a chemoattractant. Treatments were examined in triplicate and each experiment was repeated three times. The fluorescence of cells, which had migrated into the lower chamber, was read after a 24 h culture period on a Cytoflour 4000 bottom reading plate reader at A530 (Bio-Rad, Hercules, CA).

Analysis of T cell function

To determine the relationship between K6-immunization and Th1 differentiation, we examined Th1 responses in K6-immunized mice in vivo and in vitro.

Delayed-type hypersensitivity responses (DTH)

DTH responses were quantified using a 24 to 48 h ear-swelling assay at 9 and 18 days post-PLP139-151 priming. Prechallenge ear thickness was determined using a dial gauge micrometer. DTH responses were elicited by injecting 10 μg of the priming peptide PLP139-151 in 10 μl saline into the ventral surface of the right ear and OVA323-339 peptide at the same concentration into the left ear, using a 500 μl Hamilton syringe (Hamilton Co., Reno, NV) fitted with a 30 gauge needle. At 24 and 48 h after ear challenge, the increase in ear thickness over prechallenge measurements was determined. Results were expressed in units of 10^{-4} inches ± SE.

In vitro T cell proliferation assays

Spleens were removed at 12 or 21 days post-EAE priming, and proliferation assays were carried out in flat-bottomed 96-well microculture plates (Falcon Labware, Oxnard, CA) in a total volume of 200 μl of complete Click's medium, containing 5 x 10^5 splenocytes per well. Antigen-specific proliferation was assessed in triplicate by the incorporation of $^{[3H]}$TdR (1 μCi/well) during the final 18 h of a 96 h culture period, using a PLP139-151 peptide dilutions starting at 10 μg/ml. Parallel concentrations of a nonspecific peptide of ovalbumnin, OVA323-339 (ISQAVHAAHAEINEAGR), were used as a negative control. As a positive control, ConA was used at dilutions starting at 10 μg/ml. $^{[3H]}$TdR uptake was detected using a Topcount microplate scintillation counter (Packard Instruments, Meriden, CT). Results were expressed as stimulation index = mean cpm of Ag containing cultures/mean cpm of control cultures without added antigen.

Cytokine secretion

For cytokine analysis, 2.5 x 10^6 spleen cells were cultured at 12 days post-EAE induction in a total volume of 1 ml complete Click's media, supplemented with 10 μg/ml of PLP139-151, or with Click's media alone. Supernatants were harvested after 72 h and analyzed for IFN-γ and IL-2 production by capture ELISA, using capture and detection antibodies, in addition to cytokine standards, from PharMingen, following the recommendations of the manufacturer.

Statistical analysis

Where data were nonlinear, as in the case of behavior scores, the significance of differences between K6-immunized mice and controls was determined by nonparametric Mann-Whitney U-test. Parametric unpaired Student's *t* test was used for evaluation of differences in histological scores, DTH, T cell proliferation, cytokine production, and K6 mRNA expression, except when data were not normally distributed in which case the Mann-Whitney U-test was used. Comparison of percent survival between groups was made using X^2 employing Fisher's exact test. Statistical significance was set at $P \leq 0.05$.

RESULTS

K6 expression by immune cells and regulation by activation

CNS perivascular and parenchymal inflammatory cells in PLP139-151 primed mice were associated with dense K6-immunoreactivity. This corresponded to K6 production by both CD3-immunoreactive T cells and by isolectin-B_4 positive macrophages (Fig. 1*a–d*).

To assess whether K6 expression by infiltrating CNS inflammatory cells occurs above basal levels, we examined K6 mRNA expression in resting and activated mouse splenocytes (Fig. 1*e* and *f*). Specific activation of T cells (Con A and CD3 antibody) produced a twofold increase in K6 mRNA expression, compared with those cells grown in media containing PBS alone (unpaired Student's *t* test, P<0.006). In LPS-stimulated cultures, there was a trend toward an increase in K6 expression, but this did not reach the level of statistical significance. These results indicate that activated T cells, such as those seen in CNS-inflammatory lesions, express higher levels of K6 than resting cells, which we propose, contributes to pathogenesis.

K6 levels were evaluated in the sera of PLP139-151 primed mice (Fig. 4*e*). Capture ELISA demonstrated that the mean level of K6 in sera did not differ between control mice (10.6±2.1 ng/ml) and mice at 12 days post-PLP139-151 priming (8.3±3.0 ng/ml). By 21-days postpriming, however, K6 levels detected in sera were elevated by approximately sevenfold (73.5±28.0 ng/ml; Mann-Whitney rank sum test, $P< 0.008$).

K6-immunization generates K6-specific antibodies that block K6-enzymatic activity in vitro

The role of K6 in the development of clinical and histological manifestations of EAE was investigated by inhibiting K6 enzymatic activity using an active immunization approach. The sera from K6-immunized mice were analyzed by ELISA for levels of K6 antibody production before the induction of EAE in the case of K6-preimmunized mice and at the conclusion of each experiment. In all animals, K6 antibody titers were significantly above background out to a dilution of 1:16,000, and this was true even when examined in animals at 90 days post-K6-immunization. ELISA was also used to demonstrate that the antisera from K6-immunized mice bound specifically to K6 but not to other related kallikreins for which we have recombinant protein, that is, hK1, hK2, hK3, or hK13.

The capacity of antiserum from K6-immunized mice to block K6-specific enzymatic activity was evaluated in vitro (Fig. 2). The IgG fraction was isolated from the sera of mice immunized with

K6, or with adjuvant alone, before EAE induction. The IgG fraction from K6-immunized mice, but not that from control mice, or normal mouse IgG obtained commercially (data not shown), blocked K6-mediated degradation of MBP in vitro (Fig. 2*a*), and significantly reduced the rate of K6-mediated AcATRpNA substrate hydrolysis (92.3% decrease; Fig. 2*b*). These experiments demonstrate that K6-immunization resulted in the production of K6-function blocking antibodies.

Antibodies generated by K6-immunization inhibit immune cell invasion in vitro

Immune-mediated CNS demyelination is initiated after the extravasation and migration of immune cells from the vascular system into the CNS. We have demonstrated in vitro that IgG isolated from K6-immunized mice, but not control IgG, inhibited the invasion of activated splenocytes by ~20%, in a Boyden Chamber invasion assay (unpaired Student's *t* test, $P \leq 0.005$) (Fig. 2*c*). These findings suggest the efficacy of K6-immunization in attenuating clinical disease and spinal cord pathology may be due in part to a decrease in inflammatory cell CNS invasion mediated by K6-specific antibodies.

