

A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity

Ethan Stolzenberg^a Deborah Berry^b De Yang^e Ernest Y. Lee^f
Alexander Kroemer^c Stuart Kaufman^c Gerard C.L. Wong^f
Joost J. Oppenheim^e Supti Sen^b Thomas Fishbein^c Ad Bax^g Brent Harris^d
Denise Barbut^h Michael A. Zasloff^c

^aDepartment of Pathology, Section of Neuropathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, ^bHTSR, Lombardi Cancer Center, ^cMedStar Georgetown Transplant Institute and ^dDepartments of Neurology and Pathology, Georgetown University School of Medicine, Washington, DC, ^eCancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD, ^fBioengineering Department, Chemistry and Biochemistry Department, California Nano Systems Institute, University of California, Los Angeles, CA, ^gLaboratory of Chemical Physics, National Institutes of Diabetes, Digestive Diseases and Kidney, National Institutes of Health, Bethesda, MD, and ^hEnterin Inc., Philadelphia, PA, USA

Keywords

Alpha-synuclein · Cell activation · Innate immunity · Parkinson disease

Abstract

Background: Alpha-synuclein (α S) is a nerve cell protein associated with Parkinson disease (PD). Accumulation of α S within the enteric nervous system (ENS) and its traffic from the gut to the brain are implicated in the pathogenesis and progression of PD. α S has no known function in humans and the reason for its accumulation within the ENS is unknown. Several recent studies conducted in rodents have linked α S to immune cell activation in the central nervous system. We hypothesized that α S in the ENS might play a role in the innate immune defenses of the human gastrointestinal (GI) tract. **Methods:** We immunostained endoscopic biopsies for α S from children with documented gastric and duodenal inflammation and intestinal allograft recipients who con-

tracted norovirus. To determine whether α S exhibited immune-modulatory activity, we examined whether human α S induced leukocyte migration and dendritic cell maturation. **Findings:** We showed that the expression of α S in the enteric neurites of the upper GI tract of pediatric patients positively correlated with the degree of acute and chronic inflammation in the intestinal wall. In intestinal allograft subjects who were closely monitored for infection, expression of α S was induced during norovirus infection. We also demonstrated that both monomeric and oligomeric α S have potent chemoattractant activity, causing the migration of neutrophils and monocytes dependent on the presence of the integrin subunit, CD11b, and that both forms of α S stimulate dendritic cell maturation. **Interpretation:** These findings strongly suggest that α S is expressed within the human ENS to direct intestinal inflammation and implicates common GI infections in the pathogenesis of PD.

© 2017 S. Karger AG, Basel

Introduction

Alpha-synuclein (α S) is a neuronal protein that has excited widespread interest because of its role in the pathogenesis of Parkinson disease (PD) and the related alpha-synucleinopathies, Lewy body dementia, and multiple system atrophy. Of particular interest over the past decade or so has been the scientific debate spurred by the work of Braak, who has shown through his studies of pathological specimens from autopsied individuals at various stages of PD that the accumulation of α S actually begins in the enteric nervous system (ENS) [1]. Utilizing the vagus as an escalator, α S produced in the ENS traffics from the gut to the brain, spreading to centers within the central nervous system (CNS) that are ultimately destroyed. α S has no known function. Because it physically associates with vesicular structures, such as synaptic vesicles, it is described as a protein that is somehow involved in neurotransmitter release [2]. Why it would accumulate in the ENS is entirely mysterious. Two studies conducted in rodents suggest that microbes in the gastrointestinal (GI) tract accelerate α S aggregation, one through nucleating a prion-like reaction [3], the other suggesting that bacterial metabolites promote microglial activation [4]. Without a clear understanding of the function of α S and why it would accumulate in a nerve cell, we cannot unravel the pathophysiology of diseases such as PD. Recently, human α S was shown to chemoattract rodent microglia suggesting that α S could directly promote neuroinflammatory damage within the CNS [5]. In addition, α S has also recently been shown to be protective in mice infected with neurotropic RNA viruses, such as West Nile virus (WNV) and Venezuelan equine encephalitis [6], suggesting that it can play a beneficial role in immune defense. Based on these studies we hypothesized that α S in the ENS might play a role in the innate immune defenses of the GI tract.

Methods

Patients

Biopsies were obtained as part of standard clinical practice. Protocols were approved by the institutional review board at each participating center. We retrieved endoscopic biopsy specimens from 42 children (mean age 12.4 years) with upper GI distress with the pathological diagnoses of duodenitis, gastritis, *Helicobacter pylori*, or reactive gastropathy at 1 academic center over a 9-year period (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000477990). A second population included endoscopic biopsies obtained from 14 pediatric (mean age 3.4 years) and 2 adult intestinal transplant recipients (mean age

40 years) who developed a documented norovirus infection ($n = 16$; see online suppl. Table 2). In this immunosuppressed population, viral infections occur frequently, and biopsies of the allograft are obtained prospectively at specific times following transplantation and when clinically indicated.

