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TITLE: Generation of a Mouse Model to Investigate IL-6 Trans-Signaling in ALS

PRINCIPAL INVESTIGATOR: Gregory Hawkins

CONTRACTING ORGANIZATION: Wake Forest University Health Sciences
Winston-Salem, NC

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
1) IL6 transsignaling plays a potential protective role for motoneurons in the periphery, while later when extracellular levels of IL6 increase with increased muscle atrophy and decreased lung function, transsignaling promotes a breakdown in the blood brain barrier that fosters IL6 transsignaling in the CNS that can promote disease progressions through glial activation. 2) Individuals with increased levels of soluble receptor such as those with enhanced shedding due to IL6R polymorphism will be more susceptible to IL6 transsignaling and will have faster disease progression. 3) Blocking the effects of IL6 transsignaling will reduce disease progression rates and disease severity.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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1. INTRODUCTION:

We hypothesize that IL6 transsignaling plays a role in the progression of ALS by affecting the rate of NMJ denervation, glial cell activation, and MN degeneration. Given the difficulty studying humans with ALS and collecting samples critical to studying active disease, we are proposing to utilize the $SOD1^{G93A}$ mouse model of ALS to study the effects of IL6 transsignaling on disease severity and progression. Successful execution of this study will define the role of IL6 as an effector of ALS severity and progression, and will provide new information on how to target and treatment ALS using therapeutics that target and block the detrimental effects of IL6 transsignaling.

2. KEYWORDS:

Amyotrophic lateral sclerosis, ALS, CRISPR mouse model, IL6 trans-signaling, SOD1

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Goals:

1. Perform a systemic examination of IL6 transsignaling in both initiation and progression of ALS
2. Create an ALS mouse model where IL6 transsignaling is increased, thus modeling those individuals that have inherited the IL6R polymorphism, and determine if disease pathology is altered.
3. Treat the ALS/transsignaling mouse model with the transsignaling inhibitor, soluble gp130, to determine if disease progression can be significantly slowed.

What was accomplished under these goals?

Aim 1: Perform a systematic examination of IL6 transsignaling in both initiation and progression of ALS.

We have initially characterized IL6 expression in the SOD1 mouse model. Our initial survey of IL6 expression suggests expression in muscle, spinal cord and lung correlates with pathological events- similarly to our results in patients as discussed above (Figure 1). We realized that continuing these experiments would waste resources because we would have to include SOD1 littermate controls in experiments with the SOD1 X IL6R^{TMD} mice. We therefore now focused on generating the crosses between the SOD1 mice and the IL6 transsignaling models.

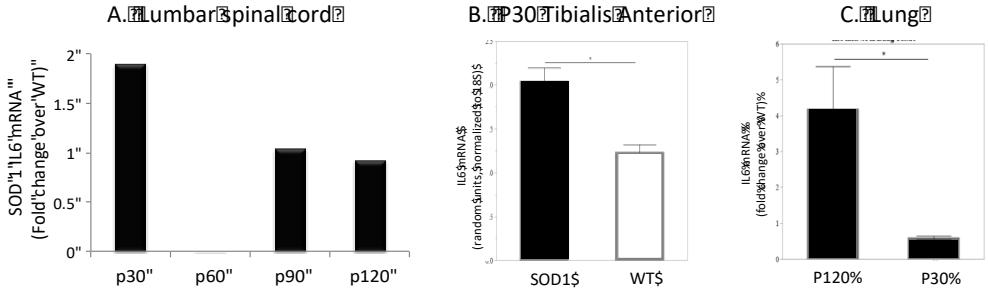

Figure 1.. IL6 mRNA levels in tissues involved in ALS pathology. A. To determine if IL6 expression levels change in ventral lumbar spinal cord with disease progression, we performed a preliminary rtPCR experiment. Message levels for cytokine are increased in SOD1 mice as compared to WT at P30, 90 and 120. Interestingly, the pattern mirrors microglial activation shown above. B. IL6 mRNA is expressed at higher levels in the SOD1 mouse as compared to wild-type age-matched controls ($p<0.001$; student's t-test; n=6 per group). C. Relative to age-matched wild type controls, IL6 mRNA is expressed at higher levels in the SOD1 at end stage (p120) than at p30 ($p=0.028$; student's t-test; n=4 per group). Proposed ELISA and immunohistochemistry experiments will provide more information regarding protein expression and cellular localization.

Aim 2. Create an ALS model where IL6 transsignaling is increased and determine if disease pathology is altered. The progress of this Aim was greatly affected by the COVID-19 pandemic (See section on Changes and Problems for more details). Briefly, we began the performance of Aim 1 in the Spring of 2020. Immediately after initiation of this Aim, we were forced to reduce our animal colony to minimal breeding pairs in March of 2020. This Aim was re-initiated in July/August 2020. The SOD1 mouse colony had to be re-established, re-genotyped and re-characterized. An important component of this project was that we characterize the effects of IL6 transsignaling on SOD1 mice concurrently with studying the effects of IL6 transsignaling in our mouse models of IL6 transsignaling crossed with the SOD1 mouse model. We have currently regenerated the SOD1 mouse colony and have successfully crossed the SOD1 mouse with our IL6 transsignaling mouse models.

Note: In 2018, right after we received notice of the DoD award, we were notified that we were also receiving a NIH R03 to generate a second mouse model of IL6 transsignaling generated by a single nucleotide polymorphism that would mirror the single nucleotide polymorphism IL6R Ala358 that occurs in humans (see Figure 2). As both grants were awarded at the same time, we worked in parallel to generate both models of IL6 transsignaling. This was fortuitous because if one model failed to produce the desired genotype and functional phenotype, we would have the second model in hand. Importantly, we have been quite successful. Following initial characterization, both models behave as expected in terms of levels of soluble receptor. This provided two valuable experimental approaches to investigate IL6 transsignaling. The TMD model that was proposed and generated with the AL170130 award allows us to determine specific pathological changes that are due solely to IL6 transsignaling because in the homozygous animal there is no membrane bound receptor. We are continuing to perform our studies using both mouse models

The IL6R E357A model generated with funds provided by the RO3 award has a very unique value because these mice model human subjects who inherit the IL6R Ala358 allele. Over the past three years, we have shown that ALS patients who inherit this allele appear to have fast disease progression and different inflammatory/glial responses in the CNS (see Appendix). By crossing the IL6R E357A mice with the

SOD1 mice, we can begin to explore specific cellular mechanisms that may mediate these differences. *We have secured institutional funds to continue to support the IL6R E357A model for the 2020-2021.*

As we are completing our characterization of these models, we began to cross both mouse models with $SOD1^{G93A}$ (C57BL/6 background). We had only begun to collect the offspring from the initial crosses (SOD1 males X heterozygous IL6raTMD or E357A females) when our institution asked us to "pause" ongoing research because of SARS-CoV-2 and COVID-19. We maintained the crosses, but at requested minimum. *Nonetheless, our preliminary data are very encouraging because SOD1/TMD (n=3; 1F, 2M;* ***100% with deficits****) and SOD1/E385A (n=9; 5M, 4F;* ***89%*** *with deficits) mice showed earlier disease onset compared with SOD1 (n=8; 6M* ***25%*** *with deficits.* All *SOD1/ Il6ra*^{E357A} and *SOD1/TMD* mice resulting from these initial crosses are heterozygous for the IL6R genotype

Figure 2 ELISA measurement of soluble IL6 receptor in (A) humans (left; n=471), (B)*Il6ra*^{E357A} mice at P90 (Ala/Ala n=13; Ala/Glu/n=35; Glu/Glu n=19; p<0.001 across genotypes; one way ANOVA), and (C) TMD mice at P90 (TMD/TMD n=17; WT/WT n=16; p<0.001 across genotypes; one way ANOVA) plotted. NOTE: Y axis scales the same for A and B, different scale for C.

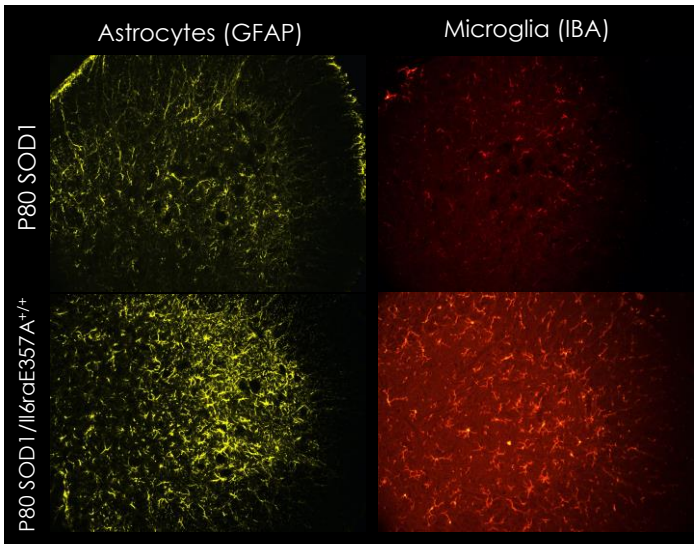

Figure 3. Glial Responses are enhanced in SOD1 with the IL6R E357A mice.

Our preliminary data suggest that in the SOD1 mouse model of ALS, enhanced IL6 transsignalling (SOD1 X $Il6ra^{E357A}$) promotes greater microglial and astrocyte activation (lower panels in image above) compared to classical IL6 signaling that would occur in the SOD1 mouse (upper panels).

Aim 3. Treat the mouse models with transsignaling inhibitor, soluble gp130 to determine if disease progression can be significantly slowed

This aim has been significantly delayed and may not be completed due to the effects of the COVID-19 on our ability to complete animal work. (See section on Changes and Problems for complete details)

What opportunities for training and professional development has the project provided?

Daniel Joshua Quillen is a MS graduate student in our Neuroscience Program. For part of his thesis project he is working with us to characterize the role of IL6 transsignaling in the ALS mouse model.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Our plans for the next reporting period are:

Our goal for the additional year of this project is to complete our crosses to generate ALS mice that experience enhanced IL6 transsignaling to determine

- if there are differences in neuromuscular junction denervation
- different glial responses in the CNS as suggested in the Tocilizumab clinical trial,
- and faster disease progression.

- **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

By determining if IL6 transsignaling is critical in promoting ALS progression and severity, we will have identified a critical pathway for ALS treatment. The success of this study will also give us new insights into how the inflammation, and most specifically IL6 signaling, may contribute to ALS initiation.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Below are the responses to Dr. Dougherty submitted in October 2020 regarding our grant AL170130. This narrative below describes the effects of the COVID-19 pandemic on the work performed.

On March 16th, 2020, Dr. Julie Freischlag, CEO and Dean, Dr. Greg Burke, Senior Associate Dean for Research and Chief Science Officer, and Chris O'Byrne, VP and Associate Dean, Research Administration notified the institution with the following to be implemented starting March 18th, 2020:

- *Pause all in-person clinical research activities not linked directly to the health of the research participant.*
- *Reduce on-site staffing for animal and mechanistic research by focusing on activities directly linked to essential functions, such as maintaining the health of animals involved in research studies.*

On March 18th, an additional notice clarifying basic science and animal research planning stated:

Basic Science and Animal Research Teams

- *Apply your lab-specific pandemic plan.*
- *Research teams should seek to reduce on-site staffing and activities to the absolute minimum possible. Please work* ***to complete ongoing experiments by close of business on March 25.***

- ***It is suggested to only pair breeder animals that will provide the number of offspring needed to continue the breeding colony. The number of offspring should not include animals for experimentation.***

- *Research teams should provide for animals as required in the approved IACUC protocol.*
- *Environmental enrichment may be provided at minimum levels, unless the specific environmental enrichment is prescribed per a special considerations plan, i.e., for some nonhuman primates.*
- ***Minimize ongoing work as much as possible now**.*

Animal Resources Program

- *ARP is working to reduce staff to those essential to maintaining the health and wellbeing of the research animals. The ARP will pause on non-essential activities effective March 25, including procedures that are not mandatory for the health of the animals.*
- *Veterinary clinical support will be reduced to essential activities. Veterinarians will remain available for consultation.*
- *ARP is stopping animal procurement, effective immediately.*
- *Transport will be available on a very limited basis until close of business March 25.*

How is variance in schedule going to impact cost, and can these impacts be quantified?

Mouse Colony

In accordance with our institutional guidelines shown above and the governor mandates, faculty and staff were allowed onsite after March 25th for only timed experiments that had already been established. With this in mind, we were able to sacrifice and collect tissue from animals that had already been assigned to specific ages for collection. We were able to freeze or preserve this tissue for experiments when we could return to the labs on a regular basis. Unfortunately, we did lose some tissue when our lab refrigerator malfunctioned and we were not aware for several days because we were not to be on site daily. In addition, while considered a minimal loss, it was compounded due to the animals lost could not be quickly replaced.

In accordance with the pandemic guidelines, we could not continue our breeding colony to generate animals for new experiments. We therefore had to sacrifice several pre-weaned litters of mice. We had to reduce our colony to only a few breeding cages to maintain the colony.

How did this impact our experiments?

We were able to finish collecting tissue from the IL6R mouse models at postnatal day 90 and 180 to characterize IL6R receptor expression in serum and tissues.

We were not able to continue to characterize the model in terms of responses to LPS stimulation because we were requested to "pause all experiments" and the litters of mice that had been generated for these experiments has to be sacrificed.

We were further set back as we were beginning to establish the crosses of the SOD1 X IL6R mice, and had to discontinue the colony expansion and reduce back to only cages that could maintain the colony

In July 2020, when we could return to the labs, we increased our number of breeding cages to expand the colony and began to resume experiments. With the COVID-19 pandemic, our research was set back by 6-9 months.

Personnel

During the shut-down, per Medical School guidelines, faculty and technical staff that were fully supported on research grants were not furloughed, but could have reduced hours. From March 25- July 1 our lab technician's time was reduced to 30 hours/week to account for reduced effort. She was primarily responsible for maintaining the colony during the "pause in research" and worked at home to maintain and update colony and lab records. Drs. Hawkins and Milligan continued to meet virtually to plan for experiments upon return to the labs as well as continue data analysis and manuscript preparation.

COVID-19 Cost Effects

The cost effects were multi-fold: 1.) cost to rebuild breeding colony, including animal per diem and reagents and supplies; 2.) unplanned cost to pay technical staff to expand animal colonies (Ms. Sabrina Lambeth). If desired, we calculate estimated losses.

Of note, during the COVID shutdown, we have transitioned into a no cost extension (NCE) on our grant. Dr. Milligan and I have significantly reduced our salary support under the NCE, however we continue to devote the required effort to the project. Because of the initial delay in hiring staff while the animals were being generated at UNC, we are able to continue with salary for our lab technician.

Have there been research-associated costs that were unanticipated due to COVID-19?

Yes. As mentioned above, we did not anticipate having to cull our colony, thus the regeneration costs of the mouse colony was a direct effect COVID 19. As a result, all of the mouse per diem, reagents, and supplies used to regenerate and genotype our colony will have to be extended, and thus can be contributed to the COVID 19 shutdown. As per our original communication, while we are in the process of restoring our research productivity, with the previous delays and concerns of another possible shutdown as we enter the fall and winter months, we thought it best to question if another NCE is possible. It is difficult for us to predict at this time anticipated remaining funds, but it is likely we may not be able to continue to support technical staff.

Actual or anticipated problems or delays and actions or plans to resolve them

Are there impacts to performance that will affect cost and/or schedule?

Yes to both. Before the COVID shutdown, the project was successfully progressing towards developing the IL6 transsignaling mouse/SOD1 mouse cross. Because of previous delays obtaining the IL6R mice at Wake Forest (3 months), we knew we would have to take our project into at least a 1 year NCE and had created a timeline to finish our work during the 1 year NCE. Now with the COVID shutdown, we will have to repeat or delay multiple research steps, including regeneration of the IL6 breeding colony, delayed generation of the IL6 transsignaling/SOD1 mouse cross, and critical functional experiments that we expected to be performing at this time, but which will now be delayed at least 6-9 months.

Changes that had a significant impact on expenditures

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Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Winston-Salem, NC

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.
  Nothing to report

  Books or other non-periodical, one-time publications.

  Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

We have developed a mouse model where the transmembrane domain or the IL6 receptor has been removed.

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Gregory Hawkins
Project Role: PI
Nearest person month worked: 2.7 months
Contribution to Project: Dr. Hawkins is a Professor of Biochemistry with experience in IL6 transsignaling research. Dr. Hawkins is working closely with Dr. Milligan in designing and testing the mouse model produced in this proposal and will be involved in data interpretation and manuscript preparation.

Funding support:

P30 CA012197-43
Wake Forest Baptist Comprehensive Cancer Center - Cancer Center Support Grant
The Wake Forest Baptist Comprehensive Cancer Center is a multidisciplinary interdepartmental research center, organized into four divisions: Two Basic Science Programs, Clinical Research, and Cancer Prevention and Control.
Role: Co-Investigator

R01 NS036695-15A1
Genetic Environmental Risk Factors for Hemorrhagic Stroke
To determine the gene expression and epigenetic factors that contribute to hemorrhagic stroke.
Role: Co-investigator

1R01HL142992-01
Effects of Rare Variants and Ancestry on Beta Agonist Response in Asthma and COPD
Surveillance trials suggest that the risk for life-threatening asthma exacerbations and asthma-related deaths are increased with long-acting beta$_2$-adrenergic receptor (β2AR) agonist (LABA) therapy; however, large clinical safety trials have not confirmed these observations despite studies showing that African Americans with asthma are more likely to respond adversely to LABA therapy. We have shown that ancestry-specific rare variants in the β2AR gene are associated with worse asthma control in people using LABA and that African genetic ancestry associates strongly with lung function in African Americans with severe asthma and COPD suggesting that genetic variants could play a role in drug response and disease severity. We propose genetic studies based on β2AR pathway gene variants and whole-genome studies of rare variants and genetic ancestry to identify novel mechanisms for inter-ethnic differences in drug response and disease severity.
Role: Co-Investigator

1R21 CA253362-01
Tumor Microenvironment at Single Cell Level in Black and White NSCLC Patients
Major Goals: To test our hypothesis that distinct tumor immune cell ecosystems exist between AA and CA lung cancer patients, which may be caused by differential tumor genetic mutation patterns, and which may explain observed patterns of disparate lung cancer outcomes that may be partially overcome in the era of immunotherapy.
Role: Co-Investigator

1P30 DK124723-01
North Carolina Diabetes Research Center
Major Goals: The prevalence of diabetes mellitus in the United States is reaching epidemic proportions and accounts for a huge national burden of morbidity, mortality, and health care expenditures. The mission of the Diabetes Research Centers is to serve as a key component of the NIDDK-supported research effort to

develop new therapies and improve the health of Americans with, or at risk for, diabetes and related endocrine and metabolic disorders.
Role: Co-Investigator

R01 MD015395
Social Factors, Epigenomics, and Lupus in African American Women (SELA
Major Goals: We will specifically seek to identify and characterize the epigenetic mechanisms by which positive and negative social experiences affect gene function and thereby influence the risk of lupus in African American women.
Role: Co-Investigator

R01 DK120667-02
Systems Genetics to Identify Neuronal Genes for Diet-Induced Obesity
Major Goals: The goal of this project is to identify genetic loci and underlying genes that regulate adiposity in response to a high fat diet. In addition to genetic mapping, we will use RNAseq to determine transcript abundance of genes in the hippocampus and hypothalamus and integrate these data with the physiological mapping.
Role: Co-Investigator

P30 AG049638
WF Alzheimer's Center Pilot Award
Genetic and Biomarkers of IL6 Transsignaling in Alzheimer's Disease
We propose to investigate if increases in CSF IL6 and soluble IL6 receptor are exhibited by AD patients, and if patients who inherit the IL6R variant allele Asp358Ala demonstrate faster conversion from MCI to dementia. Data from this pilot award will serve as preliminary data for RO1 application.
Role: PI
Overlap: None

Name: Carol Milligan, PhD
Project Role: co-I
Nearest person month worked: 2.7 months on AL170130
Contribution to Project: Dr. Milligan is a Professor in Neurobiology and Anatomy with experience and expertise in neurodegenerative processes, notably those that occur in ALS. She is working with Dr. Hawkins in designing and testing the mouse model produced in this proposal, evaluating the role of IL6 transsignaling in the ALS mouse model and will be involved in data interpretation and manuscript preparation.

Funding support:

DOD W81XWH2010265
Novel Cas9/gRNA Ribonucleoprotein Bionanoparticles for Safe and Efficient Inactivation of ALS Disease-Causing Mutations
We propose to engineer an AAV capsid-based bionanoparticle to achieve efficient CRISPR/Cas9 RNA delivery to the CNS leading to only transient expression of Cas9. We will use these novel bionanoparticle to deliver Cas9 mRNA and gRNAs to remove expanded G4C2 repeats from the C9ORF72 gene in a mouse model of ALS
Role: co-PI
Overlap: None

P30 AG049638
WF Alzheimer's Center Pilot Award
Genetic and Biomarkers of IL6 Transsignaling in Alzheimer's Disease
We propose to investigate if increases in CSF IL6 and soluble IL6 receptor are exhibited by AD patients, and if patients who inherit the IL6R variant allele Asp358Ala demonstrate faster conversion from MCI to dementia. Data from this pilot award will serve as preliminary data for RO1 application.
Role: co-PI
Overlap: None

1T32NS115704-01A1
Neuroscience Training at Wake Forest
The funds will support the broad-based, interdisciplinary training of our PhD students in the Wake Forest Neuroscience Program.
Role: PI
Overlap: None

Hope for Tomorrow ALS Foundation (Milligan)
The funds provided support for preliminary studies of IL-6 signaling in ALS patients.
Role: PI (effort as needed)
Overlap: None

Brian White ALS Foundation Funds
These funds are a contribution by the Department of Neurology for the Project "Hsp70 as a Potential Treatment for ALS"
Role: PI (effort as needed)
Overlap: None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

During the past three years Drs. Milligan, Hawkins and colleagues have investigate the potential influence of IL-6 transignaling in ALS patients. We have reported the following results.

1. ***The IL6R Ala358 variant influences IL6 signaling in the CNS that is unique to ALS. IL6R Ala358 allele also associates with serum levels of IL6R in all subjects. ALS subjects with the IL6R C allele have faster disease progression.***

   Wosiski-Kuhn M, Robinson M,* Arounleut P, Strupe J, *Martin M,* Caress JB, Cartwright M, Bowser R, Cudkowicz M, Langefeld C, Hawkins G, Milligan C (2019). The Interleukin-6 Receptor Coding Change Asp358Ala and Central Interleukin-6 Transsignaling as a Disease Modifier in Amyotrophic Lateral Sclerosis. *Neurology: Neuroimmunology & Neuroinflammation* 6(6):e631.

Classical IL6 signaling occurs when IL6 binds the membrane bound IL6 receptor on specific, limited cell populations (e.g., T cells and hepatocytes) and triggers intracellular signaling through the membrane bound glycoprotein 130 (gp130) co-receptor. In contrast, a second IL6 signaling paradigm termed IL6 transsignaling is driven by the availability of extracellular soluble IL6 receptor (sIL6R). The sIL6R is generated by receptor "shedding," a process where metalloproteinases cleave IL6 receptors from the cell surface (1-3). Once cleaved from the cell surface, sIL6R can form an active complex with IL6 and engage membrane bound co-receptor gp130. Since gp130 is constitutively expressed on all cells, IL6 transsignaling can activate IL6 dependent cell signaling in cells that do not express IL6 receptor. In humans, up to 99% of serum sIL6R results from proteolytic cleavage (shedding), and >50% of the sIL6R level variability is attributable to a common *IL6R* coding variant (rs2228145; Asp^{358}>Ala^{358}; referred to as IL6R Ala358 or C allele) (2-4). The *IL6R* Ala358 variant occurs at a frequency of ~10% in African, ~40% in European, and ~50% in Native American ancestries (4). ***In subjects inheriting the IL6R Ala358 variant, localized and systemic IL6 transsignaling is increased in the presence of IL6.*** We similarly found the same percentages

in three cohorts of ALS patients suggesting presence of the variant does not predispose one to ALS, but may influence disease progression. Therefore, disease progression in more than half of ALS patients may be influenced by transsignaling mechanisms. Understanding how, and when and where IL6R blocking strategies may prove beneficial will provide critical information to better design additional clinical trials.

2. ***IL6 and functional status in ALS patients***

In a second, longitudinal study, we show that serum IL6 levels negatively correlate both with the patient's functional status as measured by the overall ALSFRS-R and subscores, and with respiratory function as measured by the percent predicted FVC (ppFVC). The correlations in the cases of ALSFRS-R limb and respiratory subscores, and ppFVC are only present in the two-thirds of patients who carry the IL6R *Ala358* variant that mediates IL6 transsignaling

Wosiski-Kuhn M*, Caress JB, Cartwright M, Hawkins GA, Milligan C (2020). Interleukin 6 (IL6) Level is a Biomarker for Functional Disease Progression Within IL6R358Ala Variant Groups in Amyotrophic Lateral Sclerosis Patients. *Amyotroph Lateral Scler Frontotemporal Degener*. 2020 Sep 14:1-12. doi: 10.1080/21678421.2020.1813310. Online ahead of print.

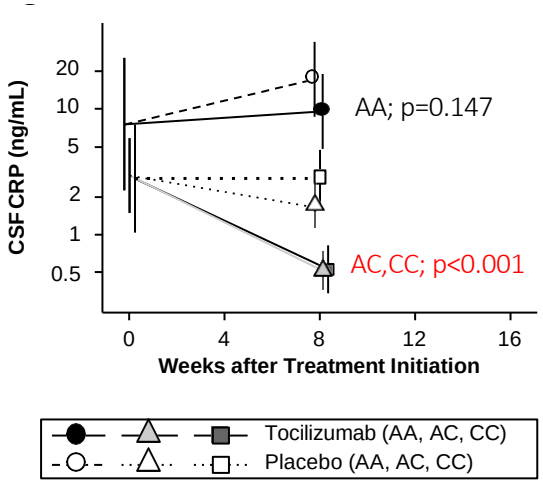
3. ***The IL6R Blocker Tocilizumab Clinical Trial in ALS***

Milligan C, Bowser R, Atassi N, Babu S, Barohn R, Caress J, Cudkowicz M, Evora A, Hawkins G, *Wosiski- Kuhn* M*, Macklin E, Shefner J, Simmons Z, Ladha SS (202X). Tocilizumab is safe and reduces C-reactive protein concentrations in the cerebrospinal fluid of patients with the interleukin 6 receptor Asp358Ala variant. *Muscle and Nerve.* 2021:1-12. https://doi.org/10.1002/mus.27339

Our results suggest individuals of specific genotypes for *IL6R* may be ideally responsive to IL6 signal blocking therapies. We teamed with Shafu Ladha and Bob Bowser at the Barrow Neurological Institute (see letter) who directed the tocilizumab (Actemra) in ALS (clinicaltrials.gov number NCT02469896; NEALS, 2019; Milligan et al., in revision). Tocilizumab (Actemra®) is a humanized monoclonal antibody to IL6R and already commercially available to treat refractory moderate to severe rheumatoid arthritis (5) that could be repurposed to study IL6 inhibition in ALS. Study results indicate:

- Tocilizumab exhibits anti-inflammatory actions by preventing both classical and trans-signaling effects of the cytokine (6). We found tocilizumab (TCZ) treatment was safe, and did not adversely alter disease progression.
- PBMC gene expression profile was inadequate as a predictive or pharmacodynamic biomarker and that expression patterns did not correlate to plasma cytokine levels.
- Increases in sIL6R were observed in all TCZ-treated patients (3.4-fold (95% CI 3.1 to 3.6 fold) in the tocilizumab group vs. a 0.02-fold decline (95% CI -0.44 to +0.40)) that likely reflect altered catabolism of the sIL6R/IL6/TCZ complexes as reported in other patient populations treated with the drug (38).
- In CSF sIL6R also increased 0.6-fold (95% CI 0.4 to 0.8 fold, adj $p < 0.001$) suggesting the peripherally administered agent can have desired physiologic effects in the CNS. There were no differences across genotypes for these measures.
- Plasma levels of the inflammatory marker, C-reactive protein (CRP) was reduced 88% (95% CI -94% to -76%) among all tocilizumab participants from baseline to the average of all follow-up assessments vs. a 4% increase (95% CI -59% to +259%) among placebo participants (-3.0-fold relative change, 95% CI -4.6 to -1.5-fold, adjusted p=0.003), and support a functional effect of the drug as IL6 itself appears to regulate CRP and other acute phase protein production in the liver (7). The role of peripheral CRP in ALS remains unclear.

- ***An unexpected (not a predefined study outcome biomarker), but nonetheless, interesting result was the reduction of CSF CRP only in tocilizumab-treated ALS patients with at least one IL6R Ala^{358} allelle*** *(Figure 3). There was a 2.5 fold reduction in CSF CRP in CC individuals treated with tocilizumab as compared to those who were note (p <0.001; 95%CI: -3.3 to -1.7). Tocilizumab treated AC individuals showed a 1.7 fold reduction (p <0.001; 95% CI: -2.4 to -1.1), while there was no significant change in the AA individuals (p=0.147; 95% CI: -2.0 to 0.3).* ***We believe these results suggest IL6 trans-signaling mediates a distinct CNS response in individuals inheriting the IL6R Ala358 allele****.*



Tocilizumab Study. Statistical differences determined by shared base-line, repeated measures ANOVA. **Study design**: Twenty-two participants with amyotrophic lateral sclerosis whose peripheral blood mononuclear cell (PBMC) gene expression profile reflected high mRNA expression of inflammatory markers were randomized 2:1 to either three monthly treatments (weeks 0, 4, 8) of 8 mg/kg IV tocilizumab or placebo. Participants were followed every 4 weeks for 16 weeks and assessed for safety, tolerability, plasma and cerebrospinal fluid (CSF) inflammatory cytokines, C reactive protein (CRP), interleukin 6 (IL6), soluble interleukin 6 receptor (sIL6R), and soluble gp130, and standard clinical measures. Plasma was collected at each visit. CSF was collected at baseline prior to first dose of study drug and again after the third dose (week 8). At the end of the study, all participants underwent genotyping for the Asp^{358}Ala polymorphism (C allele) of the *IL6R* gene.