K6-immunization before, or at the time of, PLP139-151 priming inhibits the development of clinical signs of EAE

K6-immunization at the time of (Fig. 3), or before EAE induction (Figs. 4 and 5), each delayed the onset of disease and reduced clinical disease scores in SJL mice with PLP139-151-induced EAE (refer to Table 1). In both cases, the mean day of disease onset, the mean time to peak disease, the mean daily clinical score after the onset of clinical signs, and the mean maximal clinical disease score observed were significantly reduced in K6-immunized mice relative to controls (Mann-Whitney U test, $P<0.04$). K6-immunization was also associated with an increase in the number of days free of clinical signs (unpaired Student's *t* test, $P<0.001$). SJL mice with high dose PLP139-151 EAE, used in the K6 coimmunization experiments, experienced severe clinical disease with significant mortality. Notably, in this regard, K6-immunization was associated with a significant increase in survival to the 21 day end point compared with controls (X^2, $P=0.003$, Fig. 3*a*, Table 1). The incidence of disease and survival in the lower dose PLP-EAE, used in the preimmunization experiments, did not differ significantly between the groups examined (Figs. 4 and 5, Table 1). No significant differences in the timing or severity of clinical disease were observed between nonimmunized and PBS-immunized mice. Collectively, these results indicate that K6-immunization was effective in delaying and attenuating clinical signs of PLP139-151-induced EAE.

Administration of K6-specific antibodies attenuates the development of clinical EAE

The attenuation of clinical disease observed by co- or preimmunization with K6 in the PLP139-151 model of EAE was observed in an identical fashion in mice administered K6-specific monoclonal antibodies 1 day before and every 48 h over the course of development of MOG35-55-induced disease. As detailed in Table 1, in the MOG35-55 model, the day of disease onset (unpaired Student's *t* test, $P<0.001$) and mean clinical score (Mann-Whitney U test, $P<0.002$) were significantly reduced in mice given K6-specific monoclonal antibodies, relative to that observed in immunoglobulin or PBS control groups, while the number of disease free days was significantly increased (Mann-Whitney U test, $P \leq 0.001$). Although more extensive studies are

warranted regarding the mechanism of action of K6-specific monoclonals, the current findings indicate that the K6-specific IgG generated by direct immunization was likely sufficient to account for the disease ameliorating effects observed.

K6-preimmunization reduces the development of histological EAE

Detailed histological examination of inflammation and pathological changes in the spinal cord was made in the K6-preimmunized and control groups of mice. Given the excessive incidence of mortality in the high dose PLP experiments used in the coimmunization experiments, histological examination of CNS tissue was not performed. In K6 preimmunized mice, quantitative evaluation of spinal cord sections showed that reduced clinical deficits were associated with reduced meningeal inflammation and white matter parenchymal pathology when examined either at day 12 or 21 after PLP139-151 priming. A comparison of the percentage of spinal cord quadrants with meningeal inflammation, or frank parenchymal pathology, is shown in Figs. 4*c*, as are representative examples of the extent of lesions observed at day 21 in Fig. 4*b*, *d* and *f*. The mean percentage of spinal cord quadrants with meningeal inflammation/pathology at 12 days post-EAE induction was significantly reduced in the K6-preimmunized mice (37.3%±13.9/15.9%±7.1, n=6), compared with those mice immunized with PBS alone (78.8%±7.3%/40.1±19.9, n=6) and with those receiving no prior immunization (77.6%±17.5/47.5%±10.4, n=6) (unpaired Student's t test, $P \leq 0.05$). A similar reduction in the percent of spinal cord quadrants with meningeal inflammation/pathology was also apparent between K6-preimmunized mice on day 21 post-EAE induction (44.6%±6.3/12.5%±3.7, n=11), compared with those mice immunized with PBS alone (77.1%±4.4/36.6%±5.2, n=13) and with those receiving no prior immunization (76.5%±6.2/47.5%±10.4, n=12) (unpaired Student's t test, $P \leq 0.007$). This was the case, even though clinical deficits were not statistically different between the different groups of mice at the 21 day time point. No significant differences were observed between mice preimmunized with PBS, compared with those receiving no immunization, at any of the time points examined.

Role of K6 in the development of T cell-mediated immunity

PLP139-151-specific DTH reactivity in K6-immunized mice

To further our understanding of the mechanisms by which K6-immunization ameliorates PLP139-151-induced disease, we examined the effects of K6-preimmunization on T cell function, using in vivo and in vitro approaches. In vivo, PLP139-151-induced ear swelling (DTH) was suppressed in K6-immunized mice when initiated on day 9 (Fig. 5*b*) but not on day 18 (not shown), postpriming. The development of DTH was reduced by 2.6-fold in K6-immunized, relative to nonimmunized, or adjuvant control immunized littermates, at the 48 h time point (unpaired Student's t test, $P \leq 0.003$). A significant DTH response was not observed after the injection of OVA peptide in any of the groups, indicating the specificity of the assay. These results suggest that K6-immunization acts in part by inhibiting the effector function of myelin-specific Th1 responses during the acute phase of the disease.

PLP139-151-specific T cell-proliferative responses in K6-immunized mice

To examine the potential effects of K6-immunization on T helper 1 (Th1) cell expansion and/or differentiation, we assessed the level of PLP139-151-specific T cell proliferation in treated mice.

Recall responses of splenocytes isolated from PLP139-151-primed mice were measured by in vitro proliferation assay at the end of each experiment, that is, on day 12 or 21 postpriming upon restimulation in vitro. Splenocytes from all PLP139-151 primed mice (Figs. 4 and 5), proliferated in response to PLP139-151 peptide in a dose-dependent fashion. The proliferative response to PLP139-151, but not the irrelevant antigen OVA peptide, was significantly decreased in splenocytes isolated from mice immunized with K6 (n=14) compared with PBS immunized mice (n=14) or mice receiving no prior immunization (n=14), when examined on day 12 after PLP139-151 priming (Fig. 5*a*, unpaired Student's t test, $P \leq 0.005$). More than 10-fold more PLP-peptide was required to produce equivalent proliferation by splenocytes isolated from K6-immunized mice, compared with each of the control groups. Similar examination on day 21, when clinical scores did not differ between the groups, revealed no significant differences in PLP139-151-induced T cell proliferation between K6-immunized and control mice.