Immunohistochemistry and Scoring

The method of tissue preparation followed published procedures [7, 8]. Serial sections were cut at 4 μ m thickness. The primary antibodies used were: α S (LB 509, Abcam ab27766), PGP9.5 (Agilent Z5116), and CD68 (Abcam ab955). The secondary antibody was Envision Plus polymer anti-mouse conjugated to HRP (Agilent K4001). HRP was visualized using DAB chromogen (Agilent K3468) following the manufacturer's protocol.

Histological scoring was performed on 2 sections per block for each stain by 2 pathologists blinded to the diagnoses. Mucosa from each biopsy was assessed by HE stain for acute (polymorphonuclear) and chronic (mononuclear) inflammation (1, minimal inflammatory change; 2, moderate inflammatory change; 3, marked inflammatory change), and for PGP 9.5-positive neurite density (1, minimal; 2, moderate; 3, high), neurite α S presence and intensity (1, slight; 2, moderate; 3, high), and CD68-positive cell number (1, rare cells; 2, low cell number; 3, moderate cell number; 4, high cell number). The scoring of each pathologist was averaged.

Chemotaxis and Dendritic Cell Maturation

Chemotaxis assays were performed according to published procedures [9]. Neutrophils and monocytes were isolated from the blood of healthy human donors as described [9]. Recombinant human α S (lot No. 20162050, endotoxin contamination 18×6 EU/mg) was from Proteos Inc. via the Michael J. Fox Foundation. The α S aggregates were made following a protocol provided by Proteos. N-terminal peptides were synthesized by solid-phase chemistry and purified to >98% purity by high-performance liquid chromatography. Male CD11b^{-/-} (B6.129S4-Itgam^{tm1Myd/J}) and wild-type C57B6 control mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Anti-human CD11b antibody (clone M1/70, ultrapure rat IgG) was from BioLegend (San Diego, CA, USA). Dendritic cell maturation studies were conducted as reported [10].

Results

To determine if α S expression in the ENS is associated with intestinal inflammation, we examined upper GI endoscopic biopsies taken over a 9-year period at 1 hospital center from 42 children who exhibited pathological evidence of acute and/or chronic mucosal inflammation, as determined by the presence of neutrophil or mononuclear cell infiltrates, respectively. The population included subjects with *H. pylori* gastritis, a common cause of upper GI distress in children. The upper GI tract was selected because numerous reports have demonstrated that expression of α S in the human ENS in the proximal portion of the GI tract is minimal except in the setting of PD [8, 11, 12]. For example, α S staining in the ENS of the gastric

Fig. 1. Representative biopsies of pediatric duodenum immune-stained for α S. The 3 top panels are serial sections ($\times 20$), stained with HE, immunostained for α S, or for CD68. The specimen exhibits severe acute and chronic inflammation. Lower panels: α S colocalizes with PGP 9.5 by double immunofluorescence demonstrating expression within neurons. A representative biopsy from the population in online suppl. Table 1 is shown. Primary antibodies: α -syn, ab27766 from Abcam; PGP 9.5, Z5116 from DAKO. Secondary antibodies: α -syn, Envision anti-mouse HRP polymer from Agilent; PGP 9.5, goat anti-rabbit biotin conjugated from Vector. Tertiary (fluorescent tag): α -syn, PerkinElmer Tyramide Signal Amplification with Cy5; PGP 9.5, Life Technologies streptavidin conjugated with AlexaFluor488. Procedures were as specified by the protocols provided with the reagents. Cy5 was imaged at 555 nm and GFP at 488 nm. Images were taken using Slidebook 6.0 and the overlay was an output from the Slidebook System.

