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A cell penetrating peptide from SOCS-1 prevents ocular damage in experimental autoimmune uveitis

Chulbul M. Ahmed[a], Michael T. Massengill[a], Emily E. Brown[a], Cristhian J. Ildefonso[b], Howard M. Johnson[c], Alfred S. Lewin[a,*]

[a] Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL, 32610 USA
[b] Department of Ophthalmology, University of Florida, Gainesville FL, 32610, USA
[c] Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, 32611, USA

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ABSTRACT

We describe an immunosuppressive peptide corresponding to the kinase inhibitory region (KIR) of the intracellular checkpoint protein suppressor of cytokine signaling 1 (SOCS-1) that binds to the phospho-tyrosine containing regions of the tyrosine kinases JAK2 and TYK2 and the adaptor protein MAL, and thereby inhibits signaling downstream from these signaling mediators. The peptide, SOCS1-KIR, is thus capable of down-regulating overactive JAK/STAT or NF-kB signaling in somatic cells, including those in many compartments of the eye. Attachment of poly-arginine to this peptide (R9-SOCS1-KIR) allows it to penetrate the plasma membrane in aqueous media. R9-SOCS1-KIR was tested in ARPE-19 cells and was found to attenuate mediators of inflammation by blocking the inflammatory effects of IFNγ, TNFα, or IL-17A. R9-SOCS1-KIR and also protected against TNFα or IL-17A mediated damage to the barrier properties of ARPE-19 cells, as evidenced by immunostaining with the tight junction protein, zona occludin 1 (ZO-1), and measurement of transepithelial electrical resistance (TEER). Experimental autoimmune uveitis (EAU) was generated in B10. RIII mice using a peptide of interphotoreceptor retinal binding protein ($IRBP^{161-180}$) as immunogen. Topical administration of R9-SOCS1-KIR, 2 days before (prophylactic), or 7 days after immunization (therapeutic) protected ocular structure and function as seen by fundoscopy, optical coherence tomography (OCT), and electroretinography (ERG). The ability R9-SOCS1-KIR to suppress ocular inflammation and preserve barrier properties of retinal pigment epithelium makes it a potential candidate for treatment of autoimmune uveitis.

1. Introduction

Uveitis is a complex group of sight threatening diseases that can result from infection, systemic inflammation, or an autoimmune response. Uveitis is estimated to cause 10–15% of all cases of blindness in the United States (Miserocchi et al., 2013). In recent years, host-microbiota interaction has been shown to contribute to both infectious and non-infectious uveitis, suggesting a common source of the two types (Heissigerova et al., 2016). Autoimmune uveitis activates both the innate and adaptive immune responses in cases in which the causative ocular antigens are well characterized and the multifactorial nature of the disease has been examined (reviewed in (Perez and Caspi, 2015)). Autoimmune uveitis can be restricted to the eye (examples include idiopathic uveitis, sympathetic ophthalmia, or birdshot retinochoroidopathy), or it can be a systemic response (e.g., sarcoidosis, Behcet's disease, Vogt-Koyanagi-Harada syndrome). Experimental autoimmune uveitis (EAU) is induced in experimental animals by immunizing with uveitogenic ocular antigens. These animal models have been invaluable tools in understanding the mechanism of disease as well as in developing therapies (Caspi, 2010). It is well established that either the Th1 or the Th17 response is capable of causing autoimmune uveitis. Neutralization of Th1 response leads to an elevated Th17 response, and a deficiency of Th17 response leads to an elevated Th1 response (Luger et al., 2008). Thus, a simultaneous blockage of both Th1 and Th17 responses is crucial to the treatment of autoimmunity.

The current treatments for non-infectious uveitis include corticosteroids, general immunosuppressants, or specific antibodies. Corticosteroid treatment is given topically, through intraocular injection, or systematically. Although initial inflammation is suppressed, continued treatment with corticosteroids is associated with development of cataracts, glaucoma, retinopathy, and activation of herpes simplex virus. Immunosuppressant agents, such as cyclosporine A

* Corresponding author. University of Florida, Department of Molecular Genetics and Microbiology, P.O. Box 100266, Gainesville, FL, 32610, USA.
E-mail address: lewin@ufl.edu (A.S. Lewin).