K6-immunization blocks the differentiation of PLP139-151-specific Th1 cells

To determine the effects of anti-K6 treatment on Th1 differentiation, we assessed the ability of PLP-responsive T cells to produce IFN-γ and IL-2 (Fig. 5*c* and *d*). Secondary in vitro stimulation of PLP139-151-specific T cells, derived from the spleens of mice preimmunized with K6 or PBS, or from those receiving no prior immunization, revealed that production of the Th1 cytokine IFN-γ was decreased by threefold in the K6-preimmunized mice (unpaired Student's t test, $P<0.05$), when examined on day 12 postpriming. The level of IFN-γ production by unstimulated cells was not significantly affected, suggesting recovery of similar numbers of activated Th1 cells. Moreover, despite reduced levels of IFN-γ secretion from PLP139-151 stimulated splenocytes, IL-2 secretion was comparable between the different groups. PLP139-151-induced secretion of IL-4 and IL-10 were also assessed, but only minimal levels of secretion of these cytokines were observed in all cultures (data not shown) and this likely reflects the fact that SJL mice are poor Th2 responders (21). These results are consistent with the idea that K6-immunization reduces Th1 responses.

DISCUSSION

The primary goal of this study was to determine whether a newly identified serine protease, K6, which we have shown to be abundantly expressed by inflammatory cells at sites of CNS demyelination in animal models of MS and in human lesions, participates in pathogenesis. Our starting hypothesis was that the enzymatic actions of the "trypsin-like" K6, likely participate in CNS inflammatory disease on multiple levels. EAE is known to be a complex, multistep process that requires activation and differentiation of encephalitogenic Th1 cells in the peripheral immune compartment and their trafficking into the CNS. Within the CNS, activated T cells drive the inflammatory response and activate local and hematogenous macrophages, which mediate demyelination. We provide evidence herein that K6-immunization and the generation of function blocking K6 antibodies during the development of CNS inflammatory disease effectively modulate the peripheral Th1 immune response, suppress immune cell trafficking and down-regulate inflammatory processes in both the peripheral immune compartment and the CNS parenchyma. These findings support the idea that K6 is a pleiotropic enzyme and that inhibiting its activity effectively modulates CNS inflammatory disease at several levels of the inflammatory axis, resulting in disease attenuation.

Regulation of K6 expression

Although K6 expression predominates in the CNS, from our own, and the studies of others, it is known that K6 is also expressed by immune cells (18), including dendritic cells (22), although its potentially important function therein has not been established. We have previously shown that K6 is densely expressed by both CD4 and CD8 T cells, in addition to macrophages, at sites of active CNS demyelination in all animal models of MS examined to date, including MOG35-55-induced EAE in the Marmoset and in the Theiler's murine encephalomyelitis model (17). Here we demonstrate parallel results in murine PLP139-151 induced EAE, firmly establishing the association of K6 with neuroinflammatory disease. However, whether lesional expression represents constitutive basal levels, or a frank up-regulation, had not been previously addressed. In this study, we demonstrate in vitro that activators of immune cell function, such as ConA and CD3-receptor cross-linking, increase K6 mRNA expression within cultured splenocytes. These findings support the concept that K6 is up-regulated in activated immune cells, including those responsible for mediating and amplifying CNS inflammatory disease. Moreover, we have shown that sera K6 levels are elevated by approximately sevenfold at 21 days post-EAE induction. Although considerable future work will be necessary to understand the normal biological activity of K6 in the immune system and to fully delineate the consequences of elevated K6 at the cellular and sera levels, these current data provide additional evidence that regulated expression of K6 is likely to play important functions in activated immune cells and in the progression of EAE-induced disease. In conjunction with our previous reports (17, 18), the present studies suggest that cellular K6 up-regulation may promote the development of Th1 responses, the trafficking of immune cells to the target organ and effector functions therein, including the hydrolysis of extracellular matrix and myelin proteins.

Attenuation of autoimmune CNS disease

We demonstrate that K6-immunization is associated with the generation of K6-function blocking antibodies and that this is correlated with attenuation of clinical and histological EAE in PLP139-151-primed mice. Immunization with K6 at the time of, or before EAE induction, was more or less equally effective and resulted in a significant reduction in the time to disease onset, the time to peak disease, and the severity of clinical disease observed. In addition, we have shown that administration of K6-specific monoclonal antibodies in MOG35-55-primed mice similarly attenuates clinical signs of disease supporting the concept that disease amelioration seen with direct immunization was antibody mediated. However, neither the K6-immunization strategy nor the administration of K6-specific antibodies prevented the eventual development of disease, and clinical scores in the different groups were similar near the 21 day end point of each experiment. Nevertheless, in a total of seven separate experiments, the presence of K6 antibodies was correlated with a significant attenuation of clinical disease. Additionally, in the high dose PLP139-151 experiments, we observed a significant increase in survival of K6-immunized mice. The reduced mortality associated with the presence of K6-function blocking antibodies underscores the potential beneficial effects of K6-inhibition in reducing the progression of disease in mice with PLP139-151-induced EAE.

The inability of the K6-immunization strategy or administration of K6-specific antibodies to sustain permanent reductions in clinical disease may relate to the presence of insufficient K6-antibodies to thwart all K6 activity, to the significant elevations in sera K6 by the 21-day time

point, to the compensation for the activities of K6 by other proteolytic mediators and/or that the effector functions of K6 are most important in the early stages of EAE. We do know that the development of EAE is a complex process, involving not only proteolysis mediated by several enzyme families but also T cell receptor-major histocompatability complex interactions, costimulatory molecules, regulatory T cells, and adhesion molecules, in addition to cytokine and chemokine inflammatory mediators. The present studies do, however, indicate that K6 is likely to play important roles in the development of CNS inflammatory disease and provide the impetus for the identification of more effective K6-inhibitors. It will be important to establish in future studies, whether K6-inhibition after disease onset similarly influences clinical severity, whether more complete K6 inhibition would further inhibit disease progression, and whether K6 inhibition combined with other therapeutics would have an additive effect.