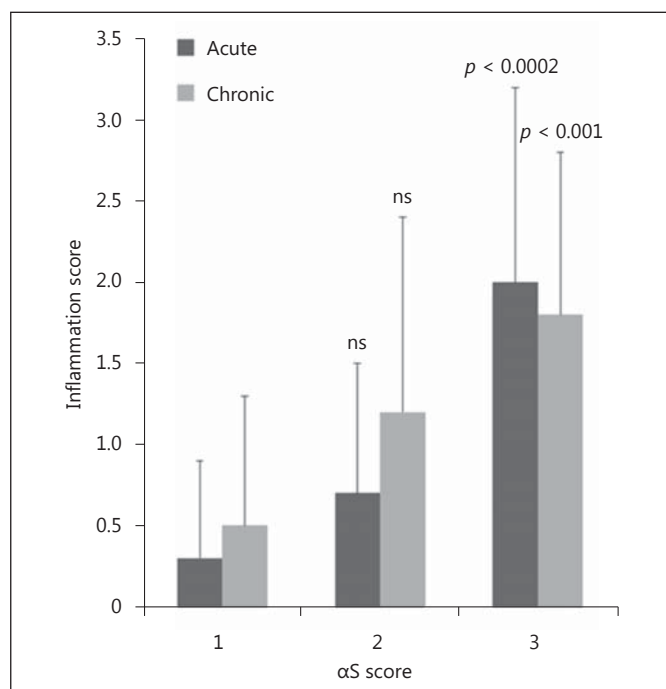
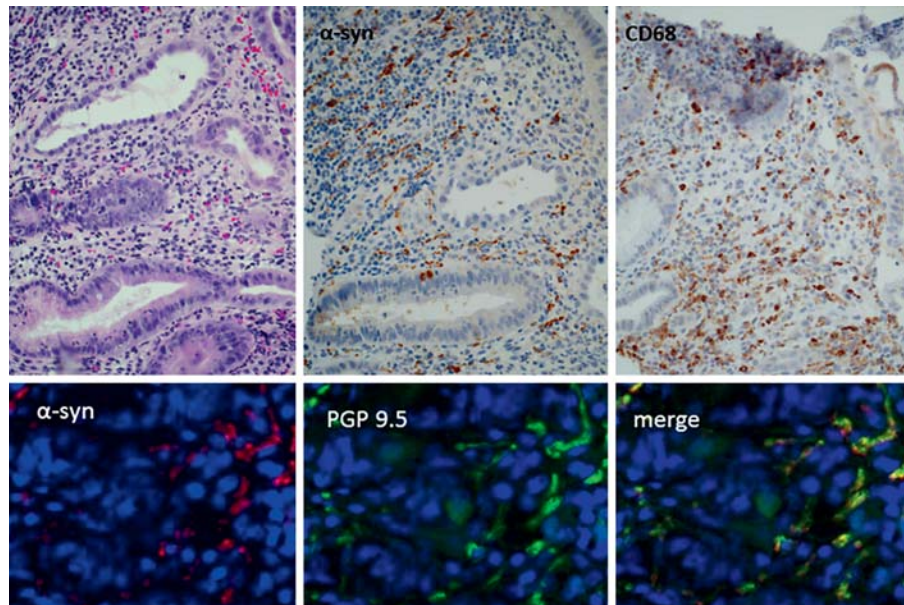


Fig. 2. The degree of acute and chronic inflammation correlates with α S immune positivity. Scores for acute and chronic inflammation for all specimens are plotted relative to the associated α S scores (listed in online suppl. Table 1). Statistical significance between the inflammation scores associated with high α S scores (2 or 3) versus an α S score of 1 were determined by a 1-way ANOVA with a post hoc *t* test, with a Bonferroni correction. ns, not significant.

mucosa of subjects over the age of 70 years was noted in about 80% of evaluable biopsies from individuals with PD, but in less than 5% of an aged-matched control population [8]. We selected a pediatric population to avoid the bias of an adult population with “pre-clinical PD” [1].

The biopsies were immunostained for α S (Fig. 1). Since the extent of α S staining depends both on its intraneuronal concentration as well as on the number of neurites present within the tissue specimen, serial sections were also immunostained for the neural protein PGP 9.5. In several specimens, we confirmed the neuronal localization of α S by immunofluorescent colocalization of α S and PGP 9.5, a neuronal marker (Fig. 1, lower panels). In general, the biopsies, being superficial, included the submucosal plexus but spared deeper structures. Because many of the gastric biopsies had insufficient neural tissue to evaluate, we focused our analyses on duodenal specimens.

In all biopsies examined from this pediatric population, immunostained α S was visible in neuronal processes within the lamina propria (Fig. 1). The intensity and extent of α S was assessed for each specimen and graded on a scale of 1–3 by 2 pathologists uninformed of the diagnosis. Similarly, the degree of acute and chronic inflammation was graded by the density of neutrophils and mononuclear cells in the biopsy specimens (e.g., minimal, 1; moderate, 2; intense, 3) visible on the HE-stained speci-

