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TRANSCRIPT

New Developments in the Prevention and Management of NEC

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We're going to talk about new developments in the prevention and management of NEC. We all know that this has been going on for a long time and we'll talk a little bit about breast milk and some things that we're doing in the lab that will hopefully, at some point in our career, translate into the clinical space.

The learning objectives of the talk is really to talk about why do we think NEC happens, what is the pathogenesis of the disease, and how can we find therapeutic targets or preventative strategies, and really how can we prevent that intestinal inflammation that happens or least dampen it so it doesn't progress on to severe NEC. I'll be talking about some new, exciting directions in NEC that we have some new funding for and talk about some new model systems. With those samples that we get that I previously spoke about, we're doing some cool things in the lab to be able to test different nutrients, but also different drugs on premature intestines, specifically, in the hope that we can develop personalized medicine approaches. My hope is actually that we'll have precision nutrition approaches with all of Dr. Belfort's work.

Going along with the theme of how do we build a world without NEC, there are several components, and so one of them starts at the mechanism level. In the lab, what is happening? And we all know that breast milk is protective and it's the best thing that we have for our babies, but then we have to think about other novel therapies or other novel strategies as to how can we make that breast milk that our babies receive better, for example, and I'll talk about some potential strategies for that. I already mentioned, collaboration and biomarker discovery, which we won't talk a lot about today. But I'll talk just a bit about what goes on in the lab and certainly, in my research program, we have the clinical and translational component, as I discussed, but we also hang out with mice every day, so I'll talk a bit about that just briefly and give you an overview.

What we know about NEC and the pathogenesis is that there's this exaggerated proinflammatory response that happens and, as I previously mentioned, we have this bacterial dysbiosis that occurs in these babies about 48 to 72 hours prior to a diagnosis of NEC. And how can we then harness that information and recapitulate what's happening at the bedside in the lab in various animal model systems or other human-derived approaches. There's so many knowledge gaps in the field, but a lot of the pathways that regulate the immune response are not known. And some of those immune responses that our babies have, and some are very exaggerated and, as you all know, they can die suddenly, how can we then modify the immune system to be able to fight off NEC or other infections. The over-arching goal of my laboratory-based research program is to design either novel nutritional epithelial-specific or immunotherapeutic strategies to prevent NEC and I'll talk just a little bit about the ways in which we do that.

At the forefront of what we do, again, is collaboration with other scientists, patient families and clinicians that are taking care of these babies at the bedside and so we're tackling this from a lot of different facets. We have a mouse model of NEC—which I'll show some pictures of and talk about—which really digs into the mechanistic studies and how can we knock out different areas in different pathways and produce responses to NEC-like phenotype. The development of novel therapies which I'll talk about and one of our patents that we have on some immunotherapy work. The goal really is preventative approaches, and I already talked about DNA methylation analysis and some other multiomic sequencing that we're doing as well.

When we think about how we are going to tackle this, as I mentioned before, we want to go from the bedside to the bench and certainly back again. The preclinical models or the models that we can recapitulate NEC in the lab go across the spectrum here and so I already talked about biobanking, but I'll just talk briefly about our mouse models, NEC and some data with one of the immunotherapies that we studied.

When we are developing model systems, as we talked about previously, there's a lot of different risk factors that predispose our infants to NEC and this is really captured in a nice review article out of UCD in which you can see there's a lot of different factors that can predispose our babies to NEC and so we can't capture that in every single model that we are testing in the lab, but we can capture a good portion of those. Certainly dysbiosis, I will say premature animals are hard to keep alive in the lab and certainly not for lack of trying, but a lot of failures in that regard from my viewpoint. Dysbiosis, certainly impaired immunity, how we can modify the feeds with various additives and others.