Mechanism of disease attenuation

Immune cell trafficking and invasion

A key event in the development of immune-mediated CNS demyelination is the extravasation of immune cells from the vascular system into the CNS. Immune cells, including lymphocytes and monocytes, can then initiate immune and inflammatory injury within the parenchyma of the brain, which in MS includes destruction of myelin sheaths, oligodendroglia, and ultimately the axons these serve to insulate. A wide variety of studies have demonstrated that treatments that block inflammatory cell extravasation are effective in attenuating disease. This includes blocking immune cell adhesion ligand pairs (23, 24), immune cell depletion (25-28), chemokine and cytokine inhibition (29, 30), and anti-inflammatory drugs (31, 32). Importantly, a recent controlled clinical trial of Natalizumab, which blocks α-4-integrin function, was shown over a 6-month period to significantly decrease the number of inflammatory brain lesions on MRI, and relapses in patients with relapsing MS (33). Together with data presented herein, these studies demonstrate the complexity of the lymphocyte homing process and suggest that combined therapeutic approaches may be valuable.

Several lines of evidence support the possibility that K6-immunization results in the generation of antibodies that block, at least in part, the extravasation of encephalitogenic T cells. First, we have previously demonstrated that components of the blood-brain barrier, that is, laminin, fibronectin, and heat-denatured collagen, serve as K6-substrates (12, 18). Since we demonstrate herein that IgG derived from K6-immunized mice, but not control mice, blocks the enzymatic activity of K6 in vitro, it follows that, in vivo, K6-specific antibodies may diminish K6-mediated breakdown of blood-brain barrier proteins and attenuate immune cell extravasation. This idea is supported by experiments examining the ability of IgG from K6-immunized mice to inhibit the invasion of activated immune cells in a modified Boyden Chamber assay. Compared with control IgG, and to PBS alone, IgG isolated from K6-immunized mice produced an ~20% reduction in immune cell invasion. Together, these findings suggest the efficacy of K6-immunization in ameliorating clinical disease and spinal cord pathology may relate to a K6 antibody-mediated decrease in inflammatory cell CNS invasion.

The idea that proteolytic enzymes mediate the entry of inflammatory cells into the brain is not a new one, and in fact, there is substantial evidence that members of the matrix metalloproteinase (MMP) family integrally participate in this process (34). It has been shown that inhibition of

MMP-9 is effective in ameliorating neurobehavioral signs of EAE (35-38). MMP-9 inhibitors have entered clinical trials for a wide range of diseases, including MS (39). A limiting factor in targeting MMPs, however, is the widespread production of these enzymes in organs and tissues throughout the body. Indeed, inhibition of MMPs can lead to the widespread deposition of ECM components resulting in adverse musculoskeletal side effects (40). In this regard, the preferential expression of K6 in the CNS, and limited expression in peripheral tissues, combined with the effectiveness of K6-inhibition in attenuating clinical and histological disease, make this newly identified enzyme an attractive therapeutic target for CNS inflammatory diseases worthy of further study.

Effector function

We have shown that immunization of mice with recombinant K6 results in the generation of antibodies that dramatically block K6-mediated hydrolysis of complex proteins as well as small peptide substrates in vitro. Since it is known that antibodies readily enter the CNS in the EAE model (41), we anticipate that autonomously generated K6 antibodies block K6-mediated proteolysis within the CNS and that this contributes to the amelioration of disease observed. Our previous studies suggest that excess K6 within the CNS parenchyma, as a result of the presence of K6 expressing inflammatory cells, likely has a wide range of degradative activities, which we have shown include the breakdown of myelin proteins. Additionally, we have demonstrated in vitro that excess K6, promotes a dying back of oligodendroglial processes (17). Thus, disease attenuation in K6-immunized mice may in part be related to the ability of K6-antibodies to inhibit the effects of excess K6 within the parenchyma of the brain. Supporting this, we demonstrate that IgG from K6-immunized mice, but not controls, effectively blocks the hydrolysis of MBP in vitro. These findings further support the hypothesis that K6-inhibition attenuates CNS inflammatory demyelinating disease on multiple levels.

Th1 differentiation

Cytokines play a pivotal role in the regulation of tissue-specific autoimmune injury. Th1 cells, and the proinflammatory cytokines they produce, are thought to be important in both the initiation and perpetuation of in EAE (42-47). The results of the present study suggest that the disease-attenuating effects of K6-immunization may be in part the result of an inhibition in the activation, expansion, and/or differentiation of Th1 cells specific for the disease initiating peptide during the acute phase of the disease. To evaluate the generation of Th1 cells in vivo, we measured DTH responsiveness to the priming antigen (48). PLP139-151-induced DTH responses were reduced in K6-immunized mice, suggesting a role for K6 in T cell activation.

Paralleling decreased Th1 responses in vivo, secondary in vitro stimulation of PLP139-151-specific T cells, derived from the spleens of K6-immunied mice during the acute phase of disease, demonstrated significant reductions in the level of secretion of the Th1 cytokine IFN-γ. Decreases in IFN-γ secretion were not seen in unstimulated cells, and secretion of the Th0 cytokine, IL-2 was not significantly affected. These findings indicate that equal numbers of PLP139-151 specific T cells were present in the splenic compartment of K6-immunized and control groups and argue against generalized immunosuppressive effects of K6-immunization.

Further evidence concerning the potential immunoregulatory activity of autonomously generated K6 antibodies comes from our examination of PLP139-151-specific T cell proliferation. The results of thymidine incorporation in splenocytes derived from PLP139-151 primed mice during the acute phase of the disease, as a measure of T cell expansion, showed that K6-immunization was associated with an inhibition in PLP-specific proliferation. These data suggest that anti-K6 antibodies may partially interfere with the clonal expansion of PLP139-151-specific T cells. In vivo, this may contribute to the reductions in meningeal and parenchymal inflammation observed in K6-immunized mice. Combined with measures of DTH response and Th1 cytokine secretion, these observations raise important questions concerning the potential activity of K6 in the development of antigen specific Th1 cell responses. These findings suggest that the ability of K6-immunization to moderate disease may have been due in part to its effects in attenuating Th1 differentiation. Additional experiments examining the effect of K6 inhibition on the differentiation of T cells in vitro will be required to increase our understanding of the role of K6 in the development of encephalitogenic T cells.