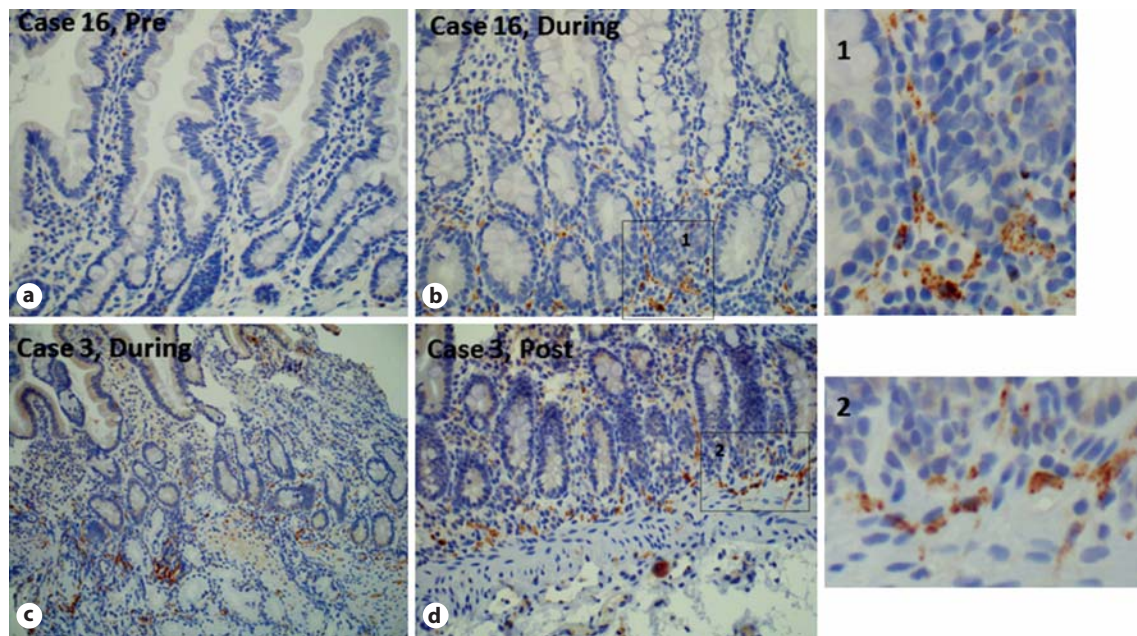


Fig. 3. Representative biopsies from intestinal transplant recipients with documented norovirus infection stained for α S ($\times 20$). **a** “Case 16, Pre” (native duodenum): a biopsy taken 1 month prior to the norovirus infection shows minimal expression of α S. **b** “Case 16, During” (native duodenum): a biopsy taken at the time of norovirus infection shows a robust expression of α S in neurites within

the lamina propria. **c** “Case 3, During” (allograft jejunum): a biopsy taken at the time of norovirus infection shows areas with high expression of α S. **d** “Case 3, Post” (allograft jejunum): a biopsy taken 4 months after the initial norovirus infection shows continued high expression of α S. The patient still tested PCR positive for norovirus. Images 1 and 2 are enlargements of the outlined areas.

mens. The number of CD68-positive cells within the lamina propria (principally macrophages and dendritic cells) varied in proportion to the degree of inflammation (Fig. 1). From the assessment of the specimens from the 42 children, the degree of inflammation within each biopsy correlated positively with the intensity and extent of α S staining, both for acute ($p < 0.0002$) and chronic inflammation ($p < 0.001$; Fig. 2). Of all of the cases, 23/42 (55%) had a confirmed upper GI tract infection: *H. pylori* in 20/23 (87%), *H. heilmannii* in 1/23 (5%), and *Candida* in 2/23 (9%). The remaining had chronic upper GI discomfort (pain, nausea, vomiting) associated with mucosal inflammation of unknown etiology, which could include some of the most common infections, such as viruses that are normally not evaluated in this clinical setting. The magnitude of α S expression did not differ significantly between subgroups that had a diagnosed bacterial or fungal infection (e.g., *H. pylori*) and those that did not.

To pursue the relationship between infection and the induction of α S, we examined intestinal biopsies taken as part of the normal standard of care of intestinal allograft recipients. Because this is an immunosuppressed popula-

tion, allograft recipients are monitored closely for bacterial, fungal, and viral infections through prospective examination of endoscopically obtained tissue specimens as well as blood, urine, and stool. We identified 14 children and 2 adults who received an intestinal transplant and had contracted a norovirus infection after the surgery, definitively diagnosed by PCR. Biopsies were examined that had been taken before, during, and after the infection. In most duodenal biopsies sampled during the infection, a robust expression of α S was seen (Fig. 3; online suppl. Tables 2A, B). In 9 of the 16 cases, biopsies had been taken shortly before the norovirus infection (1–6 months). In 4 of these 9 subjects (44%) α S was not observed in tissue from either the native or the transplanted duodenum prior to the onset of the norovirus infection, consistent with the hypothesis that the expression of α S was induced during the norovirus infection (Fig. 3; online suppl. Table 2A, B). Tissues taken between 2 weeks and 6 months following clinical resolution of the infection still exhibited the presence of α S but generally at lower levels than observed during the period of active infection (Fig. 3; online suppl. Table 2A, B).