What we know about NEC and what is happening at the cellular level, so these are epithelial cells here, just to orient you, and these darker ones are ones that are dying. What we know from a lot of good work that's been done over the last 15 or 17 years or so is that there's this innate immune receptor, specifically called toll-like receptor-4 or TLR-4, that is present on those

intestinal epithelial cells. What we think is happening in a premature infant—this is a lot of work out of David Hackam's lab out of Hopkins—is that there's elevated TLR-4 on the intestine of our premature infants and on these epithelial cells. What happens in the context of microbial dysbiosis or this microbiome shift that happens is that you have what's called lipopolysaccharide or LPS that's present on the gram-negative bacteria. And LPS binds to this receptor and then causes this significant downstream inflammatory response that occurs. You don't need to know all the different signaling pathways involved, but what happens is there's this extreme inflammatory response and there's a lot of different immune cells that come in and there's a lot of inflammation and that can really exacerbate the disease severity and then we see this epithelial cell injury.

We've all seen babies that look like they're septic and then there's a negative blood culture because what we believe is happening is that these bacteria can get across the gut barrier and then really exaggerate the immune response. They have this systemic sepsis-like picture.

What I would like you to appreciate is these neonatal mice that we take care of are similar to babies in that they need a lot of care and they need to be fed gently. I'll show you some pictures of that. They certainly don't feed themselves and so I have an awesome team that is dedicated to this work.

This is a picture of one of our neonatal mice. They're about 1.7 to 2 grams, okay, in terms of the differences in size, but what we do is we keep them with their moms, they're breast-fed until postnatal day 4. At 4 days of age, they're taken away from their mom and then we start hand-feeding them. This is one of the post-docs in the lab, this is a neonatal PIC line that they're gavaging with a special formula that's a mixture of human-term infant formula and then puppy formula to change up the osmolality of the formula. Then we add LPS to the formula and then we also add a microbiome from an infant with NEC totalis and so basically we're adding a humanized microbiome to the formula to make sure that we're changing up the microbiome of these mice.

We also subject them to hypoxia, so 5% oxygen twice a day. We all know that our babies have all those desaturations at the bedside. Sometimes we think they're nothing; sometimes they're very profound and that can really add to the additional stress of the gut of our babies and certainly our neonatal mice as well. What we do is we hand-feed these mice for 15 hours a day from 7 AM to 10 PM, again a dedicated team that does this amazing work which I no longer have to do and I'm happy about it, it was part of my fellowship and early career faculty project. At the end of 72 hours, this is what the intestines look like. Hopefully you can appreciate the necrosis or dying intestine here and there's pneumotosis intestinalis that you can see visually. We collect the intestine, we analyze it, we also collect blood and other tissues and then process them.

I'll just shift and talk about some of the novel therapies. When you're thinking about how can we test various drugs in these animal models, we have to think about what we know about NEC. So, we know that the inflammation that we see in NEC is related to gut barrier dysfunction, intestinal stem cell loss and then impaired mucosal healing. We can all see a lot of that at the bedside when our babies aren't doing well and their intestine is resected. At the time that I was an early career faculty member and was doing a lot of work on breast milk and what specifically in breast milk protects against NEC, there's a lot of cytokines that are present in breast milk and one of them is interleukin-22. There's all this data that was emerging that IL-22 was important for intestinal stem cell regeneration and regulating the gut barrier integrity and could attenuate intestinal inflammation in adult animal models of disease such as IBD, ulcerative colitis and Crohn's. And IL-22 can enhance antimicrobial defense. One of the cool things that happened is there was this awesome paper that came out in *Nature*. These are pictures of these little intestinal organoids, or what we call enteroids, that we grow up in the lab and so they're little mini guts from our babies. We get a piece of intestine into the lab and we isolate the crypts or where all the stem cells live and then feed them various growth factors and can grow up intestine. What this group did is that one of the components of the media that they add is epidermal growth factor and I don't have time to talk about it today, but some of my early work was focused on epidermal growth factor that's present in breast milk and so, they took out the EGF and added in IL-22 and the enteroids got bigger. We decided to say, okay, well maybe this makes IL-22 a nice therapeutic option for NEC and this was already in clinical trials for graft vs host disease and alcoholic hepatitis, actually, and now COVID as well. So, I'll go through these studies here.

