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Kallikrein-related peptidase 6 exacerbates disease in an autoimmune model of multiple sclerosis

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Abstract

Kallikrein-related peptidase 6 (Klk6) is elevated in the serum of multiple sclerosis (MS) patients and is hypothesized to participate in inflammatory and neuropathogenic aspects of the disease. To test this hypothesis, we investigated the impact of systemic administration of recombinant Klk6 on the development and progression of MOG35-55-induced experimental autoimmune encephalomyelitis (EAE). First, we determined that Klk6 expression is elevated in the spinal cord of mice with EAE at the peak of clinical disease and in immune cells upon priming with the disease-initiating peptide *in vitro*. Systemic administration of recombinant Klk6 to mice during the priming phase of disease resulted in an exacerbation of clinical symptoms, including earlier onset of disease and higher levels of spinal cord inflammation and pathology. Treatment of MOG35-55-primed immune cells with Klk6 in culture enhanced expression of proinflammatory cytokines, interferon-γ, tumor necrosis factor, and interleukin-17, while reducing anti-inflammatory cytokines interleukin-4 and interleukin-5. Together these findings provide evidence that elevations in systemic Klk6 can bias the immune system towards pro-inflammatory responses capable of exacerbating the development of neuroinflammation and paralytic neurological deficits. We suggest that Klk6 represents an important target for conditions in which pro-inflammatory responses play a critical role in disease development, including MS.

Keywords

cytokine; experimental autoimmune encephalomyelitis; neuroinflammation; paralysis; serine protease; T cell

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Introduction

Kallikrein-related peptidase 6 (Klk6) is a secreted ‘trypsin-like’ serine protease (Scarisbrick et al., 2012a,b; Scarisbrick and Blaber, 2012) that has been implicated in the pathogenesis of multiple sclerosis (MS) (Scarisbrick et al., 1997, 2000, 2001, 2002, 2006a,b, 2008, 2011, 2012a,b; Blaber et al., 2002, 2004; Schutzer et al., 2013; Singh et al., 2015). Multiple sclerosis is an inflammatory demyelinating disease affecting the brain and spinal cord and is a common cause of neurological disability in young adults. While the cause of MS is not known, both genetic and environmental factors have been implicated (Nylander and Hafler, 2012; Ransohoff et al., 2015). Unfortunately, there are no treatments effective in preventing the long-term progression of the disease and the development of permanent neurological deficits that many patients experience.

There is therefore an essential need to continue to identify mediators of pathogenesis with the intention that these may serve as key biological intersections for intervention to slow or block progression and foster repair and regenerative therapies. There is a growing body of evidence pointing to a pathogenic role for elevated KLK6 activity in multiple sclerosis (upper case denotes human form), although the full mechanism of action is not yet clear. The cellular mediators of MS are multifactorial, and include T cells, B cells and mononuclear cells, in addition to microglia and astrocytes resident to the CNS (Bar-Or, 2016). There is already evidence that KLK6 is altered in each of these cell types in MS lesions and in animal models of the disease (Panos et al., 2014). For example, KLK6 is up-regulated in active MS lesions (Scarisbrick et al., 2002), and in both serum (Scarisbrick et al., 2008), and cerebrospinal fluid (CSF) (Hebb et al., 2011; Schutzer et al., 2013; Singh et al., 2015) of patients with the disease. Further implicating Klk6 in key facets that drive demyelinating disease are studies demonstrating its elevated expression in activated T cells, as well as those stimulated by glucocorticoids, androgens or progesterone (Scarisbrick et al., 2002, 2006a,b; Blaber et al., 2004; Christophi et al., 2004). Klk6 also potently promotes the survival of both T and B cells, including the up-regulation of cellular survival protein BCL-XL and inhibition of those that promote apoptosis, such as BIM (Scarisbrick et al., 2011). Like T and B cells, monocytes also play key roles in the pathogenesis of MS (Scarisbrick et al., 2002; Blaber et al., 2004). Of potential importance, Klk6 is also present in macrophages/microglia in MS lesions and murine models of the disease (Scarisbrick et al., 2002; Blaber et al., 2004). Activation of THP-1 monocytes by PMA is also paralleled by increases in KLK6 (Panos et al., 2014). Activated astrocytes and microglia also up-regulate Klk6 (Scarisbrick et al., 2002, 2012a,b; Christophi et al., 2004; Radulovic et al., 2015, 2016). Much remains to be learned regarding the role of KLK6 in inflammatory pathogenesis, including that which mediates disease in MS.

Perhaps the most convincing evidence to date regarding the link between Klk6 and neuroinflammation are studies demonstrating that Klk6 neutralizing antibodies diminish clinical, histological and immunological signs of disease in autoimmune (Blaber et al., 2004) and viral (Scarisbrick et al., 2012a,b) murine models of MS, including reductions in pro-inflammatory responses (Blaber et al., 2004). Klk6 is also capable of directly impacting neural function. In excess, Klk6 contributes to oligoden-drogliopathy (Scarisbrick et al., 2002; Burda et al., 2013) and to neuron and axon injury (Scarisbrick et al., 2008; Radulovic

et al., 2013; Yoon et al., 2013). Of particular significance to MS, Klk6 rapidly hydrolyzes myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) as well as laminin, fibronectin and heat-denatured collagen, key components of the blood-brain barrier (Bernett et al., 2002; Blaber et al., 2002, 2004; Scarisbrick et al., 2002). Taken together, these data support the hypothesis that Klk6 is a key player in multiple aspects of the 'MS degradome' (Scarisbrick, 2008) and may therefore serve as a useful therapeutic target (Scarisbrick et al., 2002, 2011, 2012a,b; Burda et al., 2013; Yoon et al., 2013, 2015).