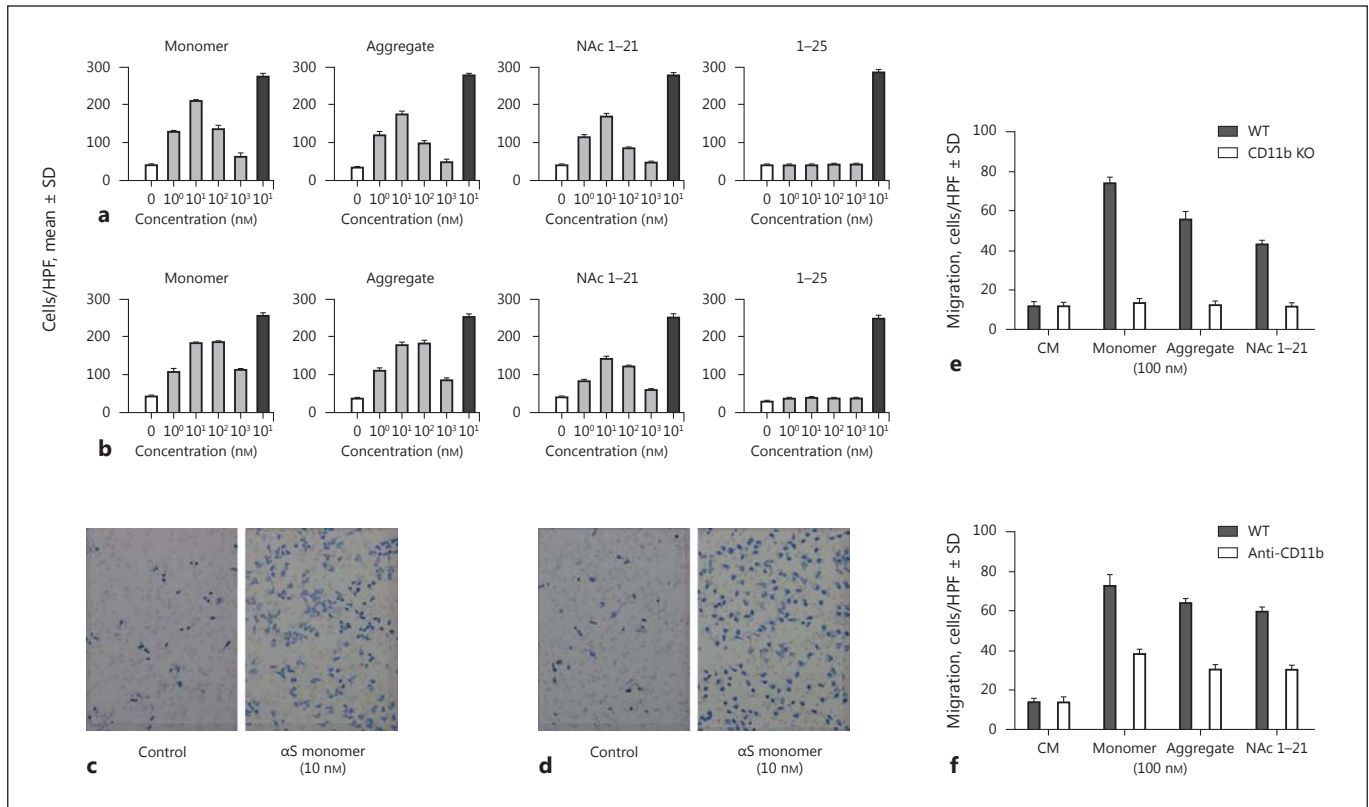


Fig. 4. α S is a chemoattractant dependent on CD11b. Assays were conducted as described in the Methods section. The average of 3 independent assays for each sample is presented. The concentration of the α S aggregate is in terms of the monomer. A positive control, FMLP, is noted in black. NAc 1-21, the N-acetylated peptide corresponding to the first 21 amino acids of α S; 1-25, a peptide corresponding to the first 25 amino acids. **a** Human neutrophils.

Our observation that the increased expression of α S within the duodenum was associated with an increased tissue density of inflammatory cells within the lamina propria prompted us to examine whether α S exhibited chemotactic activity. Indeed, both monomeric and aggregated recombinant human α S were chemotactic at nanomolar concentrations towards human neutrophils and monocytes exhibiting the classical bell-shaped concentration curve characteristic of chemoattractants (Fig. 4a-d). Furthermore, the N-acetylated peptide, corresponding to the first 21 amino acids of human α S, which is universally N-acetylated in mammalian cells [13], retained the chemotactic activity of the full-length protein. In contrast, the slightly longer peptide, extending to residue 25 but lacking the N-acetyl moiety, was inactive, implicating N-terminal acetylation as a determinant of the peptide's activity. We then explored whether peripheral white

b Human monocytes. **c, d** Representative photos of the cellular response. **e** Neutrophils from CD11b^{-/-} or wild-type mice (0.5×10^6 /mL), assayed as described in the Methods section. **f** Human neutrophils incubated in the presence of anti-CD11b antibody or the control (50 μ g/mL each). CM, control media (negative control); HPF, high-powered field.

blood cells require CD11b to respond to α S. This was prompted by the recent observation that aggregated α S stimulated the migration of rodent CNS microglia via a CD11b mechanism [5]. While both monomeric and aggregated α S exhibited potent chemotactic activity towards neutrophils from wild-type mice, no chemotactic activity was observed for cells from CD11b-deficient mice (Fig. 4e). In a separate experiment, the treatment of human neutrophils with an antibody directed at CD11b reduced the chemotactic response to α S (Fig. 4f).

