The role of the intestinal microbiota in pneumonia and sepsis
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Antibiotic-induced gut microbiota disruption decreases TNF-α release by mononuclear cells in healthy adults

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Abstract

Objectives Broad-spectrum antibiotics disrupt the intestinal microbiota. The microbiota is essential for physiological processes, such as the development of the gut immune system. Recent murine data suggest that the intestinal microbiota also modulates systemic innate immune responses; however, evidence in humans is lacking.

Methods Twelve healthy young men were given oral broad spectrum antibiotics (ciprofloxacin 500mg bid, vancomycin 500mg tid and metronidazole 500mg tid) for seven days. At baseline, one day and six weeks after antibiotics, blood and feces were sampled. Whole blood and isolated mononuclear cells were stimulated with selected Toll-like receptor (TLR) agonists and heat-killed bacteria. Microbiota diversity and composition was determined using bacterial 16S rDNA sequencing.

Results One day after the antibiotic course, microbial diversity was significantly lower compared to baseline. After antibiotic therapy, systemic mononuclear cells produced lower levels of TNF-α after ex vivo stimulation with lipopolysaccharide (LPS). This diminished capacity to produce TNF-α was restored six weeks after cessation of antibiotic therapy. In whole blood, a reduced capacity to release interleukin (IL)-1b and IL-6 was observed after LPS stimulation. Antibiotic treatment did not impact on differential leukocyte counts, phagocytosis and cell surface markers of neutrophils and monocytes.

Conclusions In this proof-of-principle study of healthy subjects, microbiota disruption by broad-spectrum antibiotics is reversibly associated with decreased systemic cellular responsiveness towards LPS. The implications of these findings in a clinical setting are remain to be determined.

Study Highlights

WHAT IS CURRENT KNOWLEDGE
✓ Antibiotics are among the most often prescribed medications
✓ The intestinal microbiota is disrupted by broad spectrum antibiotics
✓ In mice, a well-balanced microbiota appears to stimulate the systemic innate immune system

WHAT IS NEW HERE
✓ In healthy humans, antibiotic microbiota disruption is reversibly associated with decreased TNF-α production by mononuclear cells
✓ Broad-spectrum antibiotic treatment did not impact on differential leukocyte counts, phagocytosis and cell surface markers of neutrophils and monocytes
Introduction

No less than 842 antibiotic courses per 1000 Americans were prescribed in 2011 [1]. The effect of broad-spectrum antibiotics on the gut microbiota is profound: within days a marked loss of diversity and shift in community composition is seen [2]. Given the increasing knowledge on the physiological functions of the intestinal microbiota, which some now even refer to as an “organ in an organ”, the use of antibiotics may have adverse consequences that we are currently unaware of. The microbiota plays a fundamental role in metabolism and in the development of the immune system [3, 4]. The important contribution of a well-balanced intestinal microbiota in host defense against infections, e.g. by colonization resistance, is underscored by the high effectiveness of fecal microbiota transplantation as treatment for recurrent Clostridium difficile infections and potentially other diseases such as ulcerative colitis [5, 6].

Recent preclinical work has suggested that the microbiota also acts as a modulator of systemic immunity [7-11]. In mice, microbial products such as peptidoglycan were shown to prime the innate immune system, thereby enhancing responses against invading pathogens. A decreased production of neutrophils by the bone marrow was observed in mice that were treated with antibiotics or that were born germ-free [8, 9, 12]. Accordingly, host defenses against Streptococcus pneumoniae, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus and Listeria are less effective in these mice [7-10, 13]—mostly attributed to decreased phagocytosis and killing by neutrophils and alveolar macrophages [7, 10, 13].

However, evidence of the intestinal microbiota as modulator of systemic immunity in humans is lacking. We hypothesized that microbiota disruption by antibiotics has adverse consequences on the systemic cellular responsiveness towards danger signals such as microbe associated molecular patterns (MAMPs) from pathogens. As such, the use of antibiotics may lead to immunosuppression, as seen in patients with trauma, burns or sepsis [14]. In this proof-of-principle study, we investigated the effect of gut microbiota disruption by broad-spectrum antibiotics on systemic innate immune responses in healthy subjects.