CONCLUSION

This study provides the first direct in vivo and in vitro evidence, demonstrating the involvement of a newly identified serine protease, K6, in the development of CNS inflammatory disease. The data presented support several possible convergent mechanisms by which K6-mediated proteolytic cascades participate in CNS inflammatory-mediated pathology, including activities in both the priming and effector phases. The present studies firmly place K6 along-side other serine proteases and MMPs as a key effector molecule in the development of CNS-inflammatory disease. This work raises numerous questions for future investigation and underscores the need to consider K6 as a therapeutic target for inflammatory disorders, including MS.

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Table 1

Clinical disease in mice immunized with K6 or receiving K6-specific antibodies

	Incidence	Survival	Day of Onset	Disease Free Days	Mean Clinical Score	Day of Peak
Coimmunizaiton (PLP139-151)						
EAE control *n*=28	28/28	10/28	10.1 ± 0.4	9.1 ± 0.4	2.9 ± 0.2	13.1 ± 0.5
EAE with K6 coimmunization *n*=28	28/28	22/28 *P*=0.003[a]	12.7 ± 0.5 *P*=0.006[b]	11.7 ± 0.5 *P*≤0.001[b]	1.8 ± 0.2 *P*<0.01[c]	14.7 ± 0.5 *P*<0.04[b]
Preimmunization (PLP139-151)						
EAE control *n*=14	14/14	12/14	10.8 ± 0.2	9.6 ± 0.1	2.1 ± 0.2	13.7 ± 0.5
EAE Ribi-PBS preimmunization *n*=15	15/15	13/15	10.5 ± 0.1	9.5 ± 0.2	2.1 ± 0.2	14.7 ± 0.7
EAE with Ribi-K6 preimmunization *n*=13	12/13	11/13	14.3 ± 0.8 *P*<0.001[c]	13.0 ± 0.8 *P*≤0.001[b]	1.4 ± 0.2 *P*<0.005[c]	16.9 ± 0.6 *P*<0.02[c]
K6 antibody administration (MOG35-55)						
PBS *n*=23	23/23	16/23	9.7± 0.5	9.2 ± 0.6	1.7 ± 0.2	15.3 ± 0.7
L368 Ab *n*=23	23/23	20/23	9.6 ± 0.4	9.4 ± 0.3	1.5 ± 0.2	15.0 ± 0.5
K6 Ab *n*=24	20/23	19/24	13.4 ± 0.6 *P*<0.001[b]	14.1 ± 0.8 *P*≤0.001[c]	0.99 ± 0.2 *P*<0.002[c]	16.5 ± 0.8

K6-immunization or administration of K6-specific antibodies significantly attenuated development of clinical signs of EAE. K6-immunization at time of, or before EAE induction, each delayed the mean day of disease onset, the day of peak disease, and the mean daily clinical score observed, while increasing the number of days free of clinical signs in PLP-139-151-primed mice. The high dose EAE protocol used in the K6-coimmunization experiments resulted in significant mortality. In these experiments, survival to 21 day end point was significantly increased in K6-coimmunized mice compared with controls. Paralleling these findings, MOG35-55 primed mice, administered a cocktail of 2 K6-specific monoclonal antibodies, also exhibited a significant delay in onset of clinical signs, a reduction in mean clinical score, and an increase in the number of days free of disease ([a]X^2; [b]unpaired Student's *t* test; [c]Mann-Whitney *U* test).