In this study, we further evaluate the hypothesis that Klk6 directly participates in the development of MS by determining the effects of administering Klk6 systemically during the priming phase of myelin oligodendrocyte glycoprotein (MOG)35-55 induced experimental autoimmune encephalomyelitis (EAE). EAE is a T cell-mediated autoimmune disease that models several key aspects of MS pathogenesis, including perivascular $CD4^+$ T cell and mononuclear cell inflammation, demyelination, axon injury and progressive hind limb paralysis (Miller et al., 2010). Our results suggest that systemic increases in Klk6 during the early stages of disease pathogenesis have the potential to result in an earlier onset of disease and to exacerbate functional decline and CNS pathology in part by skewing the Th1, Th17 and Th2 inflammatory responses towards a pro-inflammatory profile. These findings shed additional light on the pathophysiological roles of Klk6 in the development and progression of neuroinflammatory disease and further highlight this enzyme as an important target for the development of new therapies for the treatment of neuroinflammatory disorders, such as MS.

Results

Elevations in Klk6 in the spinal cord of mice with MOG35-55 EAE occur with the peak of clinical disease

To investigate the potential for Klk6 to drive neuroinflammatory disease in the MOG35-55 EAE model we quantified the expression of Klk6 protein and RNA in the spinal cord of mice with MOG35-55-induced EAE from acute through chronic phases of disease (Figure 1). The peak of Klk6 expression occurred 21 days after MOG35-55 priming, coincident with the peak of clinical paralytic disease. By 21 days post-priming, Klk6 protein levels were 2.5-fold higher than base line. A second 2-fold increase in Klk6 protein expression over baseline occurred at 45 days post-disease induction [$p = 0.04$, Newman Keuls (NK)]. In parallel, Klk6 RNA was elevated 1.4–1.6 fold in the spinal cord at 12 and 21 days after MOG35-55 priming ($p = 0.03$, NK). These findings suggest that elevated expression of Klk6 may have a role in spinal cord pathogenesis associated with clinical disease in the MOG35-55 model.

The disease-initiating antigen promotes Klk6 expression in mixed splenocyte cultures

To begin to address the potential for Klk6 to be involved in the development and progression of EAE at the level of immune cells, we examined changes in Klk6 protein expression in cultures of MOG35-55-primed splenocytes (Figure 2). MOG35-55 stimulation of splenocyte cultures derived from mice at 21 days after MOG35-55 immunization resulted in 6.5-fold increases in a Klk6-specific band at 28 kDa ($p = 0.004$, NK). Notably the increase in MOG35-55-stimulated Klk6 production was even greater at 30 days after disease induction,

when a 9.7-fold increase over baseline was observed ($p = 0.005$, NK). This additional increase in Klk6 in splenocytes derived from mice at 30 days after MOG35-55 immunization may be accounted for, in part, by the presence of both the 28 kDa band, and an additional 25 kDa Klk6-specific band. Although more investigation will be needed to determine the significance of these unique Klk6 bands, it is possible that the 25 kDa band represents additional active protease, or its zymogen precursor, based on the expected molecular weight of the enzyme (Bernett et al., 2002; Blaber et al., 2002; Scarisbrick et al., 2012a,b). Based on descriptions by others, the 28 kDa band may be a differentially glycosylated form of Klk6 (Kuzmanov et al., 2009; Guo et al., 2014), but additional efforts will be needed to clarify this and its functional significance. As the pro-peptide for Klk6 contains only five additional amino acids that would add approximately 500 Da to its molecular weight, it is not possible to distinguish between active Klk6 and its zymogen precursor using Western blot alone. Together, findings presented show that Klk6 is up-regulated in antigen activated inflammatory cells positioning it to participate in neuroinflammatory disease pathogenesis.

Neurobehavioral deficits in EAE are exacerbated by Klk6

To test the hypothesis that increases in systemic Klk6 participate in the development and progression of neuroinflammatory disease, we investigated whether administration of exogenous recombinant Klk6 on days 4, 6, 8 and 11 after MOG35-55 immunization would impact the onset of disease, or its clinical course (Figure 3). Consistent with the hypothesis that elevations in Klk6 can exacerbate neuroinflammatory disease, we observed an earlier onset of clinical signs of disease in mice administered Klk6 relative to those administered PBS alone. In Klk6-treated mice, significant increases in clinical deficits were first observed on day 6 after MOG35-55 priming ($p = 0.03$), a full 5 days earlier than that observed in PBS-treated mice who experienced the first clinical signs of disease on day 11 ($p = 0.002$, Mann-Whitney U test).

