The role of the intestinal microbiota in pneumonia and sepsis

Lankelma, J.M.

12.
Summary

This thesis focuses on the role of the intestinal microbiota in pneumonia and sepsis. Our main hypothesis was that the gut microbiota plays a protective role in innate host defenses against systemic bacterial infections; i.e., that microbiota disruption by antibiotics would negatively affect the innate immune response during pneumonia and sepsis. In a translational fashion we used both murine and human models to test this hypothesis and also characterized the fecal microbiota in critically ill.

Murine studies previously showed that the intestinal microbiota provides signaling compounds (metabolic products such as short-chain fatty acids or bacterial cell wall components such as peptidoglycan) that activate or "prime" the innate immune system at distant sites [1-3]. Disruption of the microbiota by antibiotics could decrease the level of these circulating microbial compounds, rendering innate immunity less primed and thus less effective. In mouse models, decreased exposure to gut bacteria (mice that are born under germ-free conditions or treated with antibiotics) was associated with increased susceptibility to bacterial infections [2, 4, 5]. However, no attempt at testing this hypothesis in humans had yet been made. In chapter 3 we therefore investigated the effect of antibiotic disruption of the microbiota on innate immune responses in healthy male subjects. They were given broad-spectrum antibiotics (vancomycin, ciprofloxacin and metronidazole) for seven days and before and after treatment, we compared several *ex vivo* innate immune effector functions. We found a lower production of tumor necrosis factor (TNF)-α by mononuclear cells upon stimulation with lipopolysaccharide (LPS), one day after antibiotic treatment compared to before treatment. This was restored six weeks later, when the microbiota had also recovered. Still, most effector functions, such as phagocytosis by neutrophils, were not affected. The data partially support our hypothesis: microbiota disruption by broad-spectrum antibiotics is reversibly associated with decreased responsiveness of systemic mononuclear cells towards LPS.

In chapter 4 we aimed to test whether this effect of microbiota disruption on TNF-α production upon LPS challenge was also present *in vivo.* For this we used the human endotoxemia model, in which a septic response is mimicked through intravenous injection of LPS. Subjects become ill for a couple of hours, with symptoms such as fever, chills and headache. Several parameters of immune activation, including TNF-α production, can be measured in blood(plasma). When comparing eight control- and eight antibiotic pretreated volunteers, we could not detect any differences in cytokine production, endothelial activation, coagulation activation, neutrophil influx or LPS tolerance during endotoxemia. Gut microbiota disruption by broad-spectrum antibiotics thus does not affect systemic innate immune responses during human endotoxemia in healthy subjects. As a result, this study did not support our hypothesis.

In critically ill patients, it is difficult to dissect the differential effects of disease, antibiotics and microbiota on the innate immune system, as it is not possible to leave one variable out of the equation. However, little is known about the composition of the intestinal microbiota in patients on the intensive care. In chapter 5 we therefore characterized the fecal microbiota in 34 critically ill patients by sequencing of bacterial ribosomal RNA genes. We observed profound microbial disturbances in both septic and non-septic critically ill

patients, even though non-septic patients had received fewer classes of antibiotics than septic patients. In most critically ill patients, bacterial genera that are involved in metabolism had disappeared, as well as bacteria that are supposed to have beneficial immunomodulatory effects. The fecal microbiota was often dominated by a single bacterial genus and general bacterial diversity was low. As a previous study suggested that bacterial diversity could be used as a predictive marker for mortality following allogeneic hematopoietic stem cell transplantation [6], we explored the potential use of microbiota composition as a marker of disease severity in this small cohort of critically ill patients. We did not find any associations between microbiota diversity, Firmicutes/Bacteroides ratio or Gram positive/negative ratio and outcome measurements such as complications and survival. Large prospective studies are needed to reliably test microbiota diversity as a predictor for mortality.

Chapter 6 describes both the short- and long-term effects of the broad-spectrum antibiotic regimen that we had given to the healthy subjects described in chapter 3 and chapter 4 on the composition of the microbiota. Sequencing of bacterial ribosomal RNA genes revealed that one day after the antibiotic course, the fecal microbiota had strongly changed with significantly lower bacterial diversity and disappearance of bacterial taxa with important metabolic functions. Six weeks after the course, these parameters had largely returned to baseline, and completely so after 8 to 31 months – indicating substantial resilience of the intestinal microbiota in healthy humans.

In the second part of this thesis we used murine models to gain mechanistic insights into the effect of the microbiota on host responses during pneumonia and sepsis. We tested whether antibiotic-induced disruption of the microbiota affects innate immune responses to bacterial pneumonia-derived sepsis. To test whether our results were specific for the pathogen we had chosen, we used two models of murine bacterial sepsis with different characteristics. In chapter 7, we used Gram positive *Streptococcus pneumoniae*, the most common cause of community acquired pneumonia, and in chapter 9 we used Gram negative *Burkholderia pseudomallei*, which is a common cause of pneumonia and sepsis in South-East Asia. In addition, we looked at sterile LPS-induced lung inflammation (chapter 8). In all experiments, control mice were compared with mice that received broad-spectrum antibiotics in their drinking water for 19 days, followed by two days of normal water. Mice were sacrificed at early and late timepoints, to assess bacterial loads and markers for pathology.