Fig. 1

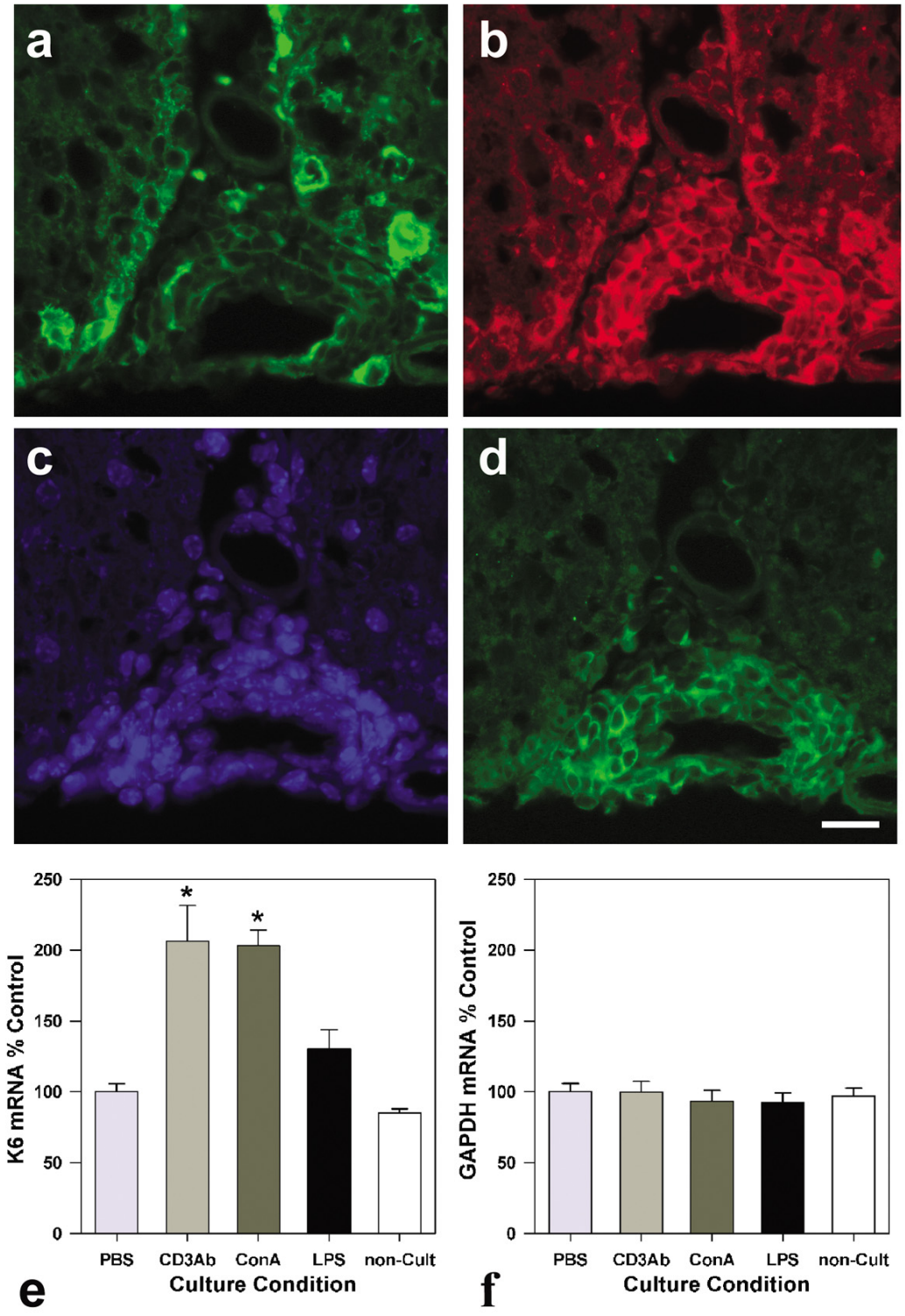

Figure 1. K6 expression by inflammatory cells in PLP139-151 induced EAE and regulation by activation. Immunohistochemical colocalization of K6 (***b***) in isolectin B_4-positive macrophages (***a***) and CD3-positive T cells (***d***). In ***c***, all cells present were visualized with the nuclear stain bisbenzamide. ***e)*** Histograms show mean percent change (±SE) in level of K6 mRNA detected in cultured splenocytes after 72 h exposure to ConA, LPS, or CD3 antibody relative to cultures exposed to PBS alone (*unpaired Student's *t* test, $P<0.006$, $n=4$). Relative level of K6 mRNA in noncultured cells (noncult) and level of GAPDH in all samples (***f***) were also assessed. Scale bar = 25 μm.

Fig. 2

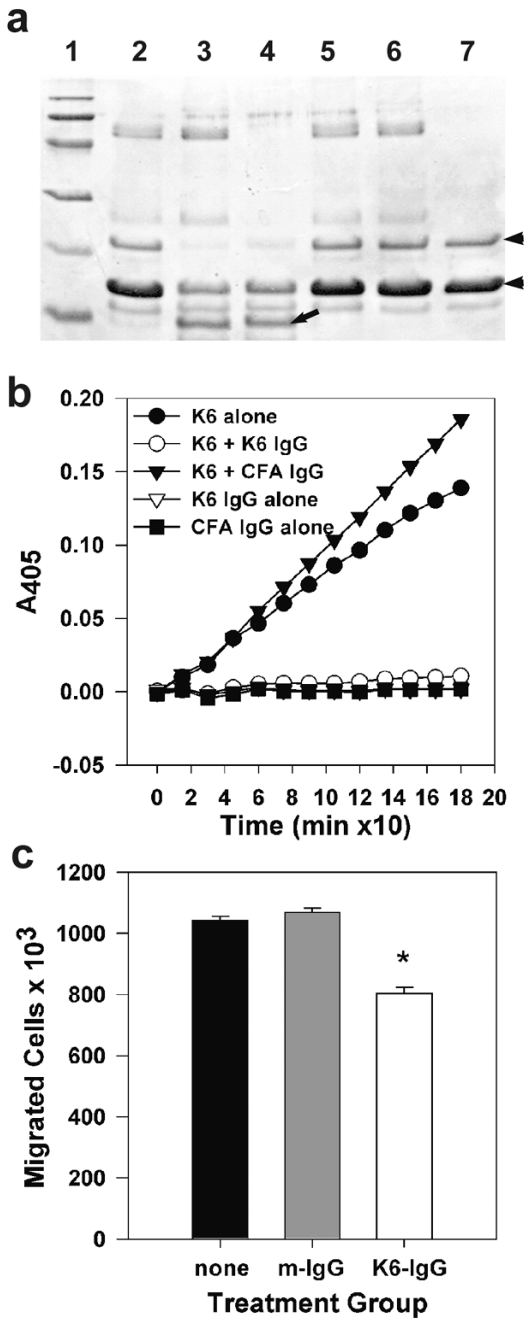

Figure 2. Immunoglobulin isolated from K6-immunized mice blocks K6-enzymatic activity and immune cell invasion in vitro. ***a*****)** 20% Tricine SDS-PAGE showing hydrolysis of rat MBP in the presence or absence of IgG isolated from K6-immunized mice or controls. Lanes: 1) molecular mass markers; 2) K6 IgG + K6 + MBP; 3) CFA IgG + K6 + MBP; 4) K6 + MBP; 5) K6 IgG + MBP; 6) CFA IgG + MBP; 7) MBP alone (2 major bands shown at arrowheads). Incubation of MBP with K6 for 3 h resulted hydrolysis (lane 4, see fragment at arrow). Preincubation of K6 with IgG isolated from K6-immunized mice effectively blocked K6-mediated hydrolysis of MBP (lane 2), whereas IgG isolated from animals immunized with CFA alone (lane 3) did not. ***b*****)** Rate of AcATRpNA-substrate hydrolysis over time by K6 in the presence of IgG isolated from K6-immunized mice or controls. The presence of K6 IgG reduced rate of substrate hydrolysis by 92.3%, that is, to a level similar to that seen in the absence of any added K6. ***c*****)** Activated splenocytes were labeled with calcein AM and cultured the in upper well of a Boyden Chamber with or without the addition of control or K6 IgG. Measurements of fluorescence in lower chamber after a period of 24 h indicated that addition of K6-IgG inhibited invasion by ~20%, compared with addition of normal mouse IgG (m-IgG) or no antibody (none) (*unpaired Student's *t* test, $P \leq 0.005$). Data are means ± SE of triplicate wells from an individual experiment, but similar results were obtained from 3 independent experiments.