Klk6-treated mice also experienced higher clinical disease scores relative to PBS-treated mice from day 6 to 12, day 17 to 19, and day 21 to 23 ($p \leq 0.05$, Mann-Whitney U test). An exacerbation of clinical signs of disease was also observed in Klk6-treated mice on days 34 and 35, and days 38 and 39 after priming, during the period of relative disease remission in vehicle-treated mice. After day 49, when clinical signs were again increased in Klk6-treated mice ($p = 0.04$, Mann-Whitney U test), no statistically significant differences were observed across groups through the 90 day endpoint examined. Quantitative analysis of histological signs of inflammation and spinal cord pathology (vacuolation) at the 90 day endpoint demonstrated significantly higher levels of pathology in those mice that had been treated with Klk6 compared to those treated with PBS alone ($^*p < 0.05$, Student t-test). These findings support the hypothesis that elevations in systemic Klk6 can exacerbate clinical and pathological signs of neuroinflammatory disease.

Klk6 promotes pro-inflammatory and inhibits anti-inflammatory immune responses

To determine whether Klk6 may alter the production of cytokines through direct effects on immune cells, we examined the impact of the addition of recombinant Klk6 (200 nm) to MOG35-55-primed splenocyte cultures (Figure 4). Kallikrein 6 enhanced expression of RNA encoding the Th1 cytokines IFNγ and TNF, as well as the Th17 cytokine, IL-17 by

1.4-fold over that seen with treatment with MOG35-55 peptide alone ($p < 0.05$, NK). Klk6 also reduced the production of Th2 cytokine RNA, IL-4 and IL-5, by 1.3–1.5-fold compared to treatment with MOG35-55 alone ($p < 0.05$, NK). These results suggest that Klk6 can modulate the inflammatory profile of immune cells.

Discussion

Kallikrein 6 is elevated in active MS lesions (Scarisbrick et al., 2002) and in MS patient serum (Scarisbrick et al., 2008), and CSF (Hebb et al., 2011; Schutzer et al., 2013; Singh et al., 2015). Furthermore, antibodies that block the activity of this secreted trypsin-like serine protease attenuate disease in autoimmune (Blaber et al., 2004) and viral (Scarisbrick et al., 2012a,b) experimental models of MS. Here we provide additional evidence that Klk6 can promote the development and progression of neuroinflammatory disease by demonstrating that systemic administration of Klk6 to mice during the priming phase of MOG35-55 EAE accelerates the onset of disease and exacerbates both clinical and pathological signs of disease in the central nervous system. Additional evidence is provided that Klk6 may exert these effects in part at the level of immune cells, as Klk6 is elevated in cultures of MOG35-55-primed immune cells and drives cytokine expression towards a pro-inflammatory profile. Taken together, these studies indicate that Klk6 is strategically positioned to be a mediator of immune function and the pathobiology of neuroinflammatory conditions, such as multiple sclerosis.

The greatest increases in Klk6 expression within the spinal cord during MOG35-55-induced EAE were detected at the peak of clinical disease (day 21), pointing to an essential link with inflammatory pathogenesis. Klk6 RNA was also elevated in the spinal cord during the priming phases of disease, further indicative of a key role in immune-mediated aspects of EAE development. In prior studies we demonstrated that Klk6 is expressed by both CD4 and CD8 T cells, in addition to the monocyte and microglial populations at sites of inflammation and demyelination in the proteolipid protein (PLP)139-151 model of EAE (Blaber et al., 2004), and in the Theilers murine encephalomyelitis virus (TMEV) model of inflammatory demyelinating disease (Blaber et al., 2002; Scarisbrick et al., 2002, 2012a,b; Panos et al., 2014). Centering in on the clinical significance of these observations, KLK6 is expressed by immune cells at sites of active demyelination in MS lesions and in the MOG-induced EAE model in marmosets (*Callithrix jacchus*) (Scarisbrick et al., 2002). The expression of Klk6 in immune cells in MS and across multiple experimental models examined to date point to likely roles in immune function that we are only beginning to discover (Scarisbrick et al., 2002, 2011, 2012a,b; Blaber et al., 2004; Radulovic et al., 2015, 2016). Current evidence suggests that Klk6 can exert its effects both by degrading extracellular matrix proteins (Bernett et al., 2002; Blaber et al., 2002, 2004; Scarisbrick et al., 2006a,b, 2012a,b) and by directing cell signaling events downstream of cleavage and activation of protease activated receptor (PAR) 1 or PAR2 (Angelo et al., 2006; Oikonomopoulou et al., 2006; Vandell et al., 2008; Scarisbrick et al., 2011; Burda et al., 2013; Yoon et al., 2013; Radulovic et al., 2015, 2016).

Klk6 remained elevated during the progressive phases of EAE, with a second peak at 45 days post disease induction. Therefore, Klk6 is positioned to exert its effects not only during

disease development, but also during disease progression that can include active and smoldering inflammation, demyelination, and axon injury. We note that prior studies showed Klk6 is elevated in the serum of MS patients experiencing progressive disease. Moreover, elevations in Klk6 at the time of serum draw correlated with future worsening of Expanded Disability Status Scale scores in relapsing-remitting patients (Scarisbrick et al., 2008). More recently, Klk6 was shown to be elevated in the CSF of MS patients (Hebb et al., 2011; Schutzer et al., 2013; Singh et al., 2015). For example, a 2.8-fold increase in Klk6 was observed in MS patient CSF with the first attack (Singh et al., 2015).

