Interviews

EMJ had the pleasure of interviewing Dennis Lee Kasper and Meghan Azad, two pioneers in the emerging field of the microbiome. Kasper discusses the fascinating interaction between the gut microbiota and immune system, highlighting the complexity of this newly discovered 'organ'. Azad explores how infant nutrition and breastfeeding shape the early microbiome, with significant implications for long-term health and development of chronic diseases.

Featuring: Dennis Lee Kasper and Meghan Azad

Dennis Lee Kasper

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Q1 Your major research focus lies in the interaction between the gut microbiota and the immune system. How have you seen the field evolve since the start of your career?

Early in my career, there was a small group of investigators interested in commensal microbes, and these were basic science-oriented microbiologists. There was a flurry of papers in the 60s and 70s about the role of commensal microbes (a.k.a. anaerobic bacteria) in disease, bringing about a more general awareness of commensals. Along with this awakening, antibiotics specifically effective against anaerobic bacteria were developed. Of interest were infections arising from commensal colonised sites where there was leakage or spread of commensal bacteria to normally sterile sites, including the peritoneum, lung, brain, and liver. These infections often manifested themselves as abscesses.

Groups in Germany and the USA who were studying microbes in the gut were able to raise germ-free mice, and they quickly learnt that these mice were very susceptible to infection. When their isolators got contaminated, these mice often died. It then became more or less known that commensal microbes had something to do with fortifying the immune system, but nothing specific was understood.

For the prior 30 years, I had been studying infection and abscess formation, and we had focused on a gut anaerobic organism called *Bacteroides fragilis*. When I looked at clinical studies that were enumerating anaerobic bacteria associated with diseases like peritonitis or lung abscesses, *B. fragilis* kept coming up as an important contributor. It did become clear very early that multiple commensal organisms were often isolated from infectious sites, unlike classic infections like pneumonia, meningitis, or sepsis, where typically one organism is responsible.

> **Our gut microbiome profoundly shapes our immune system; it both regulates our immune status and can throw it off kilter**

We made a number of observations about *B. fragilis* that clearly differentiated it from the typical gram-negative pathogen. Pathogens, such as pneumococci, meningococci, streptococci or *Escherichia coli* have one capsular polysaccharide in a given strain. When we were trying to isolate the capsule of *B. fragilis*, we were getting different chemical results from grow up to grow up, which was very confusing. Then, around the late 80s, we started to understand that each organism made several polysaccharides and could express them as capsules, which was very unusual. When *B. fragilis* was sequenced by the Sanger Centre, we learnt that it has loci to produce at least eight polysaccharides. Now, it's known that some other *Bacteroides* in the gut can make more than one polysaccharide.

A typical polysaccharide may have 3–15 genes responsible for its synthesis, and those genes occur in loci or operons. These are groups of genes that are flanked together and regulated by a single promoter. Work done with a former postdoc in my lab, Laurie Comstock, University of Chicago, Illinois, USA, showed that *B. fragilis* had at least eight loci for the production of polysaccharides, and that there was an unusual genetic mechanism that regulated it. We named these polysaccharides as A, B, C, D, E, F, G, and H. It turned out polysaccharide A (PSA) was the most important, and also the most abundant.

When we began studying PSA, we observed some unanticipated immunologic responses to this molecule. For context, all the childhood vaccines against pneumococcus, influenza, and meningococcus are conjugate vaccines: polysaccharides chemically coupled to proteins. The reason behind this is that these polysaccharides themselves, particularly in young children, were not immunogenic, so that is why they were coupled to proteins. It was a dogma in immunology that polysaccharides don't activate T cells; they were T cell-independent, and by coupling the polysaccharide to a protein, T cell help was activated.

However, former postdoc Brian Cobb, Case Western Reserve University, Cleveland, Ohio, USA, found that PSA was activating T cells in the absence of a protein, and that became a major focus of our work. PSA is processed and presented by antigen presenting cells (APCs). Former postdoc Arthur Tzianabos, Lifordi Immunotherapeutics, Inc, Boston, Massachusetts, USA, showed that these APCs induce CD4+ T cells to make a cytokine called IL-10, which actually turns off immune responses and inflammation. I thought, "This organism's induction of T cells to make IL-10 must have something to do with it living in the gut." In the gut, you have 100 trillion organisms, and you and I are sitting here talking to each other, and we're fine. But, how is it that if you had 100 trillion organisms in your blood, you would not be alive? There's an immunologic phenomenon called tolerance, which is very important to health and to preventing autoimmune disease. When you break tolerance, you start making immune responses to yourself.