Fig. 3

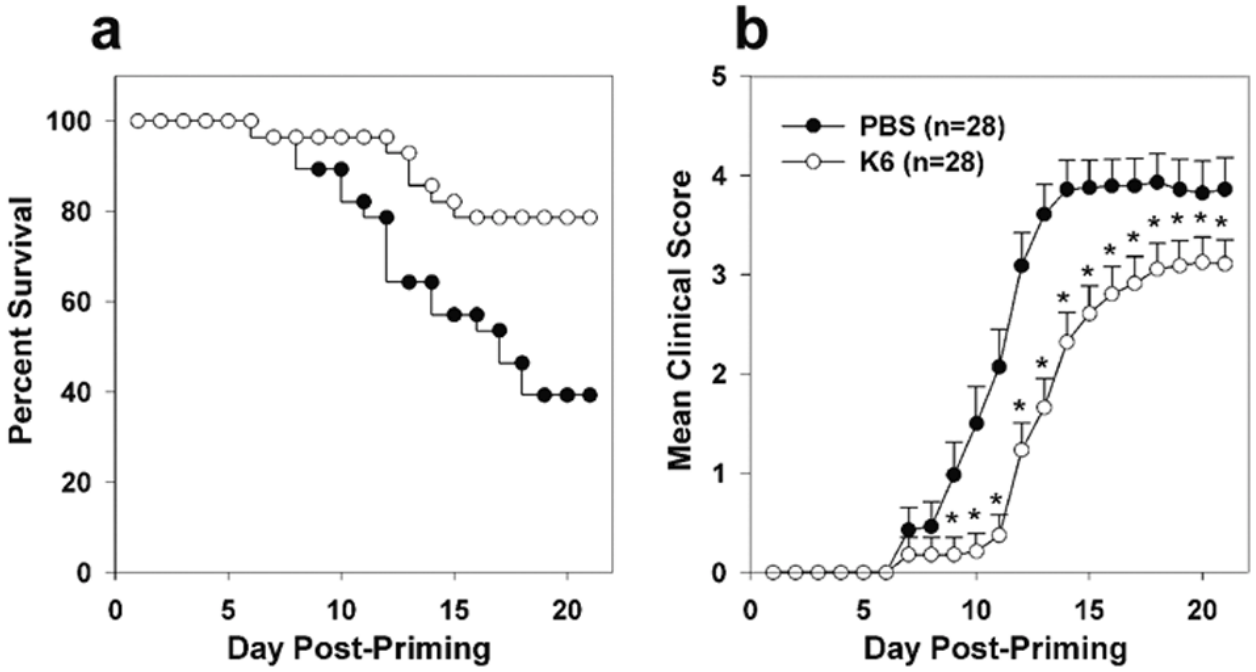

Figure 3. Immunization with K6 at time of PLP139-151 EAE induction delays onset and attenuates severity of disease. Mice were immunized with PBS or K6 at the time of priming with 100 μg of PLP139-151 in CFA. ***a*)** K6 immunization at time of priming was associated with a significant increase in survival (22/28 compared with 10/28, P=0.003 X^2 using Fisher's exact test, symbols as in ***b***). ***b*)** Data are mean clinical score (±SE) of each group plotted against time. Clinical scores of mice immunized with K6 were significantly below those of PBS-immunized mice from D9 throughout the remainder of disease course examined (*P<0.01, Mann-Whitney U test).