In this study, we directly tested whether systemic elevations in Klk6 alter the development and progression of neuroinflammatory disease. We observed an earlier onset of disease and greater clinical and neuropathological signs of disease in mice administered Klk6 during the priming phase of EAE. These studies demonstrate for the first time that elevations in systemic Klk6 can promote neuroinflammation. Additional efforts will be needed to determine whether the course of EAE would also be exacerbated with Klk6 administration during the encephalitogenic phases of disease, the impact of intracerebroventricular Klk6 administration, and any dose-dependent effects. Taken with prior evidence demonstrating that Klk6 neutralizing antibodies attenuate disease in autoimmune and viral murine models of MS (Blaber et al., 2004; Scarisbrick et al., 2012a,b), the current findings underscore the need for additional efforts to determine whether the activity Klk6 represents a new target to decrease relapses or slow disease progression in MS patients.

As administration of Klk6 to mice during the development of MOG35-55-induced EAE exacerbated the disease course, we sought to determine the potential mechanism of action. While the disease initiating event(s) in MS are not fully understood, these events are believed to activate helper T cells that recognize components of myelin. Activated helper T cells clonally expand and release cytokines that drive the chronic inflammatory response, facilitate CNS extravasation, and in turn activate B cells, cytotoxic T cells, and macrophages/microglia, all contributing to myelin, oligodendrocyte and axon injury, and the chronic evolution of the MS plaque (Bar-Or, 2016). To test the hypothesis that the disease exacerbating effects of Klk6 occur in part as a result of direct effects at the level of immune cells, we investigated whether Klk6 treatment of MOG35-55-primed splenocyte cultures would alter the production of cytokines capable of modulating the course of EAE. First, Klk6 was elevated in an antigen-specific fashion in MOG35-55-primed immune cells. This finding parallels prior studies demonstrating viral peptide-specific increases in Klk6 expression in a viral model of MS (Scarisbrick et al., 2012a,b). Th1 cytokines such as IFNγ and TNF are known to participate in the development and progression of EAE (Ferber et al., 1996; Kruglov et al., 2011) and each was elevated by the addition of Klk6 to the culture media of MOG35-55-primed immune cells. IL-17 is another pro-inflammatory cytokine established to promote the development of EAE (Komiyama et al., 2006) and this was also elevated in MOG35-55-primed splenocyte cultures after treatment with Klk6. The potential actions of Klk6 at the level of systemic immune cells in driving disease is further supported by observations that Th2 cytokines known to be elevated with the remission of clinical signs in EAE (Liblau et al., 1997), including IL-4 and IL-5, were decreased by Klk6 in the same immune cell culture samples. Together these findings suggest that Klk6 has the potential to

direct immune cell phenotype, favoring a pro-inflammatory state while suppressing anti-inflammatory activities.

As our prior studies demonstrate that Klk6 is elevated in the serum of MS patients (Scarisbrick et al., 2008), we suggest that this unique secreted serine protease may play a direct role in driving pro-inflammatory responses in patients. As the actions of Klk6 appear to include activities in the immune system as well as in the nervous system, it may be an ideal candidate for complex disorders such as MS, which have both inflammatory and neurodegenerative components. For example, the ability of Klk6 to promote antigen-specific pro-inflammatory responses demonstrated here, when taken with prior studies demonstrating its ability to promote immune cell survival (Scarisbrick et al., 2011), suggest elevations in Klk6 activity can drive inflammation by multiple mechanisms. Thus in MS CNS lesions and in MS patient sera where elevations in KLK6 have been documented (Scarisbrick et al., 2002, 2008), the protease may serve to prolong pro-inflammatory responses. Moreover, Klk6 has demonstrated biological effects on other cell types that play critical roles in the development and progression of the MS lesion. For example, Klk6 is a powerful mediator of astrogliosis, including IL-6 secretion (Scarisbrick et al., 2012a,b; Radulovic et al., 2015, 2016). Klk6 may also mediate neurotoxicity directly and exacerbate glutamate-induced excitotoxicity (Radulovic et al., 2013; Yoon et al., 2013), leaving it well positioned to help set the stage for chronic progressive inflammatory pathogenesis in the brain and spinal cord. Taken with reports that Klk6 also promotes oligodendrogliopathy and can readily cleave myelin proteins such as MBP (Bernett et al., 2002; Blaber et al., 2002; Scarisbrick et al., 2002; Burda et al., 2013), it appears that Klk6 is capable of being a multifunctional driver of MS pathogenesis. Additional efforts are needed to determine whether the activity of Klk6 can be targeted in MS patients to interrupt neuroinflammatory and neurodegenerative disease mechanisms.