In chapter 7, we observed increased bacterial growth and dissemination in antibiotic pre-treated mice compared to control mice during *S. pneumoniae* pneumonia, especially during the early phase of infection. The phenotype could be partially reversed through an oral fecal transplantation from control mice, indicating that the intestinal microbiota is indeed involved. Using transcriptional profiling and functional tests, we found evidence that alveolar macrophages in antibiotic pretreated mice are less capable of phagocytosing the pathogen, possibly related to alterations in cholesterol synthesis pathways. In addition, alveolar macrophages from antibiotic pretreated mice produced less cytokines upon *ex vivo* stimulation with LPS and lipoteichoic acid compared to controls. These data

suggested that innate immune defenses during pneumococcal pneumonia could indeed be affected by antibiotic microbiota disruption.

To further investigate this potential gut-lung axis in the host defense against pneumonia, we looked at sterile LPS-induced lung inflammation in control- and antibiotic pretreated mice (chapter 8). Toll-like receptor (TLR)-4 activation via LPS is an important mechanism in the initiation of the inflammatory response in multiple pulmonary diseases. A slight increase in the production of some proinflammatory cytokine and chemokines was found in antibiotic pretreated compared to control mice, on different time points and using different LPS dosages. This confirms an immunomodulatory effect of the intestinal microbiota, but in contrast with the previous study, a well-balanced microbiota seems to dampen pulmonary inflammatory responses.

To test whether the observations from chapter 7 were specific for pneumococci, we used the same antibiotic-induced microbiota disruption model in chapter 9 with a whole different kind of pathogen: the Gram negative, facultative intracellular bacillus *B. pseudomallei*. We found a very similar phenotype, in which bacterial growth and dissemination was increased in antibiotic-treated mice at early time points. The effects on survival and organ pathology were limited, however. Again, alveolar macrophages derived from antibiotic pretreated mice showed a diminished capacity to phagocytose the pathogen, which might contribute to the observed protective effect of a healthy gut microbiota during pneumonia-derived melioidosis. Taken together, these data identify the gut microbiota as a potential modulator of innate immunity during *B. pseudomallei* infection. In contrast with chapter 7, we did not find any differences in cytokine production by alveolar macrophages upon stimulation.

Recognition of flagellin from *B. pseudomallei* by TLR5 is important for the host response during melioidosis. Chapter 10 describes phenotypical differences between TLR5-knockout mice and wildtype control mice during melioidosis. Indeed, mice lacking TLR5 were more susceptible to infection and showed impaired survival. However, data was published showing that mice from different vendors have a different composition of their microbiota (due to different food, bedding material etc). Indeed, we found differences in fecal microbiota composition between our locally bred TLR5 knockout mice and commercially bought wildtypes. We repeated the experiment, now administering both groups our standard broad-spectrum antibiotic cocktail in their drinking water prior to bacterial challenge. Strikingly, the initial differences in susceptibility to melioidosis vanished, suggesting that they were due to a difference in microbiota as opposed to a TLR5 deficiency.

A last striking observation we made was the effect of sepsis itself on the intestinal microbiota, independent of antibiotics. In two different mouse models of pneumonia-derived sepsis (one of them the melioidosis model described in this thesis) we observed very consistent changes in the fecal microbiota, within 48-72 hours of systemic bacterial infection. During melioidosis, a strong increase in Proteobacteria was seen in all mice as well as a decrease in Actinobacteria, while no *B. pseudomallei* could be detected in these

fecal samples. The composition of Bacteroides and Firmicutes also changed in strikingly similar patterns. Pre- and post-infection samples from mice were highly dissimilar. Total microbial diversity was increased, mostly due to increased diversity of Proteobacteria. As the pathogens with which the mice were inoculated intranasally could not be detected in fecal material, the most likely explanation is that systemic immune activation affects gut bacteria. This suggests that not only the microbiota influences the innate immune system, but also vice versa: a bidirectional interplay between microbiota and immune system.

The last chapter of this thesis does not concern the intestinal microbiota, but is also about melioidosis, the infectious disease caused by *B. pseudomallei*. This pathogen is listed as a potential bioterroristic weapon, as it has a high mortality, is sensitive to few antibiotics and can easily be obtained from soil in endemic areas. However, no vaccine is currently available. In chapter 11 we tested a DNA vaccine against *B. pseudomallei* flagellin, administered either via skin tattoo or intranasal application. Strikingly, a single intranasal vaccination resulted in strongly decreased bacterial burdens and organ damage and improved survival. Intranasal DNA vaccination may therefore be a candidate for rapid mass vaccination in a bioterroristic setting and further testing is indicated.