We next asked whether α S could activate dendritic cells. Human monocyte-derived dendritic cells were exposed for 2 days to α S monomer, aggregate, and NAc 1-21 peptide, and then analyzed by flow cytometry to measure the extent of phenotypic maturation, using CD80, CD83, CD86, HLA-ABC, and HLA-DR as determinants (Fig. 5; online suppl. Fig. S1). While both monomer and aggre-

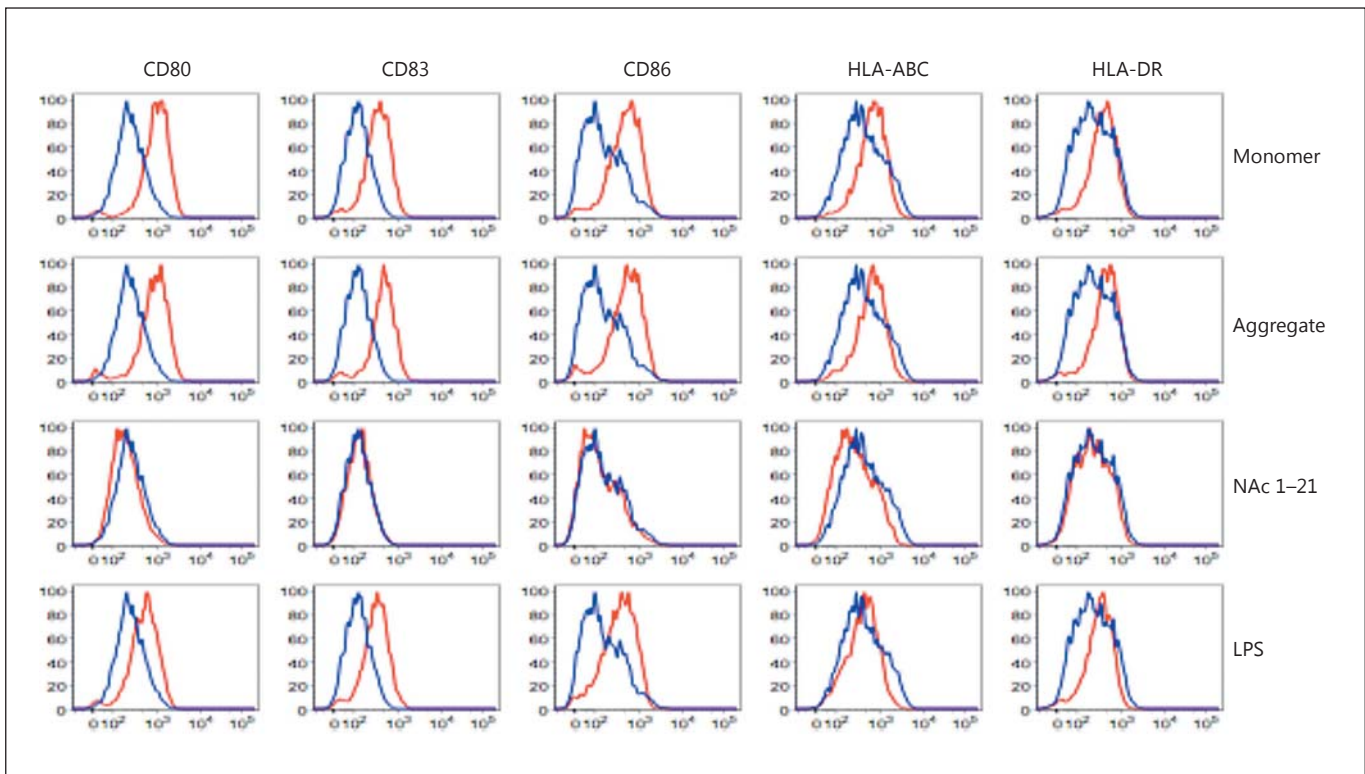


Fig. 5. α S stimulates phenotypic maturation of human dendritic cells. Human monocyte-derived dendritic cells were incubated at 5×10^5 /mL in the presence of recombinant monomeric or aggregated α S, and the NAc 1-21 amino terminal peptide of α S (each at a final concentration of $10 \mu\text{M}$), for 48 h before they were immunostained and analyzed by flow cytometry for the expression of the

indicated surface molecules. *E. coli* lipopolysaccharide (LPS; 100 ng/mL) was included as a positive control. The results of 1 experiment representative of 3 are shown. The ordinate of each flow cytometric analysis corresponds to the fluorescent intensity/cell, the abscissa to the cell number in a log scale.

gate promoted dendritic cell maturation, the N-terminal peptide did not, demonstrating that the chemotactic activity of α S and the segment involved in dendritic cell maturation reside on different portions of the molecule. Maturation of dendritic cells by the monomer was not inhibited by pretreatment of the cells with a blocking antibody directed at TLR4 (online suppl. Fig. S2), demonstrating that the cellular response was independent of the small amount of endotoxin present in the recombinant α S preparation, and that α S does not engage the TLR4 receptor to effect maturation.