Materials and methods

MOG35-55 experimental autoimmune encephalomyelitis model

The goal of these studies was to determine the effect of excess exogenous Klk6 administered during the priming phase of EAE on clinical outcome and whether a direct effect on immune function may play a role. To accomplish this we chose to study the MOG35-55 model of EAE in C57BL/6 (H-2^b) mice, which is one of the most commonly studied murine models of MS, mimicking both the inflammatory and the chronic progressive stages of disease pathogenesis (Miller et al., 2010; McCarthy et al., 2012). Active EAE is induced by immunization with myelin or myelin-specific peptides. MOG35-55 was used in the current study as this is the immunodominant peptide in C57BL/6 mice. The development of active EAE consists of both a priming (induction) phase and an encephalitogenic (effector) phase (Miller et al., 2010). The induction phase involves priming of MOG35-55-specific $CD4^+$ T cells after immunization with MOG35-55 peptide in complete Freund’s adjuvant. The effector phase includes extravasation of MOG35-55-specific T cells into the CNS where they produce chemokines and cytokines capable of activating infiltrating mononuclear cells and CNS microglia, with subsequent demyelination and axonal injury.

All studies were performed according to the guidelines of the Institutional Animal Care and Use Committee at the Mayo Clinic. Unless otherwise indicated, all reagents were obtained from Sigma (St. Louis, MO, USA).

MOG35-55 EAE disease induction

MOG35-55 EAE was induced in 12-week old female C57BL/6J mice (Jackson Laboratories) by immunization with 100 μg of the MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in incomplete Freund's adjuvant containing 200 μg of *M. Tuberculosis*. Mice were administered 100 ng of *B. Pertussis* toxin intraperitoneally (i.p.) on the day of MOG35-55 immunization and 48 h later. Mice were housed under barrier conditions, and paralyzed mice were afforded easier access to food and water. Additional groups of MOG35-55 immunized mice were also prepared to obtain MOG35-55 primed splenocytes at 21 or 30 days after disease induction.

MOG35-55 EAE clinical scores

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, non-ambulatory and moribund; grade 5, death (Blaber et al., 2004). Clinical scores were assessed daily for 90 days.

Role of Klk6 in clinical and neuropathological outcomes in the MOG35-55 EAE model

To determine the potential impact of Klk6 in the development of EAE, four groups of five mice were immunized with MOG35-55. Mice were then randomized to Klk6 treatment or vehicle control groups. Twenty micrograms of active recombinant rat Klk6 was administered to two groups of five mice i.p. on days 4, 6, 8 and 11 post-MOG35-55 priming. Intraperitoneally administered drugs are expected to rapidly enter the blood stream. Control mice received PBS alone. All cage cards were blinded to treatment group for clinical scoring. The experiment was repeated twice with similar clinical outcomes. Methods used to express, purify and activate recombinant rat Klk6 using a baculovirus expression system have been previously described in detail (Bernett et al., 2002; Blaber et al., 2002; Vandell et al., 2008; Burda et al., 2013). Rat and mouse Klk6 are highly homologous (91%) with the differing residues not located in regions critical to protease activity, and would not be expected to contribute any species-specific antigenicity (Scarisbrick et al., 2012a,b).

At 90 days post MOG35-55-induced EAE, quantitative morphologic analysis was performed on 5 μm hematoxylin and eosin (H&E) stained transverse sections through eight different spinal cord segments per mouse. Spinal segments embedded in paraffin for analysis included at least two from each major spinal cord level that is cervical, thoracic, lumbar and sacral. A pathologic 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 pathologic 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 (Blaber et al., 2004; Scarisbrick et al., 2012a,b).

Quantification of EAE-induced changes in Klk6 RNA and protein

Klk6 RNA quantification—Additional groups of MOG35-55 immunized mice were prepared to evaluate changes in Klk6 RNA or protein expression within the spinal cord at acute through chronic time points. Between three and five animals were examined per time point.

Quantitative real time RT-PCR was used to evaluate the expression of Klk6 in the spinal cord at acute (7d) through late chronic time points (90d) post MOG35-55 immunization. Specifically spinal cords were harvested from mice without EAE and those at 7, 12, 21, 46, 60 and 90 days after disease induction and snap frozen in liquid nitrogen prior to RNA isolation using RNA STAT-60 (Tel-Test, Inc. Friendswood, TX, USA). 0.5 μg of total RNA was subject to RT-PCR using the Light Cycler-RNA Amplification Kit SYBR Green I in a Roche Light Cycler apparatus following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). To control for loading the housekeeping gene glyceraldehyde phosphate 3-dehydrogenase (GAPDH) was amplified in the same RNA samples as those used to examined Klk6 expression. Primers are provided in Table 1 and are identical to those used in our prior studies (Scarisbrick et al., 2012a,b; Radulovic et al., 2013, 2015, 2016). Changes in Klk6 gene expression are reported as percentage change relative to mice without EAE.