First, we wanted to see is IL-22 important in neonates? We took neonatal mice at various gestational ages, so E15 here is embryonic day 15 and mice are typically born between embryonic day 18 and 19, so you can see here prenatally, in the small intestine, there's very low IL-22 levels and these levels stay low in the neonatal mouse until about weaning, which is postnatal day 21 to 28. At this time, they're taken off breast milk, they get chow and so these are just normal mice. These neonatal mice have a lack of IL-22 production at baseline. Then we can see, this is at the mRNA level, and this is at the protein level. Just looking at the differences between 1-week- and 8-week-old mice

That's great but we wanted to see what about NEC and so we subjected mice to our experimental NEC model. And you can

see here, DF is dam fed, so in the dam fed mice, you see nice healthy-looking intestinal villi here on this histology and then in animals that are subjected to our NEC model, you see derangement of the intestinal villi here and even loss of several of the intestinal villi. But when we looked at the IL-22 levels, we thought, well, there's this exaggerated inflammation, maybe these mice are going to have elevated IL-22. What we actually saw was the opposite. Even though IL-22 wasn't really there when we looked at homeostasis or without NEC, we saw a significant decrease in these animals in their small intestine. Then when we looked at human, small cohort, we didn't see any difference and so it raised the question, do neonates just not have the ability to make IL-22 and if we supplemented it back to them, would that then protect their intestine? That's what we did.

We evaluated treatment with IL-22 at various time points and I'll show some more pictures, but this was published a few years ago now, it is work done by a post-doc Ben Mihi in the lab, and so you see the control animals are the dam-fed animals that were exposed to either recombinant IL-22 or the vehicle PBS, so they still got an injection with saline, but not the cytokine, you see the intestine looked normal. Then when we injected PBS and subject them to our NEC model, again you see a derangement of the intestinal villi there, consistent with experimental NEC. Then when we administer recombinant IL-22, we see that treatment then attenuates that intestinal injury that we see in experimental NEC.

We then wanted to look at the proinflammatory markers that are involved in our models. One of them is interleukin-1-beta or IL-1-beta, and so you can see here at the mRNA level that the animals that received IL-22 had significant decrease in IL-1-beta at the mRNA and at the protein level as well, showing that IL-22 can attenuate the proinflammatory response in NEC. We then wanted to look at what is happening at the actual tissue level. These are pictures, confocal images, that you can see here in green, stained for proliferating cell nuclear antigen or PCNA. Green is a marker of healthy intestine, so intestine that the stem cells are healthy and they're making new either epithelial cells or absorptive cells. There's dim green here in NEC plus PBS and then when we administer IL-22, we see that there's an enhancement of epithelial cell proliferation.

What we wanted to see is what is the correct dose and we thought if you go up is it better because one of the things with immunotherapy is the dose has to be titrated appropriately. We've been working with the FDA on the different doses and what would a clinical trial look like and how can we then do this in the mice to then translate to our babies. This is some histology from several of those doses that we've looked at. You can see in this first dose of IL-22, you see that the intestines look better but still the villi are looking a little bit friable. When you get in the higher dose here, still a little bit friable, don't look as good as our breast-fed counterparts. But then in our third dose here, we start to see that the intestines look similar to what we would see in a breast-fed animal. Then in the higher doses, they looked great and I'll just fast forward to the punch line here, but when we got up to this dose, we started to see some proinflammatory cytokine response. We can see that response go down, but then we can see an increase in the proinflammatory response here. Then we did a lot of additional work looking at what is the timing. When you think about when to intervene. So if you are cooling baby, for example, and you want to get that therapy started within 6 hours, that's what we're trying to approach for babies that certainly, not at our center that we can't enroll them in clinical trial right away, what is the timing in which the cut-off, like when is too late to be able to give them an immunotherapy to be able to attenuate the disease?