Discussion

In the present study, we have demonstrated that the expression of α S in neurites and neuronal cell bodies within the gastrointestinal wall of children is associated with mucosal inflammation. Furthermore, we have

shown that α S is both a potent chemoattractant for neutrophils and monocytes, and a maturation factor for dendritic cells. Since it is known that α S is secreted from enteric nerves upon stimulation [14], it is reasonable to propose that it is the induction and secretion of α S from the ENS that promotes mucosal inflammation rather than the induction of α S having been a consequence of inflammation.

Because intestinal transplant recipients are intensively monitored prospectively for signs and symptoms of enteric infection (which can mimic allograft rejection), we succeeded in obtaining biopsies of both native and allograft tissues in 4 individuals prior to the onset of a norovirus infection, who had also remained free of any other clinically symptomatic enteric infection during that period. In these patients, no significant expression of α S could be detected until an active norovirus infection occurred and persisted months after the virus was no longer detected in feces. This observation strongly suggests that

enteric viral infection is one of the factors that can induce the expression of α S in the human ENS. Interestingly, since the α S response of the grafted (vagotomized) tissue was as robust as that of the native duodenum, it appears that α S can be produced by the ENS without input from higher neural centers.

The discovery that α S is expressed during a GI infection and mobilizes an inflammatory response leads to the conclusion that induction of this protein within the ENS is part of its normal immune defense mechanism. Even the transport of α S, as either monomer or aggregate, from the ENS to the brain appears to occur normally. Human α S injected into the gastric wall of rodents, for example, is taken up by the vagus nerve and transported to the dorsal motor nucleus within the brainstem in a time-dependent manner [15]. By virtue of this mechanism, α S produced in the ENS can be transported cranially, protecting higher centers of the nervous system. Since it is known from genetic studies that individuals with multiple copies of α S invariably develop PD, an increase in the expression of α S is sufficient to cause PD [16, 17]. A comparable increase in expression of α S might also occur as a consequence of repeated acute or chronic GI infections caused by organisms that specifically provoke the expression of α S within the ENS. Potential pathogens would include those known to infect and replicate within the ENS, such as strains of influenza [18], varicella [19], and JC virus [20]. Epidemiological studies support an association between chronic *H. pylori* infection and the risk of developing PD [21, 22]. Strikingly, individuals who have received a full truncal vagotomy (as treatment for peptic ulcer) are at a decreased risk of developing PD [23, 24].

A recent report that individuals with PD have increased intestinal permeability suggests another mechanism, in addition to infection, that might provoke the expression (or accumulation) of α S within the ENS [25, 26], namely the exposure of the ENS to commensal microbes. In another study, orally administered *Escherichia coli*-producing curli protein, a protein that facilitates bacterial attachment to epithelial cells and subsequent invasion, enhanced α S deposition in plexi in the gut and in the hippocampus and striatum in aged Fischer 344 rats (which spontaneously accumulate α S within their ENS as they age [27]) as compared to rats exposed to mutant bacteria lacking the capacity to produce curli or to rats exposed to vehicle [3]. In a study involving α S-overexpressing mice, short-chain fatty acids, produced by the intestinal microbiome, increased the presence of α S aggregates in basal ganglia and substantia nigra and enhanced the motor deficit, as did fecal transplants from patients with PD [4].

Our report provides support for the hypothesis that α S is a component of the innate immune defensive response of the ENS and might provide insight into the pathophysiology of certain human chronic inflammatory disorders. With respect to PD, the discovery reported here extends the hypothesis of Braak et al. [11] that PD begins in the ENS by proposing that PD results from the excessive response of a normal innate immune component of the ENS.

Acknowledgements

E.Y.L. acknowledges support from the T32 Systems and Integrative Biology Training Grant at UCLA (T32GM008185) and the T32 Medical Scientist Training Program at UCLA (T32GM008042). A.B. acknowledges support from the Intramural Research Program of the National Institutes of Diabetes and Digestive and Kidney Diseases (DK029047-10). E.S. acknowledges support from the University of Oklahoma HSC Department of Pathology. The Michael J. Fox Foundation facilitated the availability of the α S preparations. T.F., A.K., S.K., and M.Z. thank Dr. Joy Drass for her continued personal support of translational research within the Transplant Institute. Present address of E.S.: Nassau County Medical Examiner's Office, East Meadow, NY, USA.

Funding Sources

Funding was received from NIGMS, NIDDK, University of Oklahoma HSC.

Author Contributions

E.S., D.B., D.Y., J.O., G.W., S.S., and E.L. were responsible for the laboratory studies; S.K., T.F., and A.K. managed the transplant population and provided access to that population; E.S. and B.H. evaluated the histology of each patient sample; E.S., D.B., and M.Z. designed the study and drafted the manuscript.