Klk6 protein quantification—To determine whether transcriptional changes observed in Klk6 in the spinal cord of mice with MOG35-55-induced EAE were reflected at a protein level, we examined spinal cord Klk6 by Western blot in mice without EAE and those at 21, 35, 45 and 61 days after MOG35-55 immunization. We collectively homogenized three freshly isolated spinal cords at each time point in radioimmunoprecipitation (RIPA) buffer. Fifty microgram aliquots of each protein lysate were separated on SDS-polyacrylamide gels under reducing conditions prior to transfer onto nitrocellulose membranes. Blots were probed with a rabbit polyclonal Klk6-specific antibody (Rb008) as previously described (Blaber et al., 2002; Scarisbrick et al., 2012a,b; Yoon et al., 2013). In each case, membranes were stripped and re-probed for GAPDH (Abcam, ab9485, Cambridge, MA, USA) to control for loading, and all proteins of interest were detected on film using chemiluminescence Supersignal (Pierce, Rockford, IL, USA). For quantification, films were scanned and images quantified using Image Lab 2.0 software (BioRad). Relative changes in Klk6 protein in MOG35-55 primed relative to control spinal cords were determined by normalizing optical density measurements for Klk6 to those of GAPDH detected on the same membrane. All Western blots were repeated at least three times, providing similar results. Raw normalized relative optical density (ROD) readings across the blots are provided as mean and standard error.

***In vitro* analysis of immune cell function—**To investigate whether the expression of Klk6 is regulated in T cells in an antigen-specific manner, whole splenocyte cultures were

prepared from mice with MOG35-55-induced EAE at 21 or 30 days after disease induction. These cultures contain T and B lymphocytes, as well as monocytes capable of antigen presentation (Christophi et al., 2004; Kruisbeek et al., 2004). Purified splenocyte cultures were grown in serum free X-Vivo 15 media (Cambrex, East Rutherford, NJ, USA) supplemented with 20 mm HEPES, 50 μm 2-mercaptoethanol, 2 mm glutamine, 1 mm sodium pyruvate, 10 U/ml ampicillin and 10 μg/ml streptomycin at 37°C and 5% CO_2 in a humidified atmosphere (3×10^7 cells/ml) in the presence or absence of 10 μg/ml of MOG35-55 antigen for 72 h. Serum free media was used to avoid any protease or protease inhibitor activity present in serum supplements (Scarisbrick et al., 2006a,b, 2011). Cells were harvested into RIPA buffer and 50 μg aliquots of protein lysates separated on polyacrylamide gels under reducing conditions, transferred to nitrocellulose, and probed for Klk6 or Actin (Novus Biologicals, NB600-501, Littleton, CO, USA) as described.

Additional splenocyte cultures were prepared from mice at 21 days after MOG35-55 disease induction to determine whether Klk6 would alter cytokine RNA expression profiles elicited by the disease initiating peptide. Splenocytes were grown for 48 h in the presence of 10 μg/ml MOG35-55 and 150 nm Klk6 added to the media for the final 24 h of the culture period. All stimulation experiments were carried out in six-well plates (Corning, New York, NY, USA). RNA isolation and real time quantitative PCR was performed as described above using primers identified in Table 1. All culture conditions were performed in triplicate and the experiment repeated at least twice. Data described represent the analysis of at least three replicates of each culture condition.

Statistical analysis

Where data were nonlinear, as in the case of EAE clinical scores, the significance of differences across groups was determined by the nonparametric Mann-Whitney *U* test (Blaber et al., 2004). Parametric statistics including one way analysis of variance and the Student Newman Keuls post hoc test were used for parametric variables including RNA and Western blot analyses across multiple groups. When only two groups were involved, parametric unpaired Students *t*-test was used for evaluation of differences or for nonparametric data Dunn's test. Statistical significance was set at $p \leq 0.05$.

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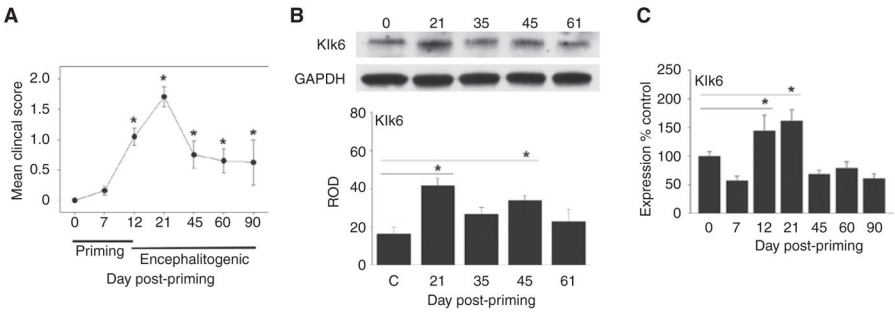

Figure 1.

Peak increases in Klk6 in the spinal cord of mice with MOG35-55-induced EAE occur at the peak of clinical disease. (A) Line graph shows the mean clinical score of mice with MOG35-55-induced EAE. The approximate time frame of the priming and encephalitogenic phases of disease are shown for reference and comparison with changes in Klk6 protein (B) and RNA (C). (B) Western blot and associated histogram show that the greatest increases in Klk6 protein (28 kDa band) occur by 21 days post-priming, at the peak of clinical disease (A). Klk6 protein levels were also increased at 45 days after MOG35-55 priming ($p < 0.04$, NK). (C) Klk6 RNA was also significantly elevated at the peak of clinical disease (21 days), and at 12 days after MOG35-55 priming. The ROD of Klk6 observed by Western blot was normalized to GAPDH to control for loading. Klk6 RNA expression levels were also normalized to those of GAPDH in the same samples and expressed as a percent control (*$p < 0.05$, NK).