Methods

Subjects

Twelve healthy Caucasian male subjects were given oral antibiotics (ciprofloxacin 500mg bid, vancomycin 500mg tid and metronidazole 500mg tid) for seven days. Before, one day after antibiotics and six weeks later, blood and feces were sampled (Figure 1a). Ethical approval was received from the Medical Ethics Committee, Academic Medical Center. All subjects gave written informed consent (Netherlands Trial Registry NTR3629). Prior to participation, healthy subjects underwent a medical screening including medical history,
physical examination and hematological and biochemical screening. Participants did not smoke or use any medication. Subjects were excluded if they had used any kind of antibiotics within one year, if they had an abnormal bowel frequency (<3 bowel movements per week or > 3 per day) or travel planned during the study period. Subjects did not use any nutritional supplements or probiotics and did not follow a vegetarian diet, nor were they given dietary instructions. Mean age was 22.2 years (standard deviation 2.5) and mean body mass index 22.6 kg/m² (standard deviation 2.2).

Stimulation assays

Whole blood and purified peripheral blood mononuclear cells (PBMCs) were used for stimulation assays. Venous blood was drawn into 10 mL heparin vacutainers (BD Bioscience). For PBMC isolation, blood was diluted 1:1 in phosphate buffered saline (PBS) and fractions were separated by Ficoll density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare, Zeist, The Netherlands) [15]. After isolation of the PBMC fraction, the cell suspension was incubated with sterile erythrocyte lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA, pH 7.4) for 15 min on ice. Cells were washed twice with PBS and resuspended in RPMI 1640 medium (Life Technologies, Invitrogen, Breda, The Netherlands). The cell number was adjusted using a particle counter (Beckmann Coulter, Woerden, The Netherlands).

Whole blood and PBMCs were mixed in 96-well plates with an equal volume of either plain RPMI or RPMI containing TLR ligands or heat-killed bacteria [16]. Final concentration of PBMCs was 2.5x 10⁶/mL. Whole blood was stimulated for 24 hours with final concentrations of lipopolysaccharide (LPS) 10 ng/mL (E. coli 0111:B4, ultrapure; InvivoGen), PAM3CSK4 1 ug/mL (InvivoGen, San Diego, CA), Flagellin 0.1 ug/mL (S. typhymurium, ultrapure; InvivoGen), heat-killed S. pneumoniae (serotype 2, D39), K. pneumoniae (serotype 2) or B. pseudomallei (clinical isolate 1026b). All bacteria were grown until mid-log phase, washed with saline, heat killed at 70°C for 30 minutes and used at an end concentration of 1x 10⁶ CFU/mL. Additionally, PBMCs were stimulated for four hours with LPS 100 ng/mL in 24-wells plates. All stimuli were pre-diluted in RPMI, aliquoted and stored at -20°C. After incubation at 37°C in 5% CO2, supernatant was obtained by centrifugation and stored at -20°C. Cytokine production was measured by cytometric bead array (Human inflammation kit, BD Bioscience, San Jose, CA) using a FACS Calibur (BD Biosciences, Mountain View, CA). Granulocyte-colony stimulating factor (G-CSF) was measured in plasma by enzyme-linked immunosorbent assay (ELISA, RnD systems, Minneapolis, MN).

Microbiota analysis

Full microbiota analyses are described in the Supplementary Material. Briefly, fresh stool samples were stored at -20°C and transferred to -80°C within 24 hours. Bacterial DNA was extracted using a bead-beating protocol and 16S rDNA was amplified with primers 27F-DegS and 338R as described before [17]. Illumina Miseq sequencing was performed.
(Illumina, San Diego, CA, USA), followed by analysis with the QIIME software package (available at http://qiime.sourceforge.net/) as described [18, 19]. All samples were processed and sequenced in one run. Unconstrained principal component analysis was performed using Canoco 5 software (Biometris, Wageningen, the Netherlands).

Statistics

Normal distribution of each dataset was evaluated using a D’Agostino and Pearson normality test. If normally distributed, comparisons between groups were made using paired t-tests and data are represented as mean ± standard deviation; if not normally distributed, data were analysed using a Wilcoxon signed rank test and data are represented as median ± interquartile range (all using GraphPad Prism 5, GraphPad Software, San Diego, CA). Values of p< 0.05 were considered to represent statistically significant differences. Analysis of fecal microbiota composition was performed with the non-linear mixed effects package in R. Differences between samples of day 0 vs. day 8 and day 0 vs. week 7 were computed using a linear mixed model and P values were corrected for multiple comparisons by the q-value package. As the present study is explorative in nature, no formal power calculation was performed. A subject number of twelve would however be sufficient to provide 80% power to detect differences of 20%, assuming a standard deviation of 20% with α= 0.05. Further methods on phagocytosis assays and cell surface markers are available in the Supplementary Material.

Results

Intestinal microbiota disruption by antibiotics

Administration of broad-spectrum antibiotics disrupted the gut microbiota in all subjects, lowering median Shannon diversity from 3.4 to 1.5 (Figure 1b) with 49 bacterial groups differentially present on day 8 compared to before treatment (Supplementary Figure 1). A strong relative increase in bacteria of the genus Streptococcus was the most striking change in all subjects. In two subjects a large proportion of bacteria consisted of Streptophyta; likewise, Lactobacilli were significantly increased in two other subjects. Genera that had disappeared compared to before treatment included Prevotella, Megamonas, Lachnospiraceae and Bacteroides. Six weeks later, the intestinal bacterial communities had almost returned to their initial diversity (median Shannon index 2.9, statistically significant compared to both day 0 and day 8), but remained different from before treatment as reflected in an unconstrained principal component analysis (Figure 1c). Apart from some gastrointestinal discomfort, none of the subjects experienced any clinically relevant consequences such as C. difficile infection.
**Figure 1.** (A) Study design; (B) Gut microbiota diversity (represented as Shannon Index) based on sequence data of bacterial 16S rRNA genes. Results are presented as median ± interquartile range; p-values are calculated between time points as indicated by lines. *** P < 0.001; (C) Principal component analysis (unconstrained) of fecal microbial communities. On the horizontal axis is Principal Component 1 and on the vertical axis Principal Component 2 with their corresponding percentages of explained variance. White bars or circles: day 0, black: day 8, grey: week 7.

**Cytokine production**

Strikingly, microbiota disruption was associated with lower interleukin (IL)-1β and IL-6 production upon 24-hour stimulation of whole blood with LPS, but not tumor necrosis factor (TNF)-α (Figure 2a-c). In line, stimulation with Gram-negative *K. pneumoniae* and *B. pseudomallei*, but not Gram-positive *S. pneumoniae* or TLR2/-5 agonists, resulted in a trend towards diminished IL-1β and IL-6 release (Supplementary Figure 2). Isolated PBMCs stimulated with LPS showed a similar reduced capacity to release the early proinflammatory cytokine TNF-α, but not IL-6 and IL-1β (Figure 2d-f). These defects were restored six weeks after antibiotics.

**Phagocytosis**

Given the findings in mice that the gut microbiota affects the phagocytic function of bone marrow derived neutrophils [7], we also performed phagocytosis assays with whole blood. However, treatment with broad-spectrum antibiotics did not affect the capacity of neutrophils and monocytes in whole blood to phagocytose heat-killed *S. pneumoniae* or *K. pneumoniae* (Supplementary Figure 3). As granulopoiesis is influenced by the microbiota in mice as well [8, 9], we looked at total blood cell counts and differential white blood cell counts, but these were similar before and after antibiotics (data not shown). Granulocyte-colony stimulating factor (G-CSF) in plasma was below detection (data not shown).

**Cell surface markers**

Lastly, we used FACS analysis to investigate whether disruption of the gut microbiota affects the activation status of unstimulated monocytes and neutrophils (reflected by their
expression of CD11b, CD62L and HLA-DR), their expression levels of TLRs (TLR2, TLR4 and TLR5) and the modulating protein triggering receptor expressed on myeloid cells-1 (TREM-1). None of these were changed after microbiota disruption (data not shown).

**Figure 2.** (A-C) TNF-α, IL-6 and IL-1β levels in supernatant after stimulation of whole blood with LPS 10 ng/mL for 24 hours; (D-F) TNF-α, IL-6 and IL-1β levels in supernatant after stimulation of isolated PBMCs with 100 ng/mL LPS for four hours. White bars represent day 0, black bars day 8 and grey bars week 7. Results are represented as mean ± standard deviation (A-D) or median ± interquartile range (E-F); p-values are calculated between groups as indicated by lines. * P < 0.05, NS, not statistically significant.

**Discussion**

Our findings suggest that gut microbiota disruption by broad-spectrum antibiotics influences TNF-α production by mononuclear cells upon stimulation with LPS. Nonetheless, the overall effect of microbiota disruption on measured systemic immune responses was limited. To our knowledge, this intervention study is the first to investigate...
the interplay between intestinal microbiota and the systemic innate immune system in healthy humans. Up to now, most human studies in the research field of microbiota and innate immune system are associative. The likelihood of microbiota disturbance has for example been positively correlated with the risk of subsequent severe sepsis in a cohort of 10,996 patients [20]. Studies in critically ill patients are limited to descriptive cohort studies, mostly associating higher bacterial diversity with better outcome [21-24].

