Rachel Caspi, Ph.D.

Chief, Laboratory of Immunology| NEI
Head, Immunoregulation Section| NEI
Acting Chief of the Clinical and Translational Immunology Unit| NEI

Mary Mattapallil, DVM; Ph.D.

Staff Scientist, Immunoregulation Section| NEI

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Will you please speak about the premise of your research that is looking at how the endogenous microbiome might affect the cytokine storm phenomenon observed in severe COVID-19 cases using a humanized mouse model?

Severity of Covid-19 symptoms and the related mortality is associated with excessive inflammation leading to cytokine storm, which is a rapid release of cytokines from inflammatory cells. In recent years we have seen an explosion of information about the role of microbiome in health and various disease conditions. Studies have shown that changes in gut microbiota can influence lung immunity through circulation of microbial metabolites or endotoxins. Altered bacterial populations in the gut (dysbiosis) can impair integrity of GI tract leading to gut "leakiness", and exit of microbes into local lymphoid tissues. Systemic release of low-grade endotoxins from these microbes into blood stream can cause increase in LPS contributing to inflammation. Studies have shown increasing levels of dysbiosis during aging process where the gut microbial community becomes enriched in proinflammatory commensals compared to beneficial microbes, which is in line with COVID being more severe in the older age group. At the time this project was conceptualized, there were also anecdotal reports that antimicrobial treatment may have efficacy in reducing symptoms, e.g., hydroxychloroquine and azithromycin, but those reports largely failed to be confirmed in clinical trials. All this information from the literature encouraged us to explore a mouse model with human immune system to study the role of gut microbiome in the induction and severity of cytokine storm.

We essentially hypothesized that the magnitude of the cytokine storm *in vivo* in a humanized model system can be affected by the microbiome resident in the host. While we favor the idea that depletion will have an ameliorating effect, the opposite is also possible. *Since there is no information currently on the effect of microbiome on cytokine storm in a humanized model, any outcome, even a negative one, would be informative.*

Will you please discuss how you structured your study including your methodology, your metrics, and some of the tools and equipment used to conduct it?

We used a ‘humanized' mouse model, where we introduced human immune cells into a genetically engineered immunodeficient strain of mice that lacks its own immune system. Prior to humanization we depleted the gut microbiota in the test group of the humanized mice by administering a combination of broad-spectrum antibiotics (Ampicillin, Metronidazole, Neomycin, and Vancomycin) through drinking water whereas the control group only received the human immune cells, but no antibiotics. Two weeks after antibiotic treatment and microbe depletion, mice received PBMCs from a single healthy human

donor. Type, dose and duration of antibiotic treatment were based on our previous experience in inbred mouse strains with normal murine immune system. Our prior experience with humanization of immunodeficient mice was an added advantage to quickly adapt the experimental conditions for this project. Level of human lymphocyte engraftment was determined by single cell analysis by flow cytometry, 2 weeks after human cell transfer. When the animals were sufficiently engrafted with human immune cells, animals with and without antibiotic treatment were either challenged or not challenged with antibody to human T cells which is known to induce cytokine storm in other animal models. A hallmark of cytokine storm is elevation of circulating cytokines in the serum, and a quick drop in body temperature, which we measured within 6 hours of challenge. A panel of 10 human cytokines and chemokines were quantitated by multiplex proteomics approach using the clinically approved Mesoscale Discovery™ system.

What have been your individual roles in this study, and how have your educational and professional backgrounds prepared you for tackling COVID?

Dr. Caspi conceptualized the project, and Dr. Mattapallil is carrying it out. We are both PhDs in immunology and MM additionally has a degree in Veterinary science. One of our interests is developing animal models for human ocular inflammatory and autoimmune disease. In recent years our work has branched into the study of commensal microbiota in this context and we showed for the first time, a role for gut microbiome in modulating inflammation in the eye. One of the methods we used is depletion of gut microbiota using short- and long-term antibiotic treatment in inbred strains of mice. In the recent past we had also optimized a model of chronic graft-versus-host disease (GVHD) in humanized mouse model for conducting preclinical trials of human biologicals.

What challenges has your team experienced thus far?

It has been a challenge to maintain immunocompromised mice on antibiotic treatment, as they are more prone to undergo temporary weight loss and dehydration as a result of their gut microbiota elimination. In addition, we are also limited by the lack of appropriate reagents to uniquely identify human cytokines or chemokines in the mouse serum. There is a great deal of homology between human and mouse proteins, resulting in cross reactivity of the reagents and this has impeded interpretation of results for some cytokines. Finally, the 'humanized' mouse model continues to be developed and brought closer to the human by improved genetic engineering for more soluble factors that support human immune cells better, so future experiments with this ever-improving model may provide additional, or better, information than the current model.

What observations have you made to date, and has anything surprised you?

This model actually involves 2 types of cytokine storm: the more chronic one elicited by the GVHD that develops in these animals, and the acute one elicited by challenge with anti-human T cell antibodies, which is superimposed on that background. Another factor that can be influenced, depending on the timing of antibiotic administration, is efficiency of engraftment (acceptance) of the human immune cells in the mouse. Thus far, we observed that depletion of gut microbiome using a combination of antibiotics can enhance engraftment or humanization of mouse immune system. We saw a strong correlation between drop in body temperature due to cytokine storm in antibiotics treated group which can be indirectly due to enhanced human cell engraftment. Quantitatively there was an increase in cytokine release in the antibiotics treated dysbiotic group of animals with respect to proinflammatory cytokines

such as GM-CSF, IFN-g, TNF-a, MIP-1a (which we can differentiate from mouse cytokines) which are also reported to be high in Covid-19 patients. However, exactly how much of this effect is due to increased human cell engraftment and how much dysbiosis will have to teased out in the future experiments.

What do you believe could some of the short-term and long-term outcomes of the study be?

Since there is no information currently on the effect of microbiome on cytokine storm in a humanized model, any information that we gather would be informative. If successful, it might help us understand better how the composition of gut microbiome affects human T cell responses to immune/inflammatory challenges during dysbiosis and identify microbial populations and/or their metabolites that may modulate cytokine storm and potentially use them as probiotic therapies

Can you envision subsequent research based on your findings?

We still have many questions in this study: Need to separate the effect of gut flora modulations from engraftment per se vs. cytokine storm, and dissect the effects on GVHD-related vs. acute challenge-related responses. Hopefully these findings can contribute to the knowledge of how gut microbiota affect cytokine storm in general. Even if it turns out that antibiotics mostly improve engraftment of foreign cells, without major effects on the cytokine storm after engraftment, there is something to study here, as hematopoietic cell transplantation is a common procedure in medicine.

What are some of the personal challenges and opportunities COVID has presented for you?

Challenges: Impairment of free interaction and effective communication with colleagues and collaborators, difficulty to train or receive training on complex techniques, difficulty in scheduling live animal experiments, tissue culture work that requires close attention on consecutive days.

Opportunities: Ability to attend seminars or conferences (even international) just a click away. Ability to discuss scientific methods and concepts with scientists around the globe through widely accepted online technology. While not a substitute for in-person meetings, current virtual technology may cause a lasting change of how we communicate science to our peers.

Thought Provoking Question: How do you hope that COVID will change the world for the better?

Hopefully it will teach us that by coming together we can overcome major existential challenges. Even young children learned some healthy habits to prevent communicable diseases. Health care system, worldwide, learned how to be vigilant and prepared for any infectious outbreak. Learned how to share resources and expertise globally in the face of a disaster. Finally, almost everyone learned resilience and quicker adaptation to new technologies for distant education and telework.