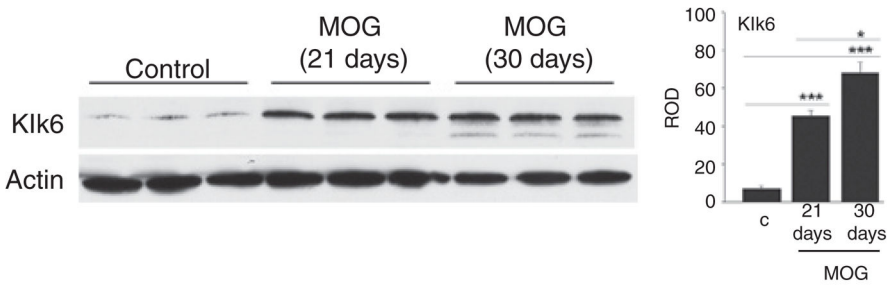

Figure 2.

Klk6 is elevated in splenocyte cultures in response to the disease-initiating antigen. Splenocyte cultures prepared from mice at 21 or 30 days after MOG35-55 immunization were stimulated with MOG35-55 *in vitro*, resulting in 6–10-fold increases in Klk6 protein expression (***$p \leq 0.005$, NK, triplicate samples shown). These cultures contain T and B lymphocytes as well as antigen-presenting monocytes. The primary Klk6 band seen under each culture condition was 28 kDa and an additional lower band at 25 kDa also observed after MOG35-55 priming of splenocytes derived from mice at the 30 day time point. The elevations in Klk6 were greater at the 30 day time point compared to 21 days (*$p < 0.05$, NK).

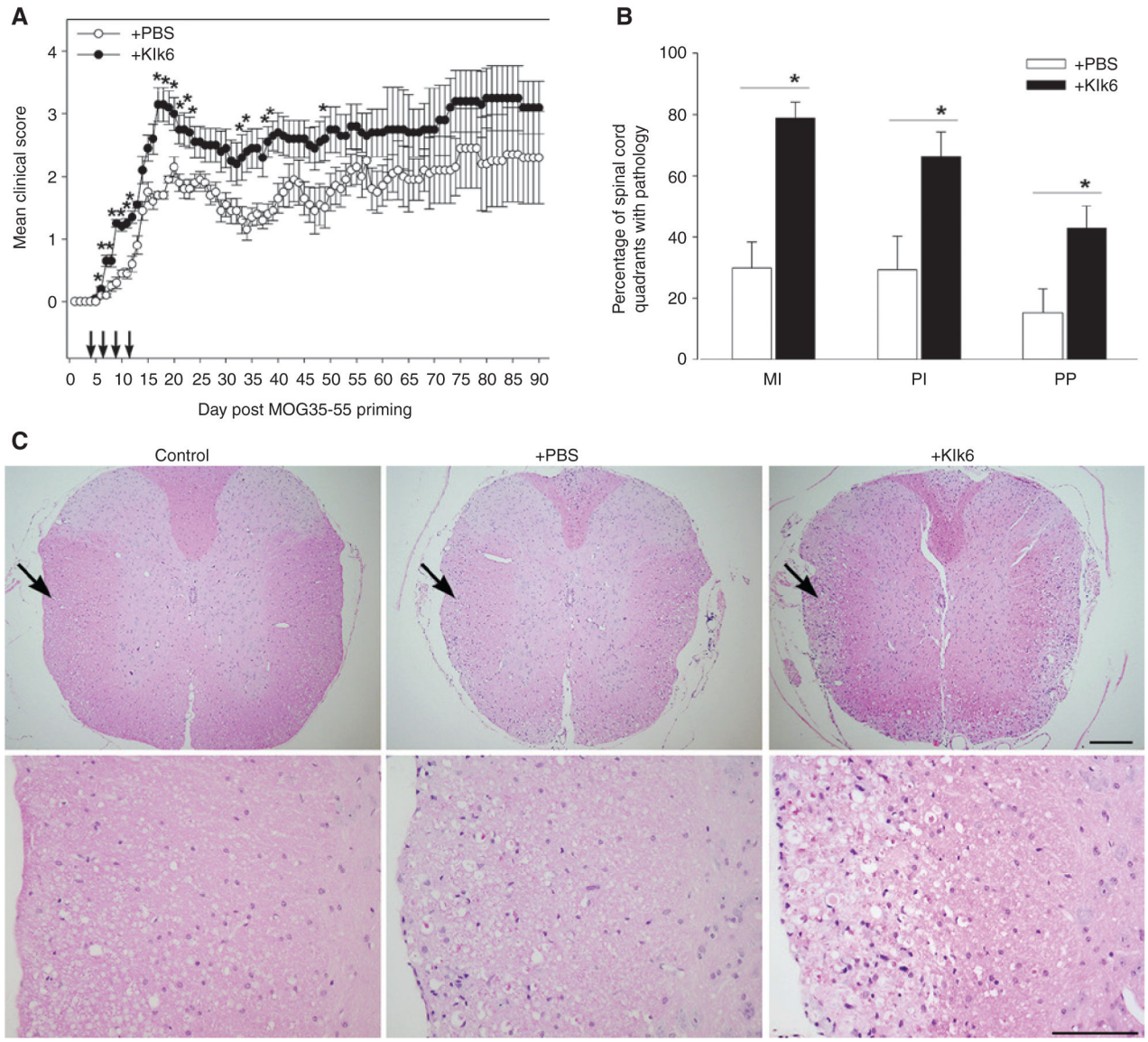

Figure 3.

