The role of the intestinal microbiota in pneumonia and sepsis

Lankelma, J.M.

13.
General Discussion

The bigger picture - Humans

The intestinal microbiota is currently a hot topic in biomedical research, with studies investigating its role in a range of diseases quickly piling up. However, many assumptions are based on associations between microbiota composition and clinical conditions or -parameters. There is a need for mechanistic studies in order to investigate causality, and subsequent clinical translation of results. The role of the microbiota in certain diseases such as severe *Clostridium difficile* infection has by now been extensively studied in both mice and humans. Research on the effect of the intestinal microbiota on the systemic innate immune system however is only just getting started.

Our human intervention studies are an attempt to dissect the interplay between intestinal microbiota and systemic innate immunity in healthy individuals. *Ex vivo*, we found that antibiotic induced microbiota disruption was associated with decreased TNF-α production by mononuclear cells upon stimulation with LPS. *In vivo* however, we did not find such an effect. This apparent discrepancy could be due to many things, among which the redundancy of the innate immune system. Even if monocytes are impaired in their cytokine production, other cell types may compensate for this deficit. Still, a relatively small decrease in TNF-α production may have more impact in for example immunocompromised patients, or those with TNF-α related disease. Although these are intervention studies, no causal relationship can be deduced from these data.

In our cohort of critically ill patients, no two cases are the same with regards to antibiotic treatment, drug use, comorbidities and underlying pathology. Accordingly, interpersonal diversity in microbiota composition was extremely large compared to healthy controls, making it hard to imagine that this does *not* in some way affect physiological and pathological processes. Opposed to classic theories, the absence rather than the presence of certain microorganisms may be indicative of a pathologic condition, such as a defect in systemic innate immune responses. We are dependent on experimental models to answer such questions, as research in critically ill patients involves many confounding factors. The high level of resilience we observed in the fecal microbiota of healthy subjects after an antibiotic regimen eradicating both Gram positive and negative bacteria, including anaerobes, does give hope that the intestinal microbiota of ICU patients can recover quickly, even after strong disruptions.

The bigger picture - Mice

The consistency between the murine pneumonia-derived sepsis models in which we tested the effect of antibiotic microbiota disruption on innate host defenses (*S. pneumoniae, B. pseudomallei* and *K. pneumoniae;* the latter not in this thesis) was striking. In all models, we observed an early increase in bacterial growth and dissemination in antibiotic pretreated mice compared to controls upon intranasal infection. At later time points, no differences in bacterial growth were detected and the effect on organ injury and survival was modest. The mechanism appeared consistent as well, with impaired phagocytosis by

alveolar macrophages of both *S. pneumoniae* and *B. pseudomallei* in antibiotic treated mice. Decreased phagocytosis could in turn lead to decreased killing of pathogens.

Alveolar macrophages are known to have a high level of plasticity, as they constantly adapt to their environment. In a healthy state, unwanted inflammatory reactions must be prevented, while in a diseased state, an effective immune response must be mounted [7]. Their activation is therefore tightly controlled through several cell–cell and soluble mediator interactions [7]. The transcriptome analysis, as described in chapter 7, pointed towards altered cholesterol metabolism in alveolar macrophages from antibiotic pretreated mice compared to controls. Cholesterol biosynthesis plays an important part in antibacterial effector functions of alveolar macrophages as cholesterol-rich membrane rafts are needed for phagocytosis [8, 9]. One could envision that the level of circulating metabolites derived from the microbiota affects the metabolism of alveolar macrophages. Apart from bacterial cell wall components such as peptidoglycan [2], short chain fatty acids (SCFA) are possible mediators between microbiota and immune cells. These are produced through microbial degradation of dietary fibers; neutrophils and monocytes are known to have receptors that interact with acetate, one of the three most common SCFA. Fibers in diet were shown to modulate immunity in both allergic airway disease and *Pseudomonas* pneumonia [10, 11]. Further studies are needed to investigate the role of SCFA and other possible mediators between microbiota and immune system in our model.

Two previous murine studies reported an effect of the intestinal microbiota on alveolar macrophage polarization, in asthma and an influenza model [3, 12]. In the first study, antibiotic treatment increased *Candida* species in the gut microbiota, which led to higher prostaglandin E_2 (PGE_2) levels in plasma. These proposedly shifted alveolar macrophages into an anti-inflammatory M2 phenotype, thereby promoting allergic airway inflammation [3]. In the second study, *Staphylococcus aureus* (pre-)infection recruited monocytes into the lung which polarized alveolar macrophages into the M2 phenotype [12]. Consequently, inflammation during a secondary influenza infection was decreased. The traditional distinction between pro-inflammatory, classically activated M1 and anti-inflammatory, alternatively activated M2 alveolar macrophages that these studies use was suggested to be abandoned, as alveolar macrophages in healthy individuals do not neatly fit into either a strict M1 or M2 classification [7]. We did not investigate markers for an alveolar M1 or M2 phenotype. Other types of tissue macrophages - Kuppfer cells in the liver and microglia in the brain - were previously reported to be affected by the host microbiota in mice as well [13-15].

The cellular mechanism behind the diminished effectivity of innate immune effector cells after gut microbiota disruption does need further attention. Chapter 7, 8 and 9 contain seemingly inconsistent results. In chapter 7, *ex vivo* stimulation of alveolar macrophages from antibiotic treated mice with LPS resulted in decreased cytokine production compared to control mice, in accordance with data from another research group [16]. In chapter 9, we did not find any differences between alveolar macrophages from control- and antibiotic pretreated mice in a similar experiment. This may for example be due to variations in microbiota in different batches of mice. In chapter 8, we found that upon intranasal

inoculation with LPS the *in vivo* cytokine- and chemokine production was even increased in lungs of antibiotic pretreated mice compared to controls. This indicates that even though effector functions of primary alveolar macrophages appear to be enhanced by a healthy, undisrupted microbiota, *in vivo* a healthy microbiota seems to dampen pulmonary inflammatory responses as a whole.

Little has been published on the effect of antibiotic microbiota disruption on pneumonia-derived sepsis in mice. One study with a design very similar to ours found increased growth of *K. pneumoniae* in antibiotic pretreated mice and also pinpointed this to decreased bacterial killing by alveolar macrophages [1]. Several other studies have been published on the role of the intestinal microbiota during (other than pneumonia-derived) bacterial sepsis [4, 5, 17]. Strikingly, these all find differences in neutrophil production or –functioning related to the intestinal microbiota. Important differences with our studies include the use of germ-free mice or neonatal mice, both of which could give very different results from specific pathogen free adult mice, as will be discussed below.

Limitations

We performed these studies in mice and healthy young men to keep the models as free from confounders as possible. Age may however be an important influential factor: the immune system of neonates receiving antibiotics may very well be affected by the absence of signals derived from the gut microbiota, whereas that of young adults is not. Elderly people may also be more prone to disturbances in microbiota derived signals, with less compensatory mechanisms present due to comorbidities and medications.

Diversity in microbiota composition between individuals and batches of mice may also be a confounding variable. In healthy humans, variation in bacterial microbiota composition is substantial [18], as is confirmed by our findings. On the other hand, it was reported that most healthy people have equal numbers of bacterial metabolic genes in important pathways such as carbohydrate metabolism and vitamin biosynthesis, even if these are coming from different kinds of bacteria [19]. As for the mice, it was previously shown that diet and supplier can be of large influence on the phenotype in malaria [20] and metabolic syndrome [21]. Our data from locally bred TLR-5 knockout mice and commercially bought wildtypes suggest that differences in intestinal microbiota may also influence observed phenotypes during bacterial pneumonia-induced sepsis. Presence of different resistant bacteria in the microbiota of a batch of mice could have a considerable influence on the observed phenotype when using antibiotics as an intervention.

In all experiments, we opted for broad-spectrum antibiotic regimens to achieve maximal changes in gut microbiota and to mimic the spectrum of antibiotics used in clinical practice in the ICU. We administered antibiotics prior to challenge with bacteria or LPS, while in a clinical setting, infectious processes will be already ongoing when antibiotic treatment is started. An important note to both the human and murine studies is that antibiotics may have direct immunomodulatory effects, as was shown for macrolides in chronic obstructive

pulmonary disease [22, 23]. We tried to obviate this through an antibiotic wash-out period before challenge, partly using non-absorbable antibiotics and by demonstrating microbiota-dependency through fecal transplantation in the murine studies. Furthermore, we cannot exclude that the airway microbiota plays a role in the murine studies, although we investigated the pulmonary microbiota by 16S sequencing in one experiment and did not find any differences in the composition of the lung microbiota between control and antibiotic treated mice (data not in this thesis). The lower airway microbiota is difficult to investigate: bacteria are not abundant and samples are easily contaminated by upper respiratory tract - or environmental bacteria. The first papers on the role of the lung microbiota during critical illness are underway: it was recently reported that intestinal tract bacteria can migrate to the airways during murine sepsis and in humans with acute respiratory distress syndrome (ARDS) [24]. Further experiments will be necessary to prove that our observations are indeed intestinal microbiota-dependent. Still, we think that our model in which we use antibiotics to disrupt the microbiota is a clinically more relevant model than germ-free mice, which develop in sterile conditions and thus have a very different immune system. For example, germ-free mice are known to have altered populations of NK-, NKT- and IFN-γ-producing T cells [25].

Fecal sampling in critically ill patients is challenging, as many patients do not defecate. Rectal swabs could serve as an alternative. One should keep in mind that fecal or rectal samples do not provide information about the microbiota in the higher parts of the gastrointestinal tract. Lastly, even though bacteria comprise the vast majority of microbes in the intestinal tract, viruses, fungi and archaea are important players as well - especially when bacteria are wiped out by antibiotics. However, sequencing techniques for investigating the viral or fungal microbiome are not yet as easily available as those for bacteria.

Implications for clinical practice

Results obtained from mice and healthy young adults cannot be directly extrapolated to patients on the ward or ICU. The experiments described in this thesis are models in which the conditions are as controlled as possible. The human endotoxemia model is the best available approximation of sepsis, but unfortunately, *in vivo* responses to LPS do not directly translate to the combat against pathogens during pneumonia and sepsis. As mentioned before, the interplay between pathogen(s), antibiotics and other therapies, microbiota, immune system and comorbidities in patients is complex.

The gut microbiota is a very promising therapeutic target for patients with sepsis, but a lot of work has to be done to determine which patients could benefit from manipulation of the intestinal microbiota and how this should be accomplished. Diet changes, probiotics and prebiotics are all possible interventions but may not be enough to accomplish changes within the extreme imbalance in microbiota we observed in critically ill patients. The positive results from fecal transplantation for recurrent *Clostridium difficile* infection raise hope for simple and effective new therapies that will improve the course of disease in

critically ill patients. At the moment, microbiota-based therapies that re-establish colonization resistance to fight multi-drug resistant bacteria are possibly the next big breakthrough [26, 27]. Fecal microbiota transplantation has a number of drawbacks, such as the risk of transplanting viruses and risk of metabolic- and autoimmune diseases, as well as lack of knowledge on how to select the optimal donor. In the future, fecal transplantation may be replaced by more refined therapies. As an example, an attempt was recently made to use bacterial spores instead of fecal transplantation as a therapy for *C. difficile* [28].

The ultimate goal would be to narrow causal effects down to a couple of metabolites or bacteria, which could then be used as therapy in septic patients. Any detrimental effects that antibiotic microbiota disruption may have on innate immune responses could thus be restored. Using mouse models, different approaches can be taken [29]. Phylogenetic sequencing and metabolomics can be used to characterize microbial communities and -metabolites in different diseases and following different microbial disturbances. Colonizing germ-free mice with single or multiple bacteria of interest allows for investigating microbe-host interactions and assessing potential for therapeutic use. In this way, a synthetic microbiota could be developed that can be cultured and transplanted easily [30]. Already in 1989, five patients with *C. difficile* infection were cured using ten selected strains that showed inhibitory effects against *C. difficile* [30, 31]. More recently, two *C. difficile* infected patients were treated with a microbiota transplantation of 33 cultured bacterial species, isolated from healthy donor feces – both successfull [32]. These results encourage further exploration of microbiota modulation as therapy for many diseases, including pneumonia and sepsis.

References

1. Clarke TB. Early innate immunity to bacterial infection in the lung is regulated systemically by the commensal microbiota via nod-like receptor ligands. *Infect Immun* 2014;82:4596-4606.
2. Clarke TB, Davis KM, Lysenko ES, et al. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med* 2010;16:228-231.
3. Kim YG, Udayanga KG, Totsuka N, et al. Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE(2). *Cell Host Microbe* 2014;15:95-102.
4. Deshmukh HS, Liu Y, Menkiti OR, et al. The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nat Med* 2014;20:524-530.
5. Khosravi A, Yanez A, Price JG, et al. Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host Microbe* 2014;15:374-381.
6. Taur Y, Jenq RR, Perales MA, et al. The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. *Blood* 2014;124:1174-1182.
7. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol* 2014;14:81-93.
8. Kannan S, Audet A, Huang H, et al. Cholesterol-rich membrane rafts and Lyn are involved in phagocytosis during Pseudomonas aeruginosa infection. *J Immunol* 2008;180:2396-2408.
9. Serezani CH, Aronoff DM, Sitrin RG, et al. FcgammaRI ligation leads to a complex with BLT1 in lipid rafts that enhances rat lung macrophage antimicrobial functions. *Blood* 2009;114:3316-3324.
10. Bernard H, Desseyn JL, Bartke N, et al. Dietary pectin-derived acidic oligosaccharides improve the pulmonary bacterial clearance of Pseudomonas aeruginosa lung infection in mice by modulating intestinal microbiota and immunity. *J Infect Dis* 2015;211:156-165.
11. Trompette A, Gollwitzer ES, Yadava K, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* 2014;20:159-166.
12. Wang J, Li F, Sun R, et al. Bacterial colonization dampens influenza-mediated acute lung injury via induction of M2 alveolar macrophages. *Nat Commun* 2013;4:2106.
13. Erny D, Hrabe de Angelis AL, Jaitin D, et al. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 2015;18:965-977.
14. Ferrere G, Leroux A, Wrzosek L, et al. Activation of Kupffer Cells Is Associated with a Specific Dysbiosis Induced by Fructose or High Fat Diet in Mice. *PLoS One* 2016;11:e0146177.
15. Wu X, Sun R, Chen Y, et al. Oral ampicillin inhibits liver regeneration by breaking hepatic innate immune tolerance normally maintained by gut commensal bacteria. *Hepatology* 2015;62:253-264.
16. Prakash A, Sundar SV, Zhu YG, et al. Lung Ischemia-Reperfusion is a Sterile Inflammatory Process Influenced by Commensal Microbiota in Mice. *Shock* 2015;44:272-279.
17. Balmer ML, Schurch CM, Saito Y, et al. Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. *J Immunol* 2014;193:5273-5283.
18. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. *Nature* 2012;486:207-214.
19. Structure, function and diversity of the healthy human microbiome. *Nature* 2012;486:207-214.
20. Villarino NF, LeCleir GR, Denny JE, et al. Composition of the gut microbiota modulates the severity of malaria. *Proc Natl Acad Sci U S A* 2016;113:2235-2240.
21. Vijay-Kumar M, Aitken JD, Carvalho FA, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science* 2010;328:228-231.
22. Albert RK, Connett J, Bailey WC, et al. Azithromycin for prevention of exacerbations of COPD. *N Engl J Med* 2011;365:689-698.

23. Hodge S, Hodge G, Jersmann H, et al. Azithromycin improves macrophage phagocytic function and expression of mannose receptor in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008;178:139-148.
24. Dickson RP, Singer BH, Newstead MW, et al. Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome. *Nature Microbiology* 2016;1:16113.
25. Hansen CH, Metzdorff SB, Hansen AK. Customizing laboratory mice by modifying gut microbiota and host immunity in an early "window of opportunity". *Gut Microbes* 2013;4:241-245.
26. Pamer EG. Resurrecting the intestinal microbiota to combat antibiotic-resistant pathogens. *Science* 2016;352:535-538.
27. Singh R, van Nood E, Nieuwdorp M, et al. Donor feces infusion for eradication of Extended Spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease. *Clin Microbiol Infect* 2014;20:O977-978.
28. Khanna S, Pardi DS, Kelly CR, et al. A Novel Microbiome Therapeutic Increases Gut Microbial Diversity and Prevents Recurrent Clostridium difficile Infection. *J Infect Dis* 2016;214:173-181.
29. Donia MS, Fischbach MA. HUMAN MICROBIOTA. Small molecules from the human microbiota. *Science* 2015;349:1254766.
30. de Vos WM. Fame and future of faecal transplantations--developing next-generation therapies with synthetic microbiomes. *Microb Biotechnol* 2013;6:316-325.
31. Tvede M, Rask-Madsen J. Bacteriotherapy for Clostridium difficile diarrhoea. *Lancet* 1990;335:110.
32. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol* 2013;13:790-801.