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Vendor effects on murine gut microbiota influence experimental abdominal sepsis

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ABSTRACT

Background: Experimental animal models are indispensable components of preclinical sepsis research. Reproducible results highly rely on defined and invariant baseline conditions. Our hypothesis was that the murine gut microbiota varies among different distributors of laboratory animals and that these variations influence the phenotype of abdominal sepsis derived from a bacterial inoculum model (intraperitoneal stool injection).

Materials and methods: Male C57BL/6 mice (8-wk old) purchased from Charles River (CR), Janvier (J), and Harlan (H) were sacrificed, and the bacterial composition of feces was analyzed using CHROMagar orientation medium. Stool was injected intraperitoneally into CR mice, followed by clinical observation and gene expression analysis. Experiments were repeated 16 mo later under the same conditions.

Results: Stool analysis revealed profound intervendor differences in bacterial composition, mainly regarding *Staphylococcus aureus* and *Bacillus licheniformis*. Mice challenged with CR as well as H feces developed significantly higher severity of disease and died within the observation period, whereas stool from J mice did not induce any of these symptoms. Real-time polymerase chain reaction revealed corresponding results with significant upregulation of proinflammatory cytokines and vascular leakage-related mediators in CR and H injected animals. Sixteen months later, the bacterial fecal composition had significantly shifted. The differences in clinical phenotype of sepsis after intraperitoneal stool injection had vanished.

Conclusions: We are the first to demonstrate vendor and time effects on the murine fecal microbiota influencing sepsis models of intraabdominal stool contamination. The

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intestinal microbiota must be defined and standardized when designing and interpreting past and future studies using murine abdominal sepsis models.

Introduction

Experimental animal models are indispensable components of preclinical sepsis research. They help to validate hypotheses generated *in vitro*, translating findings into a more complex organism. A wide range of various animal models to study bacterial sepsis have been developed over the last decades, predominantly using mice.[1] Apart from the simple injection of pathogenic ligands such as lipopolysaccharide (LPS, "endotoxin"), flagellin, or CpG oligodeoxynucleotides, the transfer of bacteria into the lung or the abdominal cavity is widely used to induce the clinical and molecular phenotype of gram-positive and -negative sepsis. Popular models to generate sepsis from an abdominal focus are, among others, the intraperitoneal stool injection (IPSI), the cecal ligation and puncture (CLP), and the colon ascendens stent peritonitis (CASP) model.[2] Although CLP and CASP are models of endogenous fecal contamination, being based on induced stool leakage into the peritoneal cavity, for the IPSI model, feces previously harvested from another living being are injected through the abdominal wall. Regardless of the model used, reproducible results rely on defined and invariant baseline conditions, that is to say primarily the bacterial composition of the feces being used to create an intraabdominal focus. In human sepsis, it was demonstrated that the bacterial spectrum isolated from the abdomen is associated with the patients' outcome.[3] Moreover, it is known that the indigenous intestinal flora itself influences the individual's susceptibility to infection.[4] On the other hand, this intestinal flora may be subject to change by varying environmental conditions.[5] Our hypothesis was that the murine gut microbial flora varies among different distributors of laboratory animals and that these variations influence the phenotype of experimental abdominal sepsis. We therefore analyzed the intestinal bacterial composition of one same inbred strain (C57BL/6 mice) from different vendors and examined the clinical as well as molecular manifestation of sepsis derived from corresponding IPSI. Our results will contribute to the definition of parameters for standardization and help designing and interpreting past and future studies using murine models of experimental abdominal bacterial sepsis.

Materials and methods

Animals

All experiments were performed on C57BL/6 mice (*n* substrain) at an age of about 8 wk. Only animals of male sex were used to control for hormonal variations that may have obfuscated the results, thus ensuring the necessary validity of the study. Stool donor mice were purchased from Charles River (CR; Sulzfeld, Germany), Janvier (J; LeGenest-Saint-Isle, France), and Harlan (H (now Envigo Research Models and Services); Indianapolis, Indiana, USA) and were sacrificed immediately after purchase without prior housing in the local animal facility. Recipient animals were purchased from CR and J and were housed for 14 d in individually ventilated, pathogen-free cages with access to water and standard rodent chow (provided from SSNIFF GmbH, Soest, Germany) ad libitum before challenge. The animal protocol of this study was approved by the local committee for animal care (LANUV, Recklinghausen, Germany; protocol no. 84-02.04.2013.A071) and was in accordance with the National Institutes of Health guidelines for the use of live animals (NIH publication No. 85-23, revised 1996).

Isolation of stool from donor mice

Donor mice were sacrificed by cervical dislocation immediately after purchase without prior housing in the local animal facility. After median laparotomy, stool from the cecum and colon was collected under sterile conditions. One animal yielded approximately 0.1-0.2 g of feces. Stool from individual animals was analyzed for bacterial composition, whereas for the challenge experiments, the stool from 20 mice per vendor was pooled.

Initial experiments were performed in 2012 (October) and were subsequently repeated 16 mo later (February 2014).

Analysis of bacterial composition of stool

For initial characterization of the microbial composition of murine feces, freshly isolated stool from donor mice was inoculated on CHROMagar orientation medium plates (CHROMagar, Paris, France) and incubated under controlled aerobic conditions at 37°C for 48 h at the Institute for Medical Microbiology, Immunology and Parasitology (IMMIP) of the University Hospital Bonn according to the local standard. This medium was designed for rapid discrimination of different bacterial pathogens by the color of the colonies formed on the agar. The differentiation was performed according to the manufacturer's description. Since a blue appearance should indicate both bacteria from the *Klebsiella (*KES) group (*Klebsiella*, *Enterobacter*, *Citrobacter,* and *Serratia* spp.) as well as *enterococci*, these groups were summarized and termed "KES-E". Random samples of colonies of all colors were furthermore analyzed using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry to verify the correct identification of the corresponding bacterial species.

Feces from 20 donor animals of each vendor were analyzed, and the number of mice showing positive results for the individual bacterial species was used to calculate the relative abundance in percentage of total readouts.

Challenge experiments: intraperitoneal stool injection

For intraperitoneal application, freshly isolated stool samples from 20 mice from each of the three different distributors were pooled within each group and diluted 1:3 using sterile 0.9% sodium chloride solution. Large particles were removed by filtration, yielding a suspension that easily passes through a

21 G injection cannula. Recipient animals were weighed, and stool suspension was injected intraperitoneally into the lower right quadrant using a 21 G cannula (8-μL suspension/g BW). Sham mice received an equivalent volume of 0.9% sodium chloride solution, whereas control animals were left untreated. According to the protocol, systemic analgesia was performed in all groups with subcutaneous administration of buprenorphine hydrochloride (Temgesic, Indivior UK Limited, Berkshire, UK) immediately following IPSI and every 12 h thereafter.

Recipient animals were purchased from CR. In experiments assessing the influence of the host microbiota, mice from J were injected in addition. In these experiments, stool from CR donors only was used.

Clinical observation

Before and after IPSI, the clinical outcome of injected animals was assessed regularly (every 6 h) by observers blinded for the treatment protocol. The severity of symptoms was assessed by applying a clinical score, adopted from the one developed by Morton and Griffiths in 1985.[6] The score used for our study describes motor coordination, grooming, and behavior. Every item was given a maximum of three points in steps of 0.5. No points were given for normal motor coordination, running, and stretching; for a smooth and shiny fur, clean orifices, and glossy eyes; and for normal behavior (sleeping, reaction to touching, curiosity, and social contacts), resulting in a minimum of 0 points for normal, healthy animals. Accordingly, up to 3 points per item were given for, for example, a cramped muscle tone, for dull fur and eyes, and for lethargy and autoaggression, resulting in a maximum of 9 points. According to the protocol, animals reaching the maximum score had to be sacrificed (humane endpoint). Correspondingly, animals that died spontaneously were generally given 9 points.

Rectal body temperature and body weight were assessed before and every 6 h following IPSI.

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

At the end of the clinical observation period, 18 h following IPSI, the animals were sacrificed by cervical dislocation, and the lungs were harvested, snap frozen in liquid nitrogen, and stored at −80°C until further use.

Frozen organs were homogenized in 4-M guanidinthiocyanat (Roth, Karlsruhe, Germany) using a stand dispersion unit (Polytron-Kinematica, Lucerne, Switzerland). Tissue debris was removed by centrifugation, and total RNA was isolated with TRIzol reagent (Fisher Scientific, Schwerte, Germany) according to the manufacturer's instructions. The air-dried RNA pellet was resuspended in RNAse-free water, and the concentration was determined spectrophotometrically (NanoDrop, Thermo Scientific, Waltham, MA, USA). Two micrograms of total RNA were reverse transcribed into complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany). Following synthesis, complementary DNA was diluted in RNAse-free water (1:5) and stored at +4°C. Gene expression analysis of Interleukin (IL)-1β, IL-6, and Tumor necrosis factor (TNF)-α; of ANG1, ANG2, and TIE2 as well as of toll-like receptor (TLR) 2 and TLR4 was performed by quantitative RT-PCR with Taqman Expression Assays for the respective genes and Taqman Gene Expression Master Mix on a ViiA7 device (all Applied Biosystems, Weiterstadt, Germany). The expression was normalized to the housekeeping gene 18S ribosomal RNA (relative quantification) and calculated as fold change expression of the respective control (delta-delta CT method). All these processes were performed by personnel blinded for the treatment protocol.

Statistics

Statistical analysis and visualization of data were performed using GraphPad PRISM 5 (La Jolla, CA, USA) and MS Office 2010 (Redmond, WA, USA). Results of the stool analysis are presented as relative abundance of the respective bacterial species in percentage of 20 readouts. Clinical data of the challenge experiments as well as PCR results are presented as mean ± standard deviation. Each experiment was repeated at least three times. Significance of vendor effects on IPSI-derived clinical symptoms was tested using one-way analysis of variance, followed by Dunnett's test to compare intragroup differences and by Tukey's test to compare between different groups of vendors. Changes of gene expression were tested using one-way analysis of variance, followed by Dunnett's multiple comparison test. To assess significance of the host microbiota, groups were compared using the unpaired, two-tailed Student t-test. The alpha level was set at 5%.

Results

The intestinal microbial flora of C57BL/6 mice varies among different distributors of laboratory animals

Male C57BL/6 mice (8-wk old, $n = 20$) from three different distributors (CR, J, and H) were sacrificed immediately after purchase without prior housing in the local animal facility. Stool was collected from the colon and spread onto CHROMagar orientation medium. This medium allowed differentiation between four main bacterial species: *Escherichia coli (E coli)*, *Staphylococcus aureus* (*S aureus*), *Staphylococcus saprophyticus* (*S saprophyticus*), and *Proteus spp*. A fifth group, showing blue spots on the culture medium, was termed "KES-E", comprising *Klebsiella*, *Enterobacter*, *Citrobacter,* and *Serratia* spp. as well as *enterococci* (Fig. 1A, B). To validate the results from the cultural analysis, random samples of colonies of all colors were furthermore analyzed using MALDI-TOF, which verified correct identification of the corresponding bacterial species.

Almost all animals from each of the analyzed distributors showed positive results for KES-E and *S saprophyticus* in their feces (Fig. 1C). *E coli* could be detected in 95% of mice from CR, whereas its relative abundance was about 60% in animals from J and H. Differences were seen in regard to the presence of *S aureus* and *Proteus* spp., that varied among the distributors, as the first was absent in feces from J, and the latter could only be detected in stool from CR mice. Interestingly, there was another group, only present in feces from all J animals, that could not be clearly identified by its appearance on the

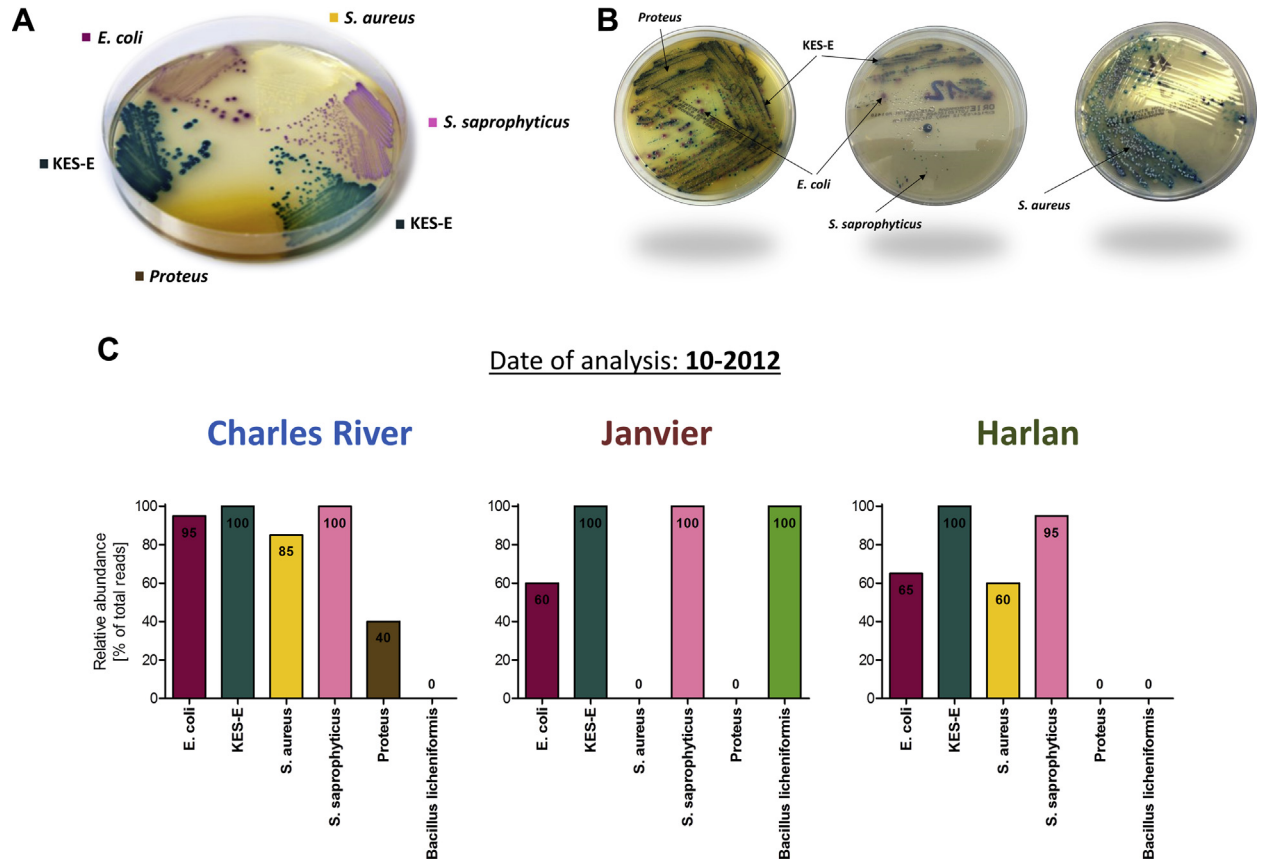

Fig. 1 – Vendor-dependent differences in the intestinal microbial flora of C57BL/6 mice. (A) Exemplary presentation of the appearance of different bacterial species on the CHROMagar orientation medium used for the analysis of murine feces. Picture with kind permission of CHROMagar, Paris, France. (B) Male C57BL/6 mice (8-wk old) were sacrificed immediately after purchase, and stool was collected from the colon and spread onto CHROMagar orientation medium. Picture shows the appearance of different bacterial species in the feces. (C) Relative abundance of the bacterial species identified in stool of mice purchased from Charles River, Janvier, and Harlan during the analysis in October 2012. ($n = 20$ for each vendor). (Color version of figure is available online.)

agar medium. However, MALDI-TOF identified these colonies as *Bacillus licheniformis*.

Varying stool microbiota of donor mice influences the clinical phenotype of sepsis derived from IPSI

Using this approach, we could clearly demonstrate differences in the fecal flora depending on the vendor. To test whether these variations in microbial stool composition of donor mice influence the clinical manifestation of IPSI-derived sepsis, the stool samples were pooled within each vendor group, diluted and injected intraperitoneally into mice purchased from CR ($n = 5$), followed by observation of the clinical outcome (Fig. 2A). Optimal stool concentration had been determined by previous dose titration experiments, the details of the model establishment will be published elsewhere.

All recipient animals showed healthy appearance as well as normal values for body temperature and weight at the beginning of the experiment (Fig. 2B). Neither the clinical score, describing motor coordination, grooming, and behavior, nor body temperature or weight significantly changed in control or sham animals (the latter having received sodium chloride injection). In mice having received stool from J animals, the clinical score was likewise unaffected, and all animals survived the observation period. In contrast, both groups being injected CR and H stool showed rapid deterioration of clinical appearance, as evidenced by significantly increased score after 12 h. Nearly, 40% (CR) and 20% of animals (H), respectively, died before the end of the observation period.

Body temperature is a very sensitive marker to determine the severity of disease of murine abdominal sepsis.[7] In the J stool receiving group, rectally measured body temperature was not significantly altered. However, mice being challenged with stool from CR or H animals rapidly developed hypothermia below critical values of 30°C within 6 h. This was persistent until the end of the observation period.

Body weight tended to decrease in all treatment groups following IPSI compared to control or sham animals (that showed tendency toward a rather increasing body weight); however, this was not significant for any of the donor groups.

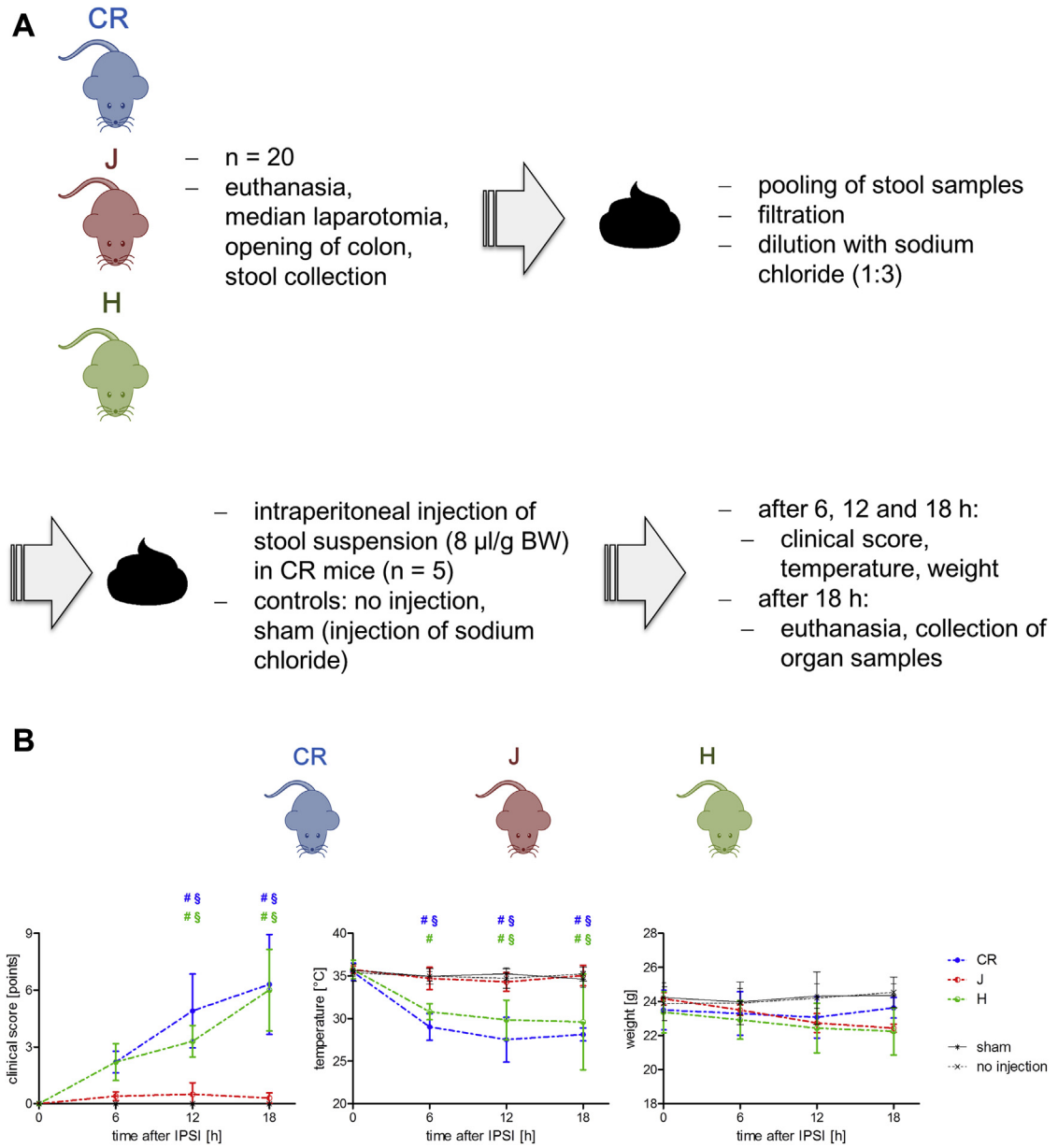

Fig. 2 – Variations in the gut microbiota of donor mice influence the clinical phenotype of sepsis derived from IPSI. (A) Male C57BL/6 mice (8-wk old, $n = 20$ for each vendor [CR, J, and H]) were sacrificed immediately after purchase. Stool was collected from the colon, and samples were pooled within each vendor group, diluted and filtered and injected intraperitoneally into mice purchased from CR ($n = 5$), followed by observation of the clinical outcome for 18 h. Control animals received no treatment, sham animals received injection of sodium chloride. At the end of the clinical observation period after 18 h, the animals were sacrificed, and organs were harvested. (B) Clinical score (describing motor coordination, grooming, and behavior; left diagram), temperature (middle diagram), and body weight (right diagram), assessed in animals receiving stool from CR (blue), J (red), and H (green) donors 6, 12, and 18 h following IPSI. ($n = 5$ per group [control and sham group: $n = 3$], mean ± standard deviation, one-way ANOVA, $^{\#}P < 0.05$ [within each group *versus* 0 h time point, Dunnett's multiple comparison test], $^{\S}P < 0.05$ [*versus* J group, Tukey's multiple comparison test]). (Color version of figure is available online.)

Bacterial composition of donor stool influences molecular markers of inflammation and vascular leakage following IPSI

At the end of the observation period, the animals were euthanized, messenger RNA was extracted from harvested lungs, and quantitative RT-PCR was performed to assess expression of proinflammatory cytokines, of markers of endothelial leakage and of TLRs. The expression of all tested genes in lungs of sham mice was unaffected compared to controls 18 h after IPSI (Fig. 3). In contrast, IL-1β, IL-6, and TNF-α were markedly upregulated in mice having received stool

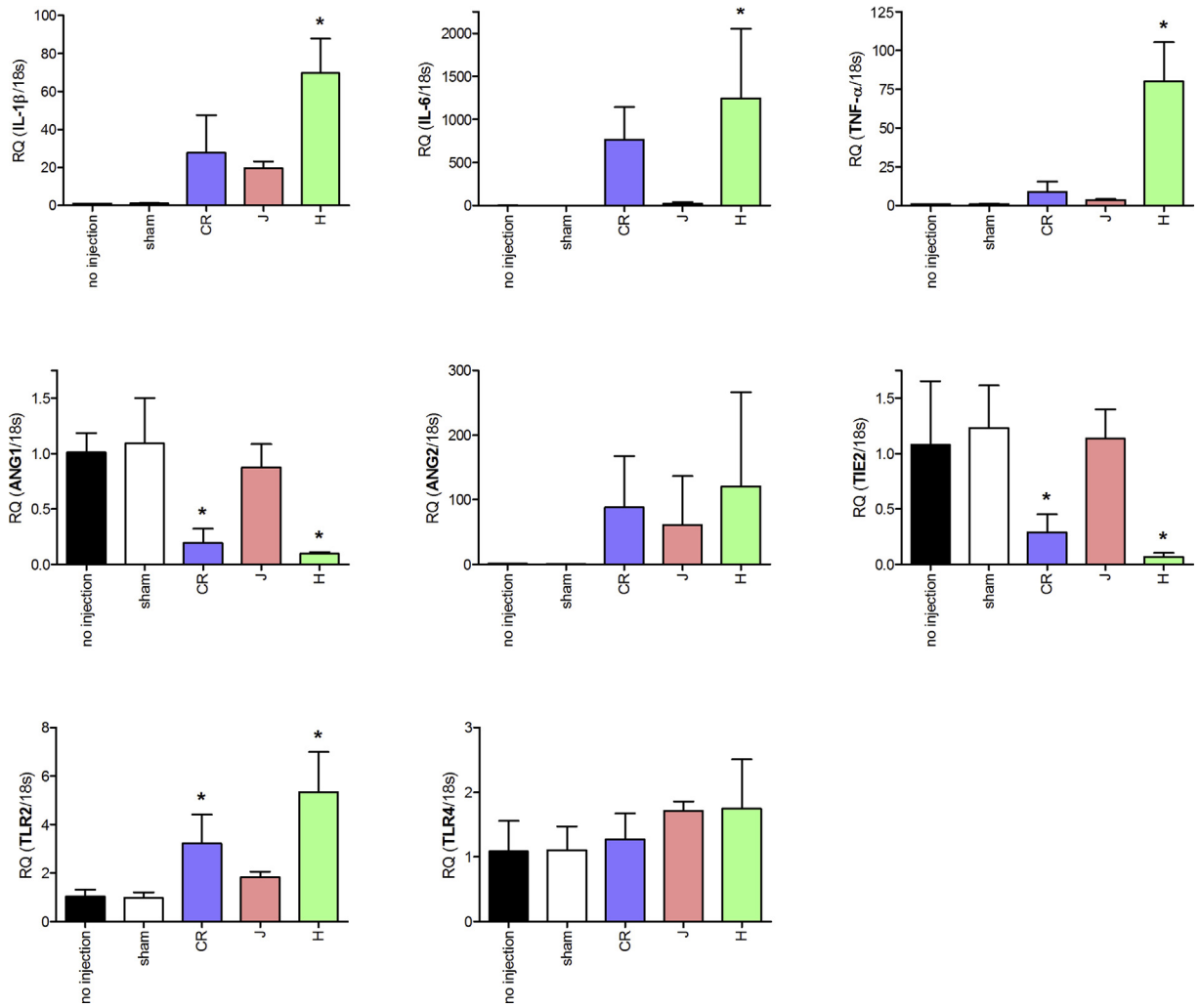

Fig. 3 – Variations in the gut microbiota of donor mice influence molecular markers of inflammation and vascular leakage following IPSI. Male C57BL/6 mice (8-wk old, $n = 20$ for each vendor CR, J, and H) were sacrificed immediately after purchase. Stool was collected from the colon, and samples were pooled within each vendor group, diluted and filtered and injected intraperitoneally into mice purchased from CR ($n = 5$). Control animals received no treatment, sham animals received injection of sodium chloride. After 18 h, the animals were sacrificed, lungs were harvested, and mRNA was extracted to perform quantitative real-time PCR. 18s ribosomal RNA was used as housekeeping gene. Figure shows relative quantification (RQ) of lung expression of proinflammatory cytokines (upper panel), of vascular leakage–related markers (middle panel), and of TLR2 and 4 (lower panel). ($n = 5$ per group [control and sham group: $n = 3$], mean ± standard deviation, one-way ANOVA, followed by Dunnett's multiple comparison test, $^*P < 0.05$ [*versus* sham group]). (Color version of figure is available online.)

from CR and H donors, whereas the expression in animals being injected J stool was not affected (Fig. 3, upper panel). The angiopoietins ANG1 and ANG2 together with their receptor TIE2 indicate and mediate altered endothelial permeability during sepsis.[8] The expression of ANG1 and TIE2 was significantly reduced in the lungs of animals that received feces from CR and H donors, whereas in J-injected mice, it remained unchanged (Fig. 3, middle panel). In contrast, although not significant, ANG2 displayed a reversed pattern, with a more pronounced expression in those animals having received stool from CR and H mice compared to the J group. Finally, although the expression of TLR4 was not altered following IPSI, that of TLR2 was significantly upregulated in the CR and H compared to the J group (Fig. 3, lower panel).

Stool quality of donor mice changes over time

To assess the stability of experimental conditions, stool extraction and IPSI were repeated approximately 16 mo after the initial experiments. Owing to the obvious and profound differences between CR and J microbiota, only donor mice from these two distributors were reassessed. Cultural analysis revealed significant shifts in bacterial composition of feces over time (Fig. 4A). E. coli, KES-E as well as *S saprophyticus* were

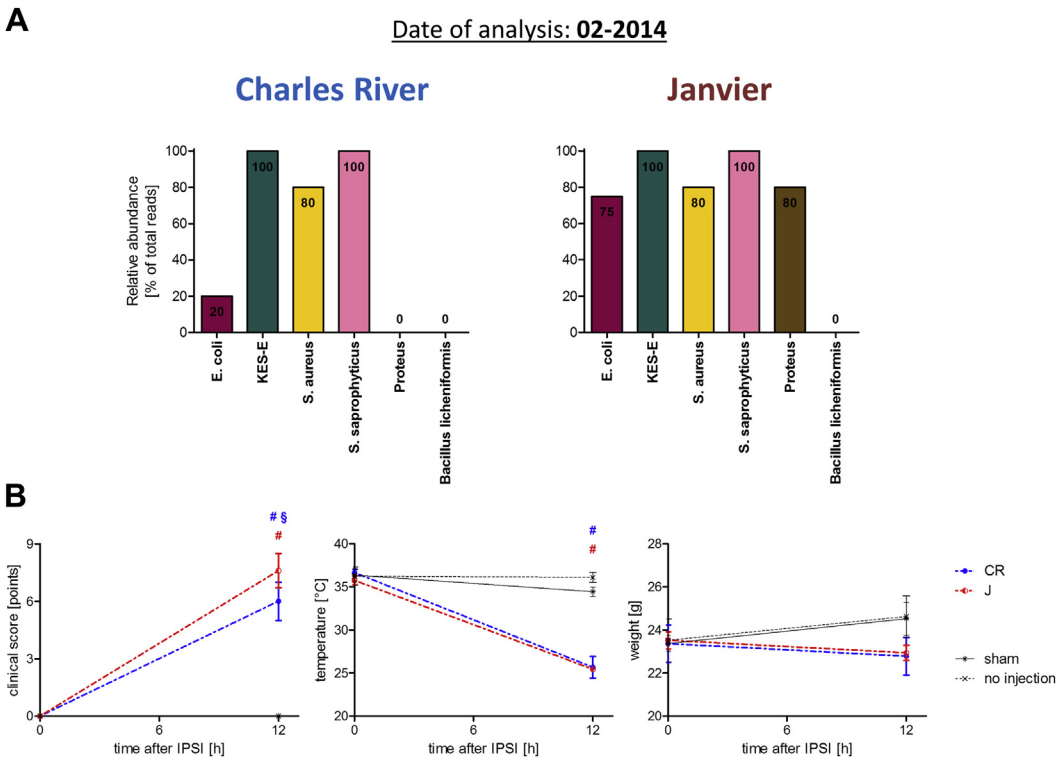

Fig. 4 – Stool quality of donor mice changes over time. (A) Male C57BL/6 mice (8-wk old) were sacrificed immediately after purchase, and stool was collected from the colon and spread onto CHROMagar orientation medium. Figure shows the relative abundance of the bacterial species identified in stool of mice purchased from CR and J during the analysis in February 2014 ($n = 20$ for each vendor). (B) Stool samples were pooled within each vendor group, diluted and filtered and injected intraperitoneally into mice purchased from CR ($n = 5$), followed by observation of the clinical outcome. Control animals received no treatment, sham animals received injection of sodium chloride. Figure shows clinical score (describing motor coordination, grooming, and behavior; left diagram), temperature (middle diagram), and body weight (right diagram), assessed in animals receiving stool from CR (blue) and J (red) donors 12 h after IPSI. ($n = 5$ per group [control and sham group: $n = 3$], mean ± standard deviation, one-way ANOVA, $^{\#}P < 0.05$ [within each group *versus* 0 h time point, Dunnett's multiple comparison test), $^{\S}P < 0.05$ (*versus* J group, Tukey's multiple comparison test). (Color version of figure is available online.)

still detected in stool of both CR and J mice; however, *E coli* was now only found in 20% of CR animals (compared to 95% during the initial analysis 16 mo ago). Both groups were now positive for *S aureus* in the majority of mice (80%). In contrast to the initial assessment, *P* spp. were now detectable in J animals, whereas they were absent in the CR group. Furthermore, the formerly detectable group of *B licheniformis* could not be detected any longer.

When CR mice were now challenged with pooled stool, the clinical manifestation after IPSI likewise changed under these shifted conditions (Fig. 4B). Injection of J stool now elicited similar disease severity as the use of CR feces did, with score values after 12 h being even significantly elevated compared to the CR group. The same applied to the body temperature, which fell to mean values below 26°C after 12 h in both groups, whereas control as well as sham animals showed stable body temperature. Again, body weight decreased in tendency with increasing values in control and sham mice. There was no difference between CR and J donor group.

To assess the role of the host microbiota for the manifestation of clinical symptoms following IPSI, we furthermore challenged animals purchased from CR as well as J with pooled stool harvested from CR mice (Fig. 5A). Twelve hours after IPSI, mice purchased from J, being injected the "foreign" stool, lost significantly more weight and developed more severe hypothermia compared to the animals from CR (Fig. 5B). Clinical score tended to be higher in J mice; however, this was not statistically significant.

Discussion

In the past decade, the vertebrates' intestinal microbiota has experienced growing interest. On the one hand, the lower gut microbial flora directly elicits, for example, immune modulating effects, and its disturbance may have severe clinical consequences.[4] On the other hand, it was recognized that this flora can intentionally be modified and shaped. New techniques such as next-generation sequencing meanwhile allow comprehensive mapping of the mammalian intestinal microbiota.[9] One fundamental principle, regardless of the species, is the existence of interindividual differences, caused by genetic background, climate or nutrition habits, making the individual's microbiota almost as representative as a

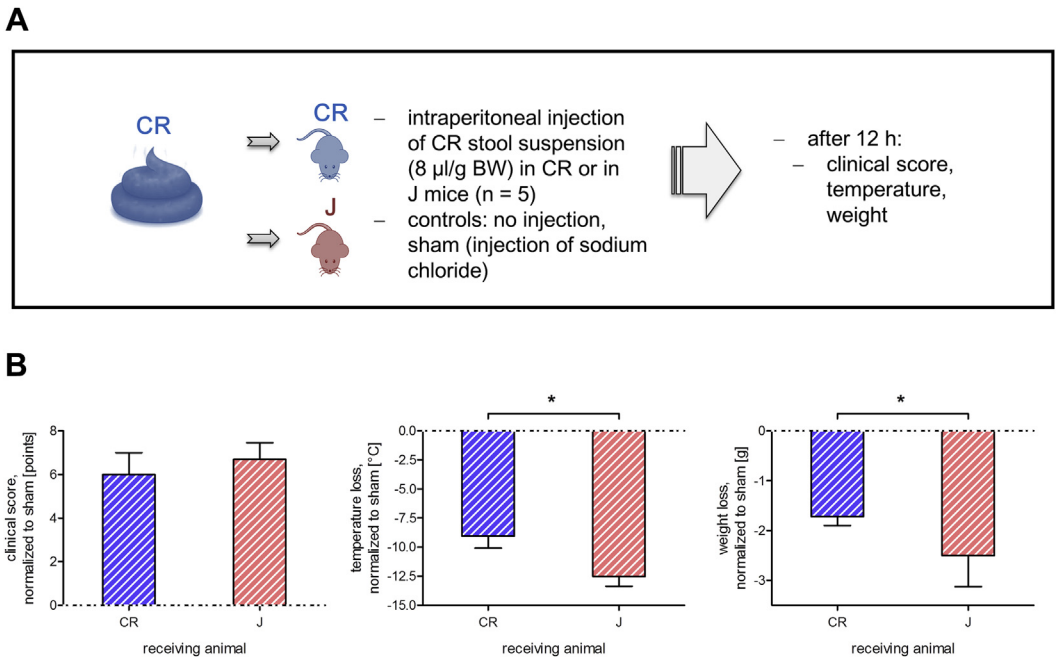

Fig. 5 – The host microbiota influences the clinical phenotype of sepsis derived from IPSI. (A) Male C57BL/6 mice purchased from CR (8-wk old, $n = 20$) were sacrificed immediately after purchase, and stool was collected from the colon. Stool samples were pooled, diluted and filtered and injected intraperitoneally into mice purchased from CR and J ($n = 5$ for each recipient group), followed by observation of the clinical outcome. Control animals from the corresponding vendor received no treatment, sham animals received injection of sodium chloride. (B) Clinical score (describing motor coordination, grooming, and behavior; left diagram), temperature loss (middle diagram), and body weight loss (right diagram), assessed in CR (blue) and J (red) recipients 12 h following IPSI. Values are normalized to sham animals of the respective time point and vendor (indicated by the dashed line). ($n = 5$ per recipient group, mean ± standard deviation, unpaired, two-tailed Student *t*-test, $^*P < 0.05$). (Color version of figure is available online.)

fingerprint.[5,10,11] We made use of a semi-quantitative, chromogenic screening agar, validated for the differentiation of urinary tract bacteria, to characterize the animals' gut microbial composition in our study.[12] Additional species identification was performed with MALDI-TOF, a technique widely used in clinical as well as research microbiological applications.[13] Although somewhat rough, our analyses revealed fundamental differences in the composition of the aerobic fecal bacterial flora of C57BL/6 mice from three different vendors (Fig. 1C). Most striking was the absence of *S aureus* in animals purchased from J, whereas this bacterium was highly present in mice from CR and H. *S aureus* is a gram-positive coccal bacterium, usually colonizing skin and mucosal surfaces of the upper respiratory tract. In healthy C57BL/6 mice, it may also be found as commensal in the intestine; however, compared to skin and nose, it is less known for being detected in the feces.[14] Since sterile sample acquisition was highly standardized in our study and *S aureus* was not detected in any of the mice purchased from J, contamination from fur or skin seems unlikely. When comparing microbial features of CR, J, and H donors with the corresponding clinical symptoms elicited on IPSI, *S aureus* may appear to be critical for the manifestation of sepsis because animals that received the *S aureus*-free stool from the J donors developed no septic symptoms (Fig. 2B). *S. aureus* is known to be particularly immunogenic due to expression of highly active TLR2 ligands and to produce a number of cytotoxins, which can drive inflammation and contribute to the emergence of lethal toxic shock syndrome with clinical symptoms similar to sepsis.[15] In the CR and H stool–receiving group, the expression of TLR2 was furthermore markedly upregulated compared to the J stool–challenged group, suggesting that intraperitoneal challenge with *S aureus*-containing stool could contribute to the induction of TLR2 expression (Fig. 3).[16] However, it has to be further evaluated whether specific staphylococcal virulence factors such as enterotoxins could significantly affect the manifestation of experimental sepsis.

A further interesting finding was the detection of *B licheniformis* in all mice purchased from J during the first observation. This gram-positive, sporulating bacterium, commonly found in soil, is usually not part of the intestinal microbiota of healthy mammals, especially when being housed under sterile conditions. Common diet for laboratory rodents mostly constitutes of cereals and other legumes such as soybean, and associated with plant matter, spores from *Bacillus* spp. may enter the gastrointestinal tract by ingestion. Contamination of laboratory animal feed has recently been reported.[17] Furthermore, probiotic-acting, *B* spp.–based feed additives are intentionally used to improve animal growth.[18] However, due to modifying effects on the immune system, they shall be avoided in laboratory animal nutrition. The origin of *B licheniformis* in the feces of our J mice remains elusive, as, on request, neither of the animal distributors provided details on the feed or any additives given to the mice. Of note,

diet-induced dysbiosis of the intestinal microbiota has been demonstrated, resulting in an overgrowth of specific bacteria.[19] Since *S aureus* belong to the same phylum as *B licheniformis* (*Firmicutes*), our results suggest that the latter might have ousted other gram-positive bacteria from the intestinal microbiota of J animals.

Our findings are confirmed by other authors. Recently, Ericsson *et al.* used next-generation sequencing to demonstrate strain- