Penn Medicine

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June 29, 2023

TO: The Job Research Foundation Board

RE: Final summary report - Nanotechnology as a novel cellular therapy for patients with Job Syndrome

Dear Dr. Fischer and Members of the Board,

We are very excited to provide you with our final summary report of the study entitled "Nanotechnology as a novel cellular therapy for patients with Job Syndrome." Our novel research findings advance our understanding of neutrophil function and demonstrate a proof-of-principle of using neutrophil-avid nanocarriers (NANs) as a novel platform to deliver cargo to neutrophils in patients with Job Syndrome. These important finding would not be possible with support from The Job Research Foundation. Here are highlighted milestones:

Procurement of blood samples from patients with Job Syndrome (JS). The goal of our study was to investigate the function of neutrophils from patients with JS. To conduct this research, we needed to procure blood samples from patients with JS (STAT3-ADDN) and control human blood. Collection of human blood samples requires a protocol and its approval by the Institutional Review Board (IRB). In January-February 2020 we wrote the protocol to obtain human blood from patients with JS and control human blood samples. In early March, the protocol was submitted for review by Allergy/Immunology group at the Children's Hospital of Philadelphia (CHOP). Unfortunately, due to COVID-19 pandemic, all non-COVID-19 related research-based work at the University of Pennsylvania and CHOP was suspended in mid-March 2020. Regardless of this challenge, the protocol was reviewed and approved by the Division of Allergy and Immunology in early May 2020. Dr. Jennifer Heimall also worked with a study coordinator on the IRB application and informed consent. During June, the protocol was moving through the institutional channels and was submitted for IRB review on July 15, 2020. The IRB protocol # 20-017679 was approved on August 21, 2020. Following up on this important approval, we initiated our study. In September 2020, we met to discuss a timeline of collection of blood samples from JS patients during their regular doctor visits. In February 2021, the first blood sample from a patient with Job syndrome was collected for neutrophils isolation. Because JS is a very rare condition, we had only two JS patients available to donate their blood for research. To address this limitation, Dr. Heimall reached out to her collaborators at the National Institute of Health (NIH) Dr. Freeman on logistics of obtaining blood samples from NIH, transport them to PENN and to conduct experiments at PENN. To receive blood samples from NIH we obtained IRB-approved protocol and MTA. We received an additional 4 serum samples from patients with JS. We also obtained 5 serum samples from healthy controls for our study. The blood samples were collected JS patients and controls listed in Table 1 in Appendix 1.

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Human neutrophils phagocytose fluorescent nanoparticles. We have recently shown in our publication in Nature Manotechnology (https://www.nature.com/articles/s41565-021-00997-y) that our lab's group of Neutrophil-Avid Nanocarriers (NANs) are phagocytosed by neutrophils, that interaction is enhanced when the particles are opsonized by serum, and they are concentrated within inflamed lungs. NANs are a potential future therapeutic that could improve pneumonia treatment for this patient population. To overcome the decreased bactericidal and fungicidal activity of neutrophils in patients with JS, we have created a new technology platform, neutrophil-avid nanocarriers (NANs), that can be used to deliver antimicrobial cargo to neutrophils. We hypothesized the NAN's can be used as a cargo for drug delivery to increase the antifungal function of neutrophils in patients with JS. We believe this technology is uniquely suited to benefit patients with JS who have hypofunctional neutrophils and difficulty tolerating antifungal medications.

Initial experiments were performed on blood from control subjects with a goal to optimize neutrophils isolation protocol. Neutrophils were isolated by density gradient followed by negative selection. CD66b staining was used to confirm neutrophils positivity. The summary results are presented in **Figure 1 Appendix 2**. Briefly, neutrophils were isolated from whole blood from healthy donors and AD-HIES patients using flow cytometry. NANs or Nanogels (NGs) were incubated for 60 min in either media or donor-matched serum. Neutrophils were then exposed to the FITC-NGs, and neutrophil uptake was quantified with flow cytometry. In the first panel, the percentage of present neutrophils that were positive for fluorescence, and therefore NG uptake, is seen. Similar to our previous work using mouse models, we found that there were more neutrophils positive for NG fluorescence when **the particles are serum-opsonized prior to neutrophil exposure**. There was no significant difference in the percentage of neutrophils with NG between the two groups. In the second panel, mean fluorescence intensity (MFI) is used as a measure of **the amount of NG** uptake by the neutrophils. We again found that human neutrophils react to NGs similar to our previous mouse work, with more NG fluorescence seen after NG serum opsonization. This suggests that not only are more neutrophils taking up particles, but there are more particles per neutrophil following serum opsonization. Unlike in the first panel, there are differences seen **between the healthy donor neutrophils and the STAT3-ADDN donor** neutrophils in the serum NG group. The MFI in STAT3-ADDN neutrophils is significantly lower than that seen in the healthy neutrophils, suggesting that while neutrophils ingest NGs after opsonization, the STAT3-ADDN **neutrophils are not able to uptake as many particles per neutrophil**. Figure 1B shows the same results as the second panel in Figure 1A. The flow cytometry histograms have the same peak when looking at the NG condition (royal blue and red lines); however, in the serum NG (maroon and navy lines) there is a peak shift. The Healthy donor neutrophils have a shift to the right, corresponding to more fluorescence, and therefore, more particle uptake by the neutrophils. In Figure 1C, confocal microscopy was used to evaluate the cellular location of NGs. Neutrophils were isolated from whole blood and incubated with serum opsonized FITC-NGs. DAPI was used to for nucleus stain (blue). Membrane was stained with red DiD. Using z-stack analysis, NGs are seen within the neutrophil, suggesting that they are phagocytosed by the neutrophils.

Serum opsonization results in a complement-predominated protein corona on the NG particles. Because serum opsonization altered NG uptake by neutrophils, we next performed mass spectrometry on NANs after exposure to serum from patients with and without JS to differentially identify soluble inflammatory factors in human serum that promote NAN-neutrophil interaction. NGs were incubated in either healthy donor or STAT3-ADDN patient serum. They were then washed 3 times and prepared for mass spectrometry and protein profiling. As shown in Figure 2A (Appendix 2), the protein signature of the NG coronas was compared and revealed 555 overlapping peptides (purple) found in the NGs prepared from both healthy and JS patient serum. There were 48 unique peptides identified on the

healthy donor serum samples (pink), and 428 unique peptides from the patient serum samples (aqua). Comparison of the 10 most abundant peptides in the protein corona of the healthy donor serum NG shown in Figure 2B on left and the STAT3-ADDN patient serum samples on the right. Both serums bound a predominance of C3, a complement cascade component, on the NG particles. They additionally both have C5 and C4, other complement cascade components, identified in the top 10 peptides per sample. Albumin, the most abundant serum protein, is the second most bound peptide in both healthy donor and patient serum exposed NGs. While the remainder of the top 10 peptides identified have a few overlapping proteins, such as actin and filamin-A, the remainder of the highest abundance peptides in the protein corona from the STAT3-ADDN patient were from cytoskeletal peptides. (Figure 2C and 2D) The normalized total spectra counts for C3 and C5, respectively, of the individual healthy and patient samples. The median counts of the groups are not statistically significantly different when compared.

STAT3-ADDN patient serum had a unique NG protein corona composition when compared to the corona of healthy donor serum. As shown in Figure 3, NGs were incubated in either healthy donor or STAT3-ADDN patient serum. They were then washed 3 times and prepared for mass spectrometry and protein profiling. Proteomic results were analyzed for peptides with a statistically significant differential between the healthy and patient samples. Shown are the results of the identified peptides that were present in at least 50% of the samples of either the healthy or patient group. Previous literature has shown that STAT3 is a negative regulator of neutrophil function, and Panel A show there is an upregulation of neutrophil proteins in the patient samples. STAT3 is a fundamental mediator of the induction of liver acute-phase genes. In panel C, we see that there is a significantly lower amount of each acute phase protein in the patient samples as compared to the healthy.

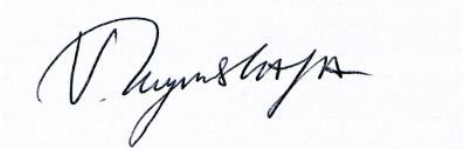
Summary:

- **Neutrophils from patients with JS demonstrate significant impairment in NGs uptake after serum opsonization.**
- **Serum opsonization results in a complement-predominated protein corona on the NG particles.**
- **STAT3-ADDN patient serum had a unique NG protein corona composition compared to the corona of healthy donor serum.**

Reporting of results of the study: Our findings were summarized in a form of abstract and were presented at the Pediatric Academic Societies (PAS) 2022 Meeting and at the 2022 American Thoracic Society (ATS) International Conference. Currently, we are working on the manuscript to summarize our study supported by this award.

Please do not hesitate to contact me if you have any questions.

Best Regards,



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Appendix 1

Table 1. Description of samples collected from control and JS patients.

Sample	Age	Sex	Race
Healthy 1	29	M	Caucasian
Healthy 2	38	M	Caucasian
Healthy 3	60	F	Caucasian
Healthy 4	33	M	Caucasian
Healthy 5	42	M	Caucasian
Patient 1	18	M	Hispanic
Patient 2	26	F	African American, non-Hispanic
Patient 3	27	F	Caucasian
Patient 4	42	F	Hispanic
Patient 5	7	M	African American
Patient 6	60	M	Caucasian

Appendix 2

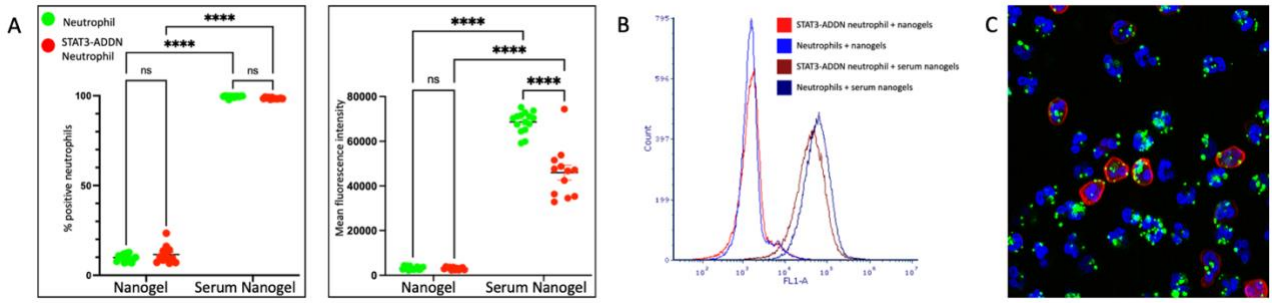

Figure 1. Differential uptake of NGs by JS neutrophils.

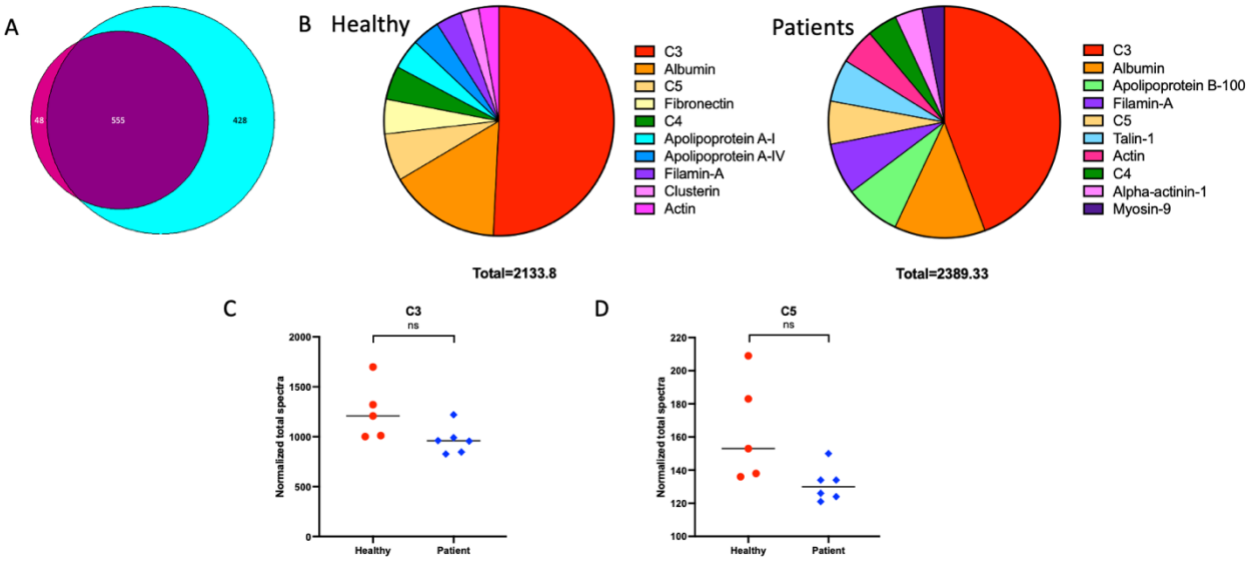

Figure 2. Serum opsonization results in a complement-predominated protein corona on the NG particles.

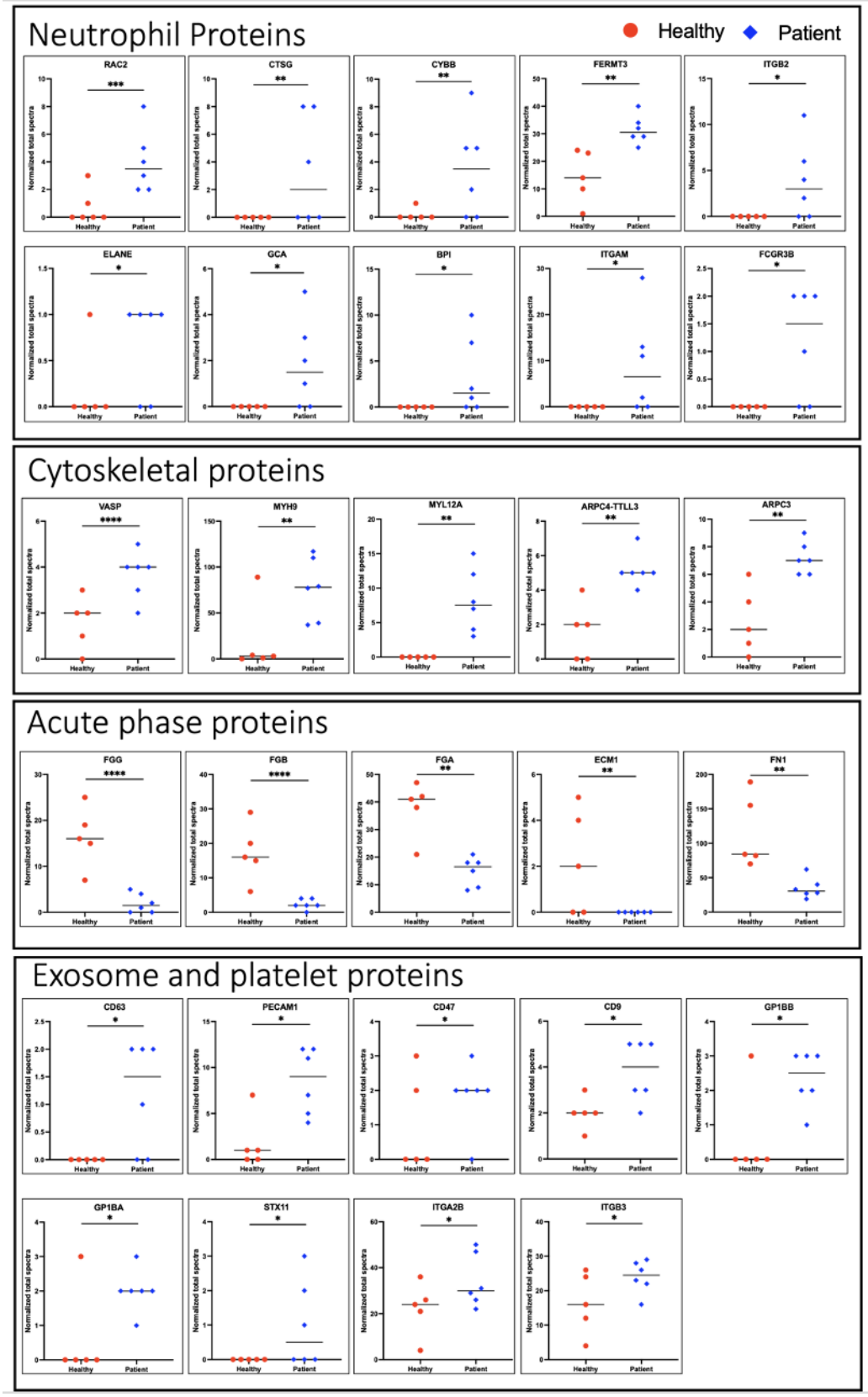

Figure 3. Differential composition of proteins opsonized by NANs exposed to JS serum.