Mouse studies have reported several mechanisms by which antibiotic microbiota disruption may negatively affect host responses against invading pathogens. Our results support some, but not all of the postulated hypotheses. Firstly, murine studies have suggested that granulopoiesis is an important aspect of microbiota-related protection from infection [8, 9, 12]. We did not find any differences in neutrophil numbers in peripheral blood after antibiotic treatment, compared to before. Secondly, a difference in phagocytic or killing functions of neutrophils has been reported in antibiotic-treated mice [7]. We tested the phagocytic capacities of neutrophils in whole blood, but again did not find any differences. Lastly, cytokine production has been reported to be affected by the microbial status of mice [8, 10]. Our results corroborate these studies, as production of TNF-α by mononuclear cells upon LPS stimulation was significantly lower after the antibiotic course. To a lesser extent, IL-1β and IL-6 production by whole blood upon Gram-negative stimulation was affected as well.

Several factors could explain the differences between the present study and previous mouse studies. Some of the reported experiments were performed in neonatal mice; the bone marrow of healthy adults may be much less sensitive to microbial signals than that of children. Also, redundancy in the innate immune system may compensate for any small deficit caused by microbiota disruption. Finally, humans are much more diverse than mice in their genetic makeup, environment and microbiota, which could make it more difficult to detect any small changes in immune effector functions. Still, this study was designed to be as controlled as possible.

This proof-of-concept study has several limitations. Firstly, it is not possible to associate the observed effects with specific antibiotics or bacterial taxa due to the broad spectrum antibiotic regimen, which was chosen to achieve the largest possible effect on the intestinal microbiota. Secondly, a 24-hour antibiotic washout period was included to avoid direct effects of antibiotics present in blood on the performed tests. We cannot exclude that partial recovery of the microbiota and immune system has taken place during this interval. Furthermore, like most human microbiota studies, we have focused on bacteria only as they comprise the vast majority of the intestinal microbiota. Fungi, viruses and archaea may however play a significant role when bacteria are eradicated by antibiotics. Lastly, interindivdual differences in microbiota composition may be an important factor, although numbers of genes for bacterial metabolic pathways are very similar among healthy people, even if those genes are coming from different kinds of bacteria [25]. Still, it is important to keep in mind that these results may not translate to people with a different diet, sex, age or genetic background - or to patients, where comorbidities and medications come into play.
TNF-\(\alpha\) is of major importance in the host defense against invading pathogens, as is evident from the occurrence of severe infections in patients who receive TNF-\(\alpha\) blocking agents [26]. Decreased TNF-\(\alpha\) production caused by antibiotic microbiota disruption may therefore have detrimental effects in for example critically ill patients, in whom immunosuppression is a common phenomenon [14]. These effects could be more pronounced in patients with any underlying immune-mediated diseases, but further research is needed to further explore this. For example, the selection of healthy donors for fecal microbiota transplantation for immune-related diseases such as inflammatory bowel disease and autoimmune diseases could be improved through further knowledge on this subject.

If our results are to be reproduced, future research should focus on the underlying mechanisms. It will be of importance to clarify which bacteria or which bacterial components of the microbiota are key players in these immunostimulatory processes. Ultimately, potential therapeutic strategies that target the microbiota in order to boost the immune system during systemic illness may be explored. Of interest, a first patient with therapy-resistant sepsis and diarrhea has recently been treated with fecal microbiota transplantation [27].

In conclusion, these data suggest the existence of systemic immunomodulation by the microbiota in humans and highlight potential adverse consequences of microbiota disruption by broad-spectrum antibiotics on TNF-\(\alpha\)-related immune defenses to infection.

**Translational impact**

Antibiotics could have unknown adverse consequences by affecting systemic innate immune defenses via microbiota disruption. Further research should elucidate the mechanisms behind this finding.

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References

Supplementary methods

Microbiota analysis

Fresh stool samples were stored at -20°C and transferred to -80°C within 24 hours. DNA was isolated using a bead-beating protocol as described before [1]. Samples were prepared for Illumina Miseq sequencing using a two-step protocol for amplification of the 16S rRNA gene. The V1-V2 region of the bacterial 16S rRNA gene fragments was amplified using universal primers 27F and 338R [1]; for the second step, barcodes Unitag1 were added on the 3' end (5'-GAGCCGTAGCCAGTCTGC-3') of the forward primer and Unitag2 (5'-GCCGTGACCGTGACATCG-3') to the reverse primer. The PCR program consisted of 98°C for 30 seconds to activate the enzyme, then 25 cycles of 98°C for 10 seconds, 56°C for 20 seconds and 72°C for 20 seconds, followed by a final extension at 72°C for 10 minutes. Hereafter, 5 μL of PCR product was used in a second PCR to add the 8 nucleotide sample specific barcodes. The PCR program started with an activation step at 98°C for 30 seconds, followed by 5 cycles at 98°C for 10 seconds, 52°C for 20 seconds and 72°C for 20 seconds, finishing with an extension step at 72°C for 10 minutes. The PCR product was purified using Highprep PCR clean-up magnetic beads (Magbio, London, UK). The concentration of the cleaned PCR product was measured with a Qubit dsDNA BR Assay Kit (Thermo Fischer, Waltham, MA, USA) in the Qubit 2.0 device (Thermo Fischer). Finally, the samples were pooled equimolarly, 48 samples per library (including 2 mock communities as an internal standard), after which the libraries were concentrated with Highprep PCR beads (Magbio). The samples were analysed on the Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA).

Data was analysed using the QIIME software package. Fasta data was demultiplexed and filtered using default settings. Sequences were denoised using Acacia, followed by chimera removal using the USEARCH algorithm. After filtering, denoising and chimera removal, UCLUST software was used to pick operational taxonomic units (OTUs) with 97% sequence similarity. A representative sequence from each OTU was picked and taxonomy assigned using the SILVA 111 reference database clustered at 97% similarity and complying with the six taxonomic levels of the Ribosomal Database Project (RDP) classifier. The obtained OTU table was filtered for OTUs with a total observation count of less than two and for OTUs that were present in less than two samples.

Phagocytosis assay

To measure phagocytic capacity, whole blood was incubated with pre- aliquoted fluorescein isothiocyanate (FITC)-labeled, heat-killed *S. pneumoniae* or *K. pneumoniae* (as described above) for 30 minutes at 37°C or 4°C and quenched with 0.4% trypan blue. After washing with ice-cold FACS buffer and erythrolysis (as described above), cells were
analysed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA).
Neutrophils and monocytes were identified by forward- and side scatter. The phagocytic index was calculated by multiplying the percentage of FITC-positive cells and their fluorescence intensity, followed by subtraction of the corresponding 4°C sample.

**Cell surface markers**

After erythrocyte lysis, whole blood was incubated with antibodies for activation markers diluted in FACS buffer. Expression of cell surface markers was analyzed using phycoerythrin-conjugated mouse anti-human TLR2, TLR4 (both eBioscience, San Diego, CA), TLR5 (Abcam, Cambridge, UK), CD11b, CD62L, HLA DR (BD Pharmingen, San Diego, CA) and TREM-1 (R&D Systems, Minneapolis, MN) with a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA). Cell populations were identified by mouse anti-human FITC-labelled CD66B, PE-Cy7 labelled CD16 and APC labelled CD14 (all from BD Pharmingen, San Diego, CA).

**References**

Figure S1. Heat maps of fecal microbiota with significantly different (adjusted p-value < 0.05) bacterial groups between day 0 versus day 8 samples. Each column represents one volunteer; the color code shows log10-fold changes. No significant changes in bacterial groups were detected between day 0 and week 7.
Figure S2. (A) IL-6 and (B) IL-1β levels in supernatant after stimulation of whole blood with heat-killed *K. pneumoniae* for 24 hours, measured by cytometric bead array; (C) IL-6 and (D) IL-1β levels in supernatant after stimulation of whole blood with heat-killed *B. pseudomallei* for 24 hours, measured by cytometric bead array. White bars represent day 0, black bars day 8 and grey bars represent week 7. Results are represented as median ± interquartile range; p-values are calculated between groups as indicated by lines. NS: P > 0.05.

Figure S3. Phagocytosis of FITC-labelled heat-killed *K. pneumoniae* (A) and *S. pneumoniae* (B) by neutrophils in whole blood after 30 minutes, expressed as phagocytosis index (% of FITC-positive cells x mean fluorescence intensity, followed by subtraction of the 4°C sample value from the 37°C sample value). Results for monocytes are similar (not shown). Results are represented as median ± interquartile range. NS: P > 0.05.