Administration of Klk6 during the priming phase of EAE exacerbates clinical disease and inflammatory pathology within the spinal cord. (A) Intraperitoneal delivery of 20 μg of active Klk6 on days 4, 6, 8 and 11 after MOG35-55 immunization (indicated by arrows), accelerated the onset of clinical signs of disease and increased the severity of disease (*$p \leq$ 0.05, Mann-Whitney *U* test, n=10 per group). Histogram (B), and photomicrographs of hematoxylin and eosin (H&E) stained sections through the thoracic spinal cord at the 90 day endpoint (C), show that both meningeal inflammation (MI), parenchymal inflammation (PI), and parenchymal pathology (PP) were all increased in the spinal cord of mice administered Klk6 compared to those given PBS alone (*$p < 0.05$, Student *t*-test). Lower panels show higher magnification view of region at arrow in upper panels. Parallel regions of a non-EAE control spinal cord are also shown for comparison. (Scale bar = 100 μm in upper and lower panels.)

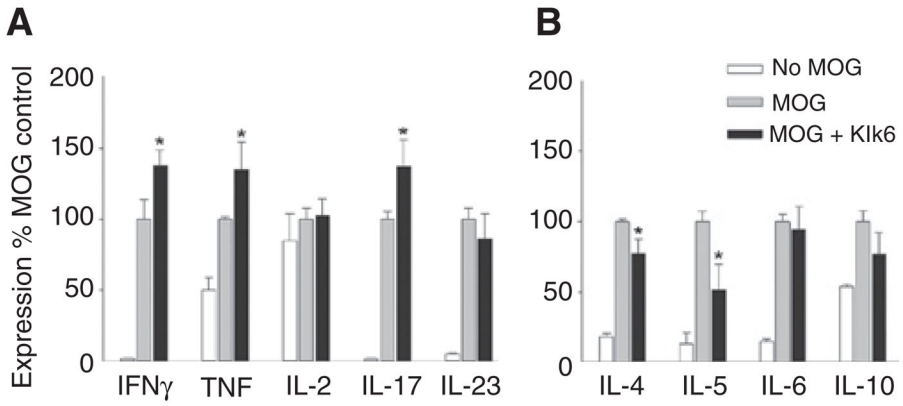

Figure 4.

Klk6 promotes pro-inflammatory and inhibits anti-inflammatory cytokine RNA expression in MOG35-55-primed immune cell cultures (*$p < 0.05$, NK). Histograms show the expression of (A) pro-inflammatory, or (B) anti-inflammatory cytokine RNA in resting (no MOG), or MOG35-55-primed splenocytes. The addition of 200 nm Klk6 to MOG35-55-treated cultures resulted in significant increases in the expression of Th1 cytokines (IFNγ, TNF), in addition to IL-17, and decreases in Th2 cytokines (IL-4 and IL-5) over that seen with MOG35-55 alone ($p < 0.05$, NK). Splenocytes were harvested from mice at 21 days after EAE induction with Klk6 added to MOG35-55-treated cultures for the last 24 h of a 48 h culture period. The expression of each cytokine was detected by real time PCR, normalized to the expression of GAPDH in the same sample to control for loading and expressed as a percent of the MOG35-55-treated control.

Table 1

Real time PCR assays used to quantify molecular changes in gene transcription in response to MOG35-55 induced EAE in the spinal cord or in antigen-stimulated splenocytes.

Gene	Accession number	Primer sequence forward/reverse
GAPDH	NM_008084.2	ACCACCATGGAGAAGGC/ GGCATGGACTGTGGTCATGA (IDT)
IL-2	NM_008366.3	Probe Assay ID: Mm00434256_m1* (AB)
IL-4	NM_021283.2	Probe Assay ID: Mm00445259_m1 (AB)
IL-5	NM_010558.1	Probe Assay ID: Mm00439646_m1 (AB)
IL-6	NM_031168.1	Probe Assay ID: Mm00446190_m1 (AB)
IL-10	NM_010548.2	Probe Assay ID: Mm00439614_m1 (AB)
IL-17	NM_010552.3	Probe Assay ID: Mm00439618_m1* (AB)
IL-23	NM_031252.2	Probe Assay ID: Mm00518984_m1* (AB)
IL-1γ	NM_008361.3	Probe Assay ID: Mm.PT.51.17212823
IFNγ	NM_008337.3	Probe Assay ID: Mm01168134_m1 (AB)
Klk6	NM_011177.2	CCTACCCTGGCAAGATCAC/GGATCCATCTGATATGAGTGC (IDT)
TNF	NM_013693.2	Probe Assay ID: Mm00443258_m1 (AB)

Primers were obtained from applied biosystems (AB) or from Integrated DNA technologies (IDT) as indicated.