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(CsA), a T cell targeting drug that blocks IL-2 signaling has been employed to combat ocular inflammation. FK-506 (tacrolimus) and rapamycin, which also target IL-2 signaling, have also been used. This line of treatment is discouraged, however, because of the involvement of IL-2 in maintenance of Tregs, since decreased levels of Tregs are undesirable. Furthermore, these treatments are also not advised for prolonged periods. Antibodies targeting tumor necrosis factor α (TNFα) or IL-1 that can suppress inflammatory signaling pathways are currently being tested (Schwartzman, 2016). Therefore, effective treatments for uveitis is an unmet need.

The extent and duration of an immune response is negatively regulated by a set of intracellular proteins called as suppressors of cytokine signaling (SOCS) (Yoshimura et al., 2012). Amongst the known 8 SOCS proteins, SOCS1 and SOCS3 are particularly relevant to nearly all the cells where JAK/STAT and NF-kB signaling is utilized. SOCS1 contains a kinase inhibitory region (KIR), which spans the residues 53 to 68 of the SOCS1 protein. We have previously shown that the peptide SOCS1-KIR binds to the kinase activation loop containing phosphotyrosine residues of such important tyrosine kinases, as JAK2 and TYK2, and the toll-like receptor adaptor protein MAL (Jager et al., 2011). Owing to this property, the SOCS1-KIR peptide is capable of suppressing signaling downstream of these molecules. Attachment of palmitoyl-lysine allowed this peptide (lipo-SOCS1-KIR) to gain access across the plasma membrane. We showed that this peptide was capable of protecting against experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (Jager et al., 2011). This form of the SOCS1-KIR peptide was also shown to be effective in preventing the onset of experimental autoimmune uveitis if given topically beginning at the time of immunization (He et al., 2016). Here, we report the synthesis and use of a water soluble version of SOCS1-KIR, denoted as R9-SOCS1-KIR. R9 represents the 9 arginines attached to SCOS1-KIR that causes both water solubility and cell penetration. R9-SOCS1-KIR is likely to be less immunogenic than lipo-SOCS1-KIR (Deres et al., 1989; Robinson et al., 1992). In this report, we describe the ability of R9-SOCS1-KIR to suppress inflammatory responses emanating from IFNγ, TNFα, or IL-17, and protection against the loss of transepithelial electrical resistance (TEER) in a human retinal pigment epithelial cell line, ARPE-19. Since multiple sclerosis and autoimmune uveitis share several common features and can in some cases be overlapping (Gordon and Goldstein, 2014; Messenger et al., 2015), we tested R9-SOCS1-KIR in a mouse model of experimental autoimmune uveitis (EAU), and found it to be capable of protecting against the ocular damage in EAU, both as a prophylactic (delivered two days before immunization), and as a therapeutic (applied 7 days after immunization) by simple application as eye drops.

2. Materials and methods

2.1. Cell culture

ARPE-19 cells (ATCC, 2302) were grown in DMEM/F-12 medium containing 10% FBS and 1% each of penicillin and streptomycin in a humidified incubator at 37 °C. THP-1 cells (ATCC, TIB-202) were grown in RPMI medium with 10% FBS and 1% each of penicillin and streptomycin.

2.2. Peptide synthesis

Conventional fluorenylmethyl-oxycarbonyl (FOMC) chemistry was used for the synthesis of peptides on an Applied Biosystems 431A automated peptide synthesizer, as described previously (Szente et al., 1994). R9-SOCS1-KIR had the following sequence: RRRRRRRRRDTH**F**RT**F**RSHSDYRRI. The critical phenylalanines shown in bold were replaced by alanines in an inactive control peptide, R9-SOCS1-KIR2A, which had the following sequence: RRRRRRRRRDTH**A**RT**A**RSHSDYRRI. Peptides were characterized by mass spectrometry and purified by high performance liquid chromatography (HPLC). Peptides were reconstituted in phosphate buffered saline (PBS) under sterile conditions before use.