Fig. 4

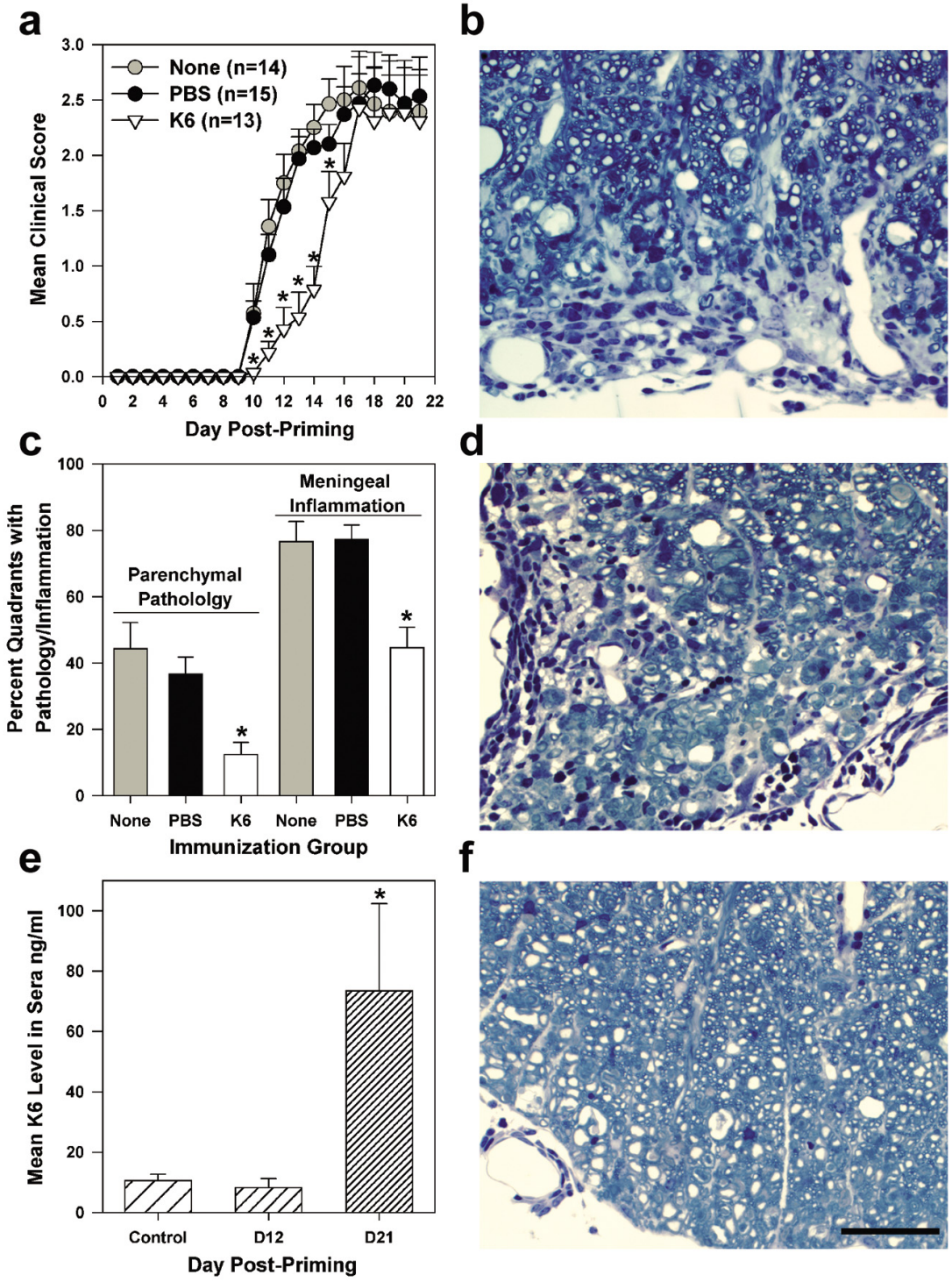

Figure 4. Preimmunization with K6 delays onset and severity of clinical and histological disease in PLP139-151 primed mice. ***a***) Mean day of onset, and time to peak disease, were delayed by 3 to 4 days, in K6-immunized mice, compared with nonimmunized, and PBS-immunized controls ($P<0.02$). K6-immunized mice also exhibited significantly reduced mean daily clinical scores up to day 15 post-PLP priming (*$P<0.005$ Mann-Whitney U test). ***c***) Detailed histological evaluation of the spinal cord at the 21 day end point indicated that K6-preimmunization (***f***) was associated with a reduction in both of the extent of parenchymal pathology and meningeal inflammation, compared with mice immunized with PBS (***d***) or with those receiving no prior immunization (***b***; *unpaired Student's t test, $P\leq0.007$). ***e***) Capture ELISA was used to demonstrate that K6 levels in sera are elevated by sevenfold when examined at 21-days post-PLP139-151 priming, relative to the levels observed at 12-days and to level in nonprimed control mice (*Mann-Whitney rank sum test, $P<0.008$). Data are means ± SE. Scale bar = 100 µm.

Fig. 5

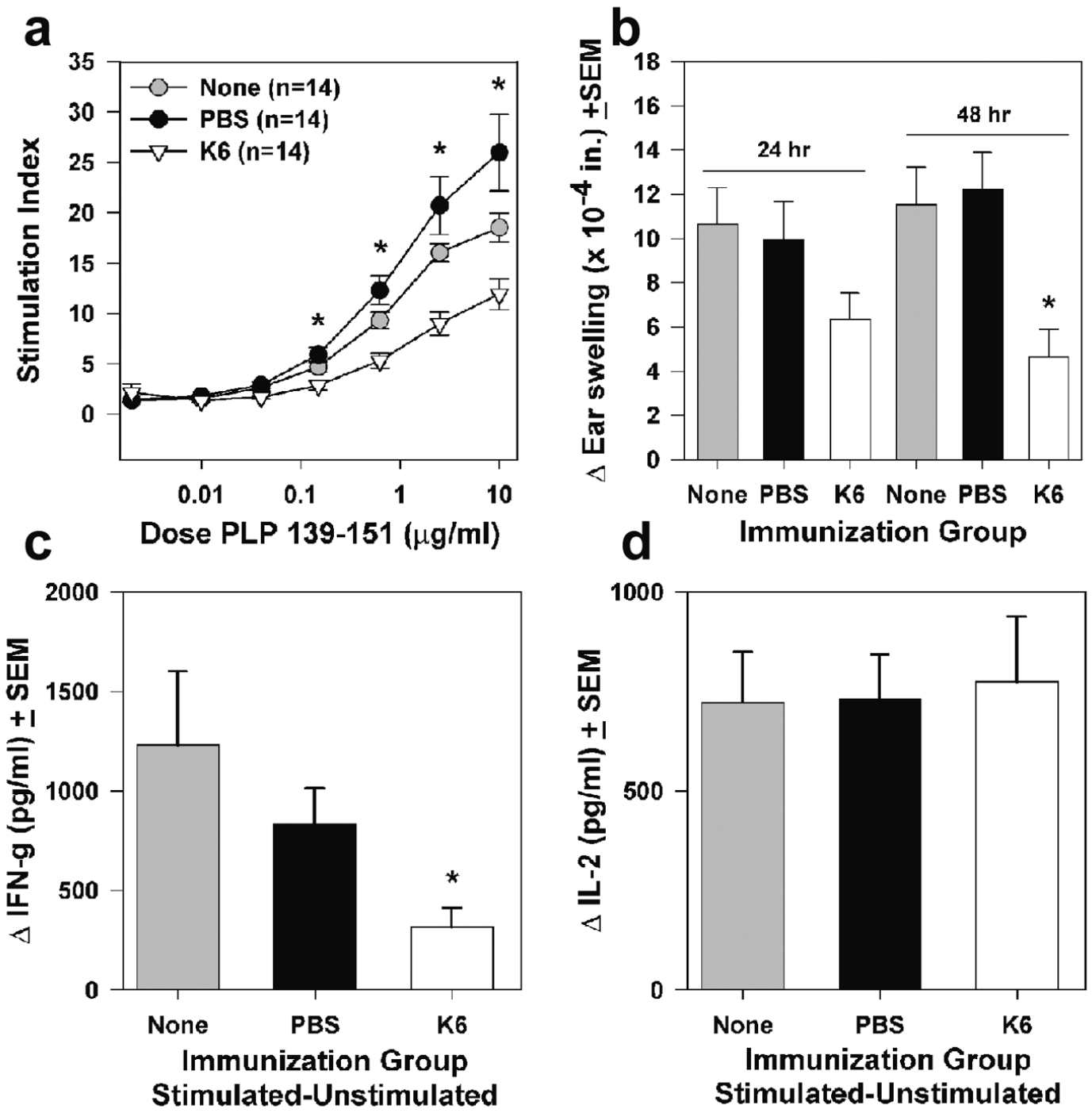

Figure 5. ***a***) PLP139-151-specific proliferation of splenocytes was reduced in K6-preimmunized mice, relative to controls when examined 12-days postpriming (*unpaired Student's *t* test, P≤0.005). ***b***) DTH responses (ear thickness ± SE, to challenge with 10 μg of the disease initiating peptide, at day 9 after priming. Significantly less swelling was observed in K6-immunized mice relative to controls at 48 h postchallenge (*unpaired Student's *t* test, *P*≤0.003). PLP139-151 induced IFN-γ (***c***) secretion was significantly reduced in K6-immunized mice relative to controls (*unpaired Student's *t* test, *P*<0.05), whereas IL-2 (***d***) secretion was unchanged. Data are means ± SE. Severity of clinical disease and number of spinal cord quadrants associated with pathology or meningeal inflammation were also examined in groups of mice used for experiments shown in ***a-d***. As observed on day 21 (**Fig. 4**), both clinical (Mann Whitney U, P<0.05) and histological (unpaired Student's *t* test, *P*≤0.05) signs of EAE were significantly reduced in K6-preimmunized mice, relative to controls, when examined at the 12 day time point.