I'll say the interim summary for this is that we have found that IL-22 expression is low in the developing mouse and human intestine and that treatment with IL-22 can attenuate some of the intestinal inflammation seen in experimental NEC. One of the things that we're really interested in, as I mentioned, was immunotherapy is great, but there has to be a way that we can potentially modify the intestinal environment with diet prior to NEC. One of the projects that we did a few years ago now since we were working on this IL-22 work, there are various dietary factors that can up-regulate IL-22, specifically these aryl hydrocarbon receptor ligands, or AhR ligands, that are present in breast milk and so they're also present in green leafy vegetables. We're really interested in can we modulate the baby's diet, but also can we modulate the mom's diet to actually either make better breast milk or be able to impact immunity on her infant.

One of the aryl hydrocarbon receptor ligands that we looked at is called indole-3-carbinol, or I3C for short, and this is some histology here that you can see. These are, again, the breast-fed animals that I talked about, but then in the NEC animals, this is the vehicle control compared to those treated with I3C. You can see in the animals that were gavaged I3C, you see nice healthy intestine. We looked at some various markers, including IL-1-beta, as I mentioned, and also lipocalin-2 which is a pro-inflammatory marker, and you see decreases in the animals that received I3C that were subjected to NEC and then Cyp1a is a marker just showing that aryl hydrocarbon receptor signaling was activated. It's a downstream marker showing that indeed the animals got the drug and Cyp1a was activated in the intestine.

We took the intestine and performed bulk RNA sequencing just to see what are the different pathways that are involved. You can see there's a lot of different—this is a volcano plot—a lot of

different up- and down-regulated genes and I just highlighted some of them here. But what I want you to appreciate is there's a lot of different pathways that are modulated with this AhR ligand, specifically up-regulation of the tryptophan metabolism pathway which there's a lot of emerging data to show now that these metabolites can certainly impact gut immunity, which is fantastic. But, in terms of down-regulation—so the cytokine receptor interaction—a lot of those pro-inflammatory cytokines that are up-regulated in NEC are down-regulated. We were excited to see that down-regulation.

What we can do in the lab with mice, but how does that really translate and, I know for all of you, you're like why is this important to me. I talked already about the biobanking and we really want to use a lot of those samples in the lab. One of the ways that we do this is in this organ-on-a-chip model. I talked about those little intestinal organoids are mini guts that we create in the lab, but I'll tell you what we do with them, and we put them on the chips.

How do we do that? We get a piece of intestine from the operating room or from the baby's bedside when they are having surgery and we isolate the crypts or the base of the intestine and then we can culture those up in the lab again with those various growth factors. Ultimately, this is just a schematic of what you saw in the previous picture, you can see these little enteroids or mini guts. Then, after we grow those up, we can break them up a little bit, dissociate them into fragments and then we can put them on this cool microfluidic chip and I'll show you some other pictures. But what this looks like in cross-section after they grow up is you can see we can grow up a nice intestinal epithelium. There's a nice extracellular matrix coated membrane here and then we can put endothelial cells or even immune cells on the bottom chamber and see how they interact.

This is just a schematic of what the chip looks like and their little holder and then you put the chip, which is here, into this pod and this pod delivers their media or whatever you'd like them to receive. Various drugs, you can feed them formula or breast milk or different components of each. You put those pods in the culture module. What the culture module does, is it actually delivers continuous media or continuous drug or continuous food, whatever you would like to do, delivers continuous flow. And then also this module, this orb module, stretches the chip to mimic peristalsis in the gut.

This is one of those very advanced cell culture models, but it's really cool. This is what those enteroids look like. Again, we dissociate them to put them on the chip, that's what that looks like. After 3 days, these cells combine here and you have this neonatal epithelial monolayer, nice and flat. But then with that continuous media flow and stretch, you see that an epithelium starts and you can see these little crevices there. By day 8, we start to get—remember I showed you those intestinal villi—we see these villus-like axes that develop and I'll show you what they look like in cross-section. This is work that was really pioneered by a premed student in my lab named Wyatt Lank.

In cross-section, they look like this. These nice intestinal villi, you can see we stained for some various epithelial markers, like villin and (inaudible), just to really show the structure of the villus. Here's an up-close picture and then when we do some staining, so these red cells are goblet cells. We really wanted to see what are all the different cell types that are there. There's epithelial cells, there's nice goblet cells that make mucus.

Now that we can grow the intestine on a chip, how can we then model NEC-on-a-chip? Thinking about what we know from the animal models that we've done, we know that dysbiosis is really important. I didn't talk about it before, but in our mouse model, if you don't have dysbiosis, they don't get NEC. If you don't have hypoxia, they have decreased intestinal inflammation. You really need all those different components. When we're modeling NEC-on-a -chip, we wanted to see what are the different components that they need, do they need LPS, do they need just bacteria, etc.

We started with bacteria because we know how important it is for the human disease. We took the same bacterial slurry, or we call them NECteria for short, but that bacterial microbiome from an infant with NEC totalis. We standardized that, we certainly culture, we have individualized cultures and we culture bacteria from the stool or the intestine, from all the babies in our studies when we can. We added that to the chip and the punch line, I'll show you some data, but the punch line is that we found up-regulated proinflammatory cytokines and then reduced epithelial proliferation which is important in NEC as I mentioned from our mouse studies, and then really disrupted gut barrier integrity. What's cool about this model is we can really dial up or down how much inflammation that we want to see and so I'll show you some of those things here.

We wanted to look at the different cellular markers. These are different epithelial cell markers and stem cell markers. So, LGR5 is a stem cell marker, MUC23 is a goblet cell marker, lysozyme is a Paneth cell marker and Chromogranin A is one of the enteroendocrine markers. You can see in human NEC, this is just what happens in the human condition in the intestine, you see decreases in a lot of those markers. As I already mentioned and what we already know, there's an increase in proinflammatory cytokines and again, decrease in PCNA proliferation, but also KI67 is another proliferation marker.

When we looked in our NEC-on-a-chip model, so the control chips are just media alone. They're not normal microbiome,

they're just media alone. We've done a lot of different studies on various microbiomes and how they impact the chip, but these are just naive chips with media. And so, in our NEC-on-a-chip model, you can see that there's decreased stem cell markers, decreased goblet cell, Paneth cell and all those different types of markers and elevated proinflammatory cytokines and decreased proliferation as well.

What that looks like by confocal microscopy, so again at the top, here is human intestine, control and NEC. This is just regular intestine that we stain for all the different markers that we just discussed, so enteroendocrine, proliferation, Paneth cells and goblet cells, and what I want you to appreciate here is you see not a lot of red. The blue is just nuclear stain. In our NEC-on-a-chip model, so you can see in the controls, they have all those different cell types that we discussed, and they have nice enterocyte proliferation as well, but in NEC you see those are significantly decreased. This is the NEC-on-a-chip model.

We wanted to look at tight junctions, so remember I talked about how the gut barrier integrity is so important in NEC and so we see a significant loss of tight junctions in NEC-on-a-chip. In our controls at various time points, you see nice green staining here of zona occludens 1 and you see a progressive decrease and the loss of those tight junctions. You can see the cells actually start to die and there's less cells there, up to 72 hours.

When we looked by sequencing again, just to look at all those pathways that are involved in the chips at various time points, a lot of the same things that we see in our human condition. Apoptosis, all the different cell, types of cell death, so necroptosis and all the different cytokine pathways, HIF signaling, IL-17 signaling, TNF signaling, etc, are up-regulated in our chips at various time points.

In conclusion, NEC-on-a-chip is a promising new patient-derived model of NEC. It does take a while, so in terms of personalized medicine, I can't sell it to you like that just yet, but it does recapitulate many of the features that are seen in human NEC. We do have a grant to look at using the NEC-on-a-chip platform and even just the neonatal intestine on a chip platform to look at how different drugs and how different nutritional additives affect those tight junctions and the different cellular markers on the chip. Basically, we don't have to always test these things necessarily in a clinical trial. We can do it in a preclinical human-derived model first. We're doing continued biomarker discovery as well. Hopefully I gave you an overview of our NEC research program and talking about our different in vitro models of NEC and then, just to put it out here because I know, I know this disease can really feel devastating and our patients and families really depend on us for hope, and so please continue to fight, fight against NEC in any way that you can.

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