In the late 90s, Jeff Gordon, Washington University, St. Louis, USA, focused on identification and enumeration of specific microbiota, and understanding microbiota populations in health and disease. He focused on genomic and metabolic relationships of the host and their commensal microbes. I think the word 'microbiome' was actually coined by Nobel Laureate Joshua Lederberg, from Stanford University, California, USA, and Gordon capitalised on the term 'microbiome'. This was a turn of the century, and a rumination was getting louder that said, "Gee, these organisms may be important."

About 25 years ago was when I decided it was time to turn our attention from pathogenesis to anaerobic organisms living in the gut. Work by my former postdoc Sarkis Mazmanian, California Institute of Technology, USA, found that our gut microbiome profoundly shapes our immune system; it both regulates our immune status and can throw it off kilter, making you more susceptible to certain diseases. Without the gut microbiome, you don't have normal development of T cell populations systemically, and immune tissues such as the spleen and lymph nodes are deficient in T cells and have abnormal histology.

Q2 You have elucidated the role of *B. fragilis*, an important intestinal commensal, in immune system modulation. Can you explain how PSA on the surface of this organism stimulates the immune system?

I often use *B. fragilis* as a model to try to understand at a mechanistic level how molecules of gut bacteria stimulate the immune system, but *B. fragilis* and PSA are just models for the interaction of microbial molecules in the gut with the immune system, they're not the whole story.

PSA stimulates both the innate and adaptive immune system. PSA binds to innate Toll-like receptors (TLR4) and C-lectin receptors (dectin-1) on APCs and is transported to the endosome,

where it gets depolymerised by nitric oxide into smaller subunits, usually about eight or 10 sugars long. The structure of PSA has one characteristic that differs it from most bacterial polysaccharides: it is zwitterionic, meaning it has positive and negative charges on each repeating unit. Most polysaccharides have no charge groups or only negative charge groups. Because of its zwitterionic nature, PSA actually binds to the major histocompatibility complex class II (MHC II) cleft on APCs. This cleft is loaded with positive and negatively charged amino acids. It's an ionic binding between the amino acids in MHCII and the zwitterionic charge of the depolymerised PSA. Most other polysaccharides get digested by nitric oxide or reactive oxygen species, but they don't bind, so they never get presented because they lack the positive and negative charge groups. PSA activates T cells because it gets presented by MHC II to the T cell receptor, and this induces IL-10 production.

Q3 You also discovered a role for PSA in shaping mammalian immune development and mediating Th1/Th2 balance. Can you elaborate on this mechanism, and its potential implications for autoimmune or inflammatory disorders?

There are several types of CD4 T cells; two of the major types are called Th1 and Th2. Th1 cells are primarily involved with cellular immunity, while Th2 cells are is primarily humoral immunity stimulating. In the immune system of a germ-free mouse, T cells are heavily Th2-skewed. If you colonise germ free mice with *B. fragilis* or give PSA orally to a sterile mouse, you actually balance Th1 and Th2 cells in the systemic immune system, bringing it back to normal. One of the other interesting things we found at that time was that, with no bacteria, the spleen and lymph nodes have abnormal histology, but when you give them PSA early in life, they end up with normal tissue in the spleen and lymph nodes. The interactions of molecules of gut bacteria with the immune system has been a primary focus of ours in the last 20 years.

There's another subset of T cells that live in the gut, called regulatory T cells, which shut off inflammation mostly using IL-10. It is thought that if you give patients with Crohn's disease a healthy dose of regulatory T cells, that would make the disease quiescent. PSA induces these regulatory T cells to make IL-10. There is another subset of T cells in the gut lamina propria called natural killer T cells (NKT) cells, and we showed that *Bacteroides* make a molecule which is a glycosphingolipid, not a polysaccharide, that actually turns off the pro-inflammatory response of NKT cells. So, those are both examples of specific T cell subsets regulated by gut bacteria.

The literature now contains papers about associations of gut bacteria with neurologic diseases like autism, Parkinson's

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disease, and even schizophrenia. Nearly all human systems have in one fashion or another been associated with the microbiome. Each of us has a few hundred microbial species in our gut, with a total organism load of around 100 trillion microbes. Interestingly, the effect of these organisms on the immune system is strain-specific. You could have a *Clostridium* that affects regulatory T cells, and I could have a different bacterial species impacting on the same cell type, making understanding this so complex. It's going to take science a long time to really understand the full scope of the microbiome. A lot of people have called the microbiome an additional organ and I believe that we haven't really come to grips with how it works or the full scope of its impact.

Q4 Group B *Streptococcus* (GBS) is the main cause of serious neonatal bacterial infections. Can you tell us about your contributions to the development of vaccines against GBS?

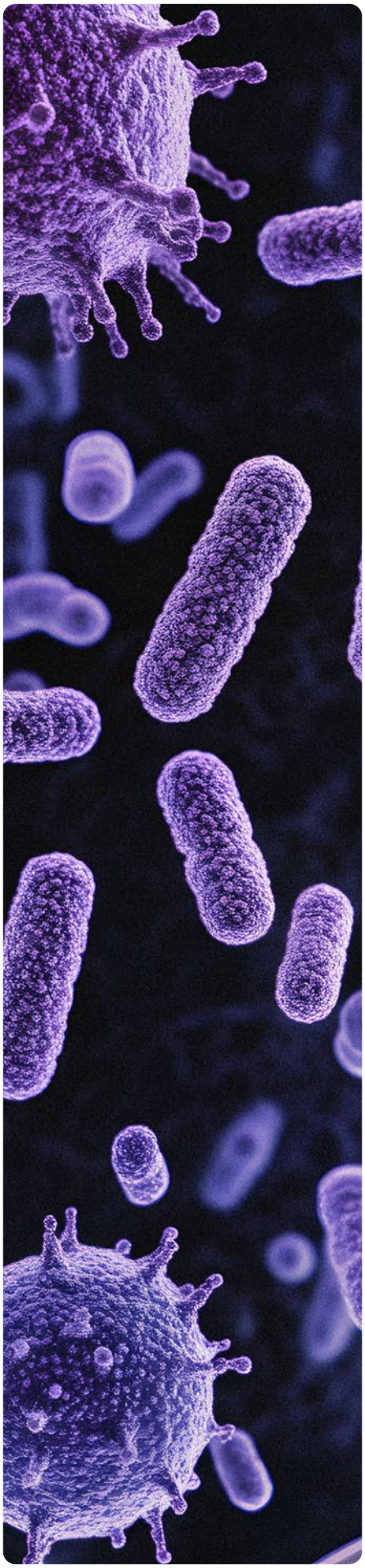
We basically conceived of the possibility of a vaccine for this devastating neonatal disease in one of my first papers. In 1975–76, we showed that women whose babies got GBS infection lacked antibody to the polysaccharide, but if the mother had antibodies to the capsular polysaccharide, the baby was protected through transplacental passage of the antibodies. So that led me on a 30-year intense effort to try to get companies interested in immunising pregnant women.

We identified the polysaccharides that represent each of different serotypes of GBS, solved the structure of all the polysaccharides, and made conjugate vaccines by covalently linking them to carrier proteins. These vaccines were immunogenic in adult humans and the plan was to immunise women and protect their neonates by transplacental passage of antibodies. Unfortunately, at the time, industry was not interested in further development because of the fear of liability associated with immunising pregnant women. Currently, however, there are some companies moving ahead with these conjugate vaccines using the capsular polysaccharides and a similar chemical approach to that we discovered.

Q5 How can your work in GBS inform the design of vaccines for other age-specific or immune-compromised populations?

We designed the vaccine for GBS and made many basic science contributions to the understanding of it, but the basic idea of taking polysaccharides and coupling them to proteins for a vaccine was developed in the 1940s with pneumococcal capsular polysaccharides. So, the idea of enhancing T cell help to make antibodies to polysaccharides was known. In fact, there was already a vaccine on the market that used that basic approach with *Haemophilus influenzae* type B capsular polysaccharides. Much of the pneumococcal vaccine development that was done by some pharma companies followed the work we did with GBS, although our vaccine never made it to the market.

One basic concept that came from our work on both *B. fragilis* PSA and GBS conjugate vaccines was that T cell receptors are able to recognise carbohydrates if they are presented in the presence of the MHCII molecule.



Q6 You have also led fascinating research on *Francisella tularensis*, a potential agent of bioterrorism. Can you tell us more about the approach you used to develop a vaccine against this pathogen?

There was a huge scare in the world about anthrax as a biologic weapon in the early 2000s, and that fear spread to all of the organisms with potential for use in warfare. Tularaemia is certainly one of those organisms

For *F. tularensis*, we took the lipopolysaccharide (LPS) of the organism, called endotoxin of the organism. We chemically cut off the toxic lipid A end of the LPS, and we coupled the polysaccharide to carrier proteins to make a conjugate vaccine. We also did studies on what optimises the polysaccharide immunogenicity in these conjugates, and found that longer polysaccharides made better conjugate vaccines than smaller polysaccharides.

Q7 Moving beyond microbiome-wide associations to identify causative microbial agents has been a significant challenge in the field. How did your team overcome this barrier?

Our work has primarily taken a reductionist approach where we have tried to solve mechanistic relationships of host and microbe. Microbiome-wide associations have not fully solved the questions that arise when studying the microbiome. For example, just because we have an important species like *B. fragilis*, it does not mean all the organisms belonging to the species *B. fragilis* will act the same. There is enough genetic variation between strains of a given species to result in different functions. So, you really have to talk about individual strains.

For example, it became known about 10 years ago that the microbiome has an effect on the efficacy of checkpoint blocking antibodies used for cancer immunotherapy. The main targets of immunotherapy currently are PD-1, PD-L1, and CTLA4. It became clear that gut colonisation with certain organisms, as well as some patient microbiomes, had a negative effect on checkpoint blockade efficacy in mouse models. This has now blossomed into an important field. When we began work in this field, it was difficult to find an organism in a given patient's microbiome that inhibited checkpoint blockade.

So, we developed a model, for which I published a paper in 2017 with my former postdoc and colleague Neeraj K Surana, Duke University, Durham, North Carolina, USA. We applied that model to this question of checkpoint inhibition working with Arlene Sharpe and Gordon Freeman, from Harvard Medical School, Massachusetts, USA. First, we found microbiomes that inhibited checkpoint blocking in a mouse model, and then, using specific antibiotics and various other manipulations, our postdocs Francesca Gazzaniga, Harvard Medical School, and Joon Seok Park, University of Chicago, sorted through microbes and came out with a small group of organisms from which we eventually could isolate one organism that had a pretty significant effect on checkpoint blocking. I hadn't even heard of this species when we found it. It's called *Coprobacillus catenaformis*.

Using models and studying the response of cells in tumours and in tumour-draining lymph nodes,

we learnt that, when mice were colonised with C. catenaformis, another checkpoint-blocking molecule called PD-L2 was actually lowered in the tumour-draining lymph nodes on dendritic cells. It was known from the literature that PD-L2 had two different binding receptors. One is PD-1, which is a main target of checkpoint blocking, and the other was called repulsive guidance molecule b (RGMb). It turned out that it was the impact on RGMb that affected the efficacy of PD-(L)1 therapy. If you took a monoclonal antibody to RGMB or to PD-L2 and used it in combination with the antibody to PD-(L)1, you actually could overcome tumour resistance. It was a pretty nifty mechanistic approach to figuring out a complex biological problem.

To return to your question, how do we overcome this barrier? One of the reasons we've been successful with this is that, even though I'm in an immunology department, I also use a lot of microbiology, chemistry, and genetics. It takes an interdisciplinary approach to solve complex problems in the microbiome because you couldn't ask for a more complex system. People who only consider the whole microbiome, to me, are missing part of the story. It's really the molecules on, or made by, the microbes in the microbiome that are having an immunological effect. The complete picture requires figuring out the mechanisms by which microbial molecules interact with the immune system. So, taking an interdisciplinary approach has been very helpful in working through these host/microbe interactions. The unfortunate thing is that it's really slow work and requires microbiology, chemistry, immunology, and cell biology tools. I'm hoping that someday someone will figure out a better way to do it.

Q8 Finally, having mentored over 100 trainees, what advice would you give to young scientists entering the fields of microbiome and immunology, especially regarding interdisciplinary collaboration?

You shouldn't be inhibited by dogma if your data tell you something different. I use PSA as the example. It was quite a bit of work to prove to colleagues that polysaccharides can activate T cells. What we did with PSA is that we've defined what it is about the structure of the molecule that allows it to activate T cells, and why most polysaccharides don't. I heard many times in the late 90s and early 2000s that PSA must be contaminated with peptides or proteins because it's activating T cells, because the dogma taught that polysaccharides don't activate T cells. So, my main advice would be to not be constrained and to keep an open mind. You have to believe in your data if you are sure it is accurate.

Another thing that I try and teach my students and postdocs by example, is that I've never considered what I do as 'work'. I go to my lab every day because what I do there is have fun solving challenging questions. I have a good time, and it's always been that way. It's something I look forward to. I get energised from our data and thinking about new experiments. So, one, enjoy your work, and do something that's exciting, and two, don't be constrained by dogma in the field.

Finally, in my experience, interdisciplinary collaboration works best when each side offers unique expertise in solving a problem.