2.3. Immunohistochemistry

ARPE-19 cells were grown in 8-well chambered slides. After overnight growth in regular DMEM/F12 medium with 10% FBS, the medium was changed to DMEM/F12 medium with 1% FBS. While testing for the effects of IFNγ, TNFα, and IL-17A, cells were incubated in serum-free medium and pre-treated with R9-SOCS1-KIR or the control peptide R9-SOCS1-KIR2A at 20 μM for 3 h followed by treatment with IFNγ (1 ng/ml), TNFα (10 ng/ml), or IL-17A (50 ng/ml) for 30 min. In experiments in which ARPE-19 cells were differentiated to cuboidal morphology, cells were grown in 1% FBS containing medium with a change of media twice per week for 4 weeks. Cells were pretreated with R9-SOCS1-KIR or the control peptide R9-SOCS1-KIR2A at 20 μM for 3 h followed by treatment with TNFα (10 ng/ml), or IL-17A (50 ng/ml) for 48 h. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed with PBS, followed by permeabilization using 1% Triton X-100 in PBS for 30 min at room temperature. Cells were then blocked in 10% normal goat serum in PBS containing 0.5% Triton X-100 for 30 min at room temperature followed by washing in 0.2% Triton X-100 in PBS (wash buffer). Rabbit polyclonal antibody to pSTAT1α, STAT1α (both from Santa Cruz Biotechnology), pSTAT3, STAT3 (both from Cell Signaling), p65 (Cell Signaling), or ZO-1 (Invitrogen) (1:200 dilution) were added and incubated overnight at 4 °C, followed by washing. Cy-3 conjugated anti-rabbit secondary antibody (Invitrogen, 1:300 dilution), or Alexa Fluor 488 conjugated secondary antibody (Invitrogen, 1:300 dilution) were added and incubated for 30 min at room temperature, followed by washing. After the addition of mounting media, cells were covered with a cover slip and imaged under a Leica DMi8 fluorescence microscope. In experiments that involved ZO-1 staining, cells were imaged in a Keyence BZ-X700 fluorescence microscope.

2.4. RNA extraction and qPCR

ARPE-19 cells were seeded in a 12 well plate at 70% confluence. The next day, the medium was changed to DMEM/F-12 containing 1% FBS. Cells were pre-treated with 20 μM R9-SOCS1-KIR for 3 h, followed by treatment with 10 ng/ml of TNFα for 1 h. Cells were then washed with PBS, and total RNA was extracted using the RNeasy Mini Kit from QIAGEN, following the manufacturer's instructions. One microgram of RNA was used to synthesize first strand cDNA using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). The following PCR primers, synthesized by IDT (Coralville, Iowa) were used. IL-1β (For: CTCGCCAGTGAAATGATGGCT, Rev: GTCGGAGATTCGTAGCTGGAT), IL-6 (For: CTTCTCCACAAGCGCCTTC, Rev: CAGGCAACACCAGGAGCA), CCL-2 (For: CTCATAGCAGCCACCTTCATTC, Rev: TCACAGCTTCTTTGGGACACTT), and β-actin (For: AGCGAGCATCCCCCAAAGTT, Rev: GGGCACGAAGGCTCATCATT). The PCR reaction mixture contained cDNA template, SsoFastEvaGreen Super mix containing SYBR green (Bio-Rad (Hercules, CA), and 3 μM gene specific primers. After denaturation at 95 °C for 2 min, 40 cycles of reaction including denaturation at 94 °C for 15 s followed by annealing at 60 °C for 30 s were carried out using C1000 thermal cycler CFX96 real-time system (Bio-Rad). Gene expression was normalized to beta actin. Relative gene expression was compared with untreated samples and determined using the CFX96 software from Bio-Rad.

2.5. NF-κB promoter activity

A plasmid, pNF-κB-Luc that contains NF-κB promoter linked to firefly luciferase and another plasmid with a constitutively expressing thymidine kinase promoter driven Renilla luciferase (pRL-TK-Luc) were

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