References

- 1 Del Tredici K, Braak H: Review: sporadic Parkinson's disease: development and distribution of alpha-synuclein pathology. *Neuropathol Appl Neurobiol* 2016;42:33–50.
- 2 Burre J: The synaptic function of alpha-synuclein. *J Parkinsons Dis* 2015;5:699–713.
- 3 Chen SG, Stribinskis V, Rane MJ, et al: Exposure to the functional bacterial amyloid protein curli enhances alpha-synuclein aggregation in aged Fischer 344 rats and *Caenorhabditis elegans*. *Sci Rep* 2016;6:34477.
- 4 Sampson TR, Debelius JW, Thron T, et al: Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell* 2016;167:1469–1480 e12.

- 5 Wang S, Chu CH, Stewart T, et al: Alpha-synuclein, a chemoattractant, directs microglial migration via H₂O₂-dependent Lyn phosphorylation. *Proc Natl Acad Sci USA* 2015; 112:E1926–E1935.
- 6 Beatman EL, Massey A, Shives KD, et al: Alpha-synuclein expression restricts RNA viral infections in the brain. *J Virol* 2015;90:2767–2782.
- 7 Fishbein T, Novitskiy G, Mishra L, et al: NOD2-expressing bone marrow-derived cells appear to regulate epithelial innate immunity of the transplanted human small intestine. *Gut* 2008;57:323–330.
- 8 Sanchez-Ferro A, Rabano A, Catalan MJ, et al: In vivo gastric detection of alpha-synuclein inclusions in Parkinson's disease. *Mov Disord* 2015;30:517–524.
- 9 De Y, Chen Q, Schmidt AP, et al: LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 2000; 192:1069–1074.
- 10 Yang D, Postnikov YV, Li Y, et al: High-mobility group nucleosome-binding protein 1 acts as an alarmin and is critical for lipopolysaccharide-induced immune responses. *J Exp Med* 2012;209:157–171.
- 11 Braak H, de Vos RA, Bohl J, Del Tredici K: Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. *Neurosci Lett* 2006; 396:67–72.
- 12 Gray MT, Munoz DG, Gray DA, Schlossmacher MG, Woulfe JM: Alpha-synuclein in the appendiceal mucosa of neurologically intact subjects. *Mov Disord* 2014;29:991–998.
- 13 Anderson JP, Walker DE, Goldstein JM, et al: Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem* 2006;281:29739–29752.
- 14 Paillusson S, Clairembault T, Biraud M, Neunlist M, Derkinderen P: Activity-dependent secretion of alpha-synuclein by enteric neurons. *J Neurochem* 2013;125:512–517.
- 15 Holmqvist S, Chutna O, Bousset L, et al: Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathol* 2014;128:805–820.
- 16 Singleton AB, Farrer M, Johnson J, et al: Alpha-synuclein locus triplication causes Parkinson's disease. *Science* 2003;302:841.
- 17 Chartier-Harlin MC, Kachergus J, Roumier C, et al: Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* 2004;364:1167–1169.
- 18 Jang H, Boltz D, Sturm-Ramirez K, et al: Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration. *Proc Natl Acad Sci USA* 2009;106:14063–14068.
- 19 Rao M, Gershon MD: The bowel and beyond: the enteric nervous system in neurological disorders. *Nat Rev Gastroenterol Hepatol* 2016;13:517–528.
- 20 Selgrad M, De Giorgio R, Fini L, et al: JC virus infects the enteric glia of patients with chronic idiopathic intestinal pseudo-obstruction. *Gut* 2009;58:25–32.
- 21 Barnum CJ, Tansey MG: Neuroinflammation and non-motor symptoms: the dark passenger of Parkinson's disease? *Curr Neurol Neurosci Rep* 2012;12:350–358.
- 22 Nielsen HH, Qiu J, Friis S, Wermuth L, Ritz B: Treatment for *Helicobacter pylori* infection and risk of Parkinson's disease in Denmark. *Eur J Neurol* 2012;19:864–869.
- 23 Svensson E, Horvath-Puho E, Thomsen RW, et al: Does vagotomy reduce the risk of Parkinson's disease: the authors reply. *Ann Neurol* 2015;78:1012–1013.
- 24 Liu B, Fang F, Pedersen NL, et al: Vagotomy and Parkinson disease: a Swedish register-based matched-cohort study. *Neurology* 2017;88:1996–2002.
- 25 Forsyth CB, Shannon KM, Kordower JH, et al: Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PLoS One* 2011;6:e28032.
- 26 Kelly LP, Carvey PM, Keshavarzian A, et al: Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease. *Mov Disord* 2014;29:999–1009.
- 27 Phillips RJ, Walter GC, Ringer BE, Higgs KM, Powley TL: Alpha-synuclein immunopositive aggregates in the myenteric plexus of the aging Fischer 344 rat. *Exp Neurol* 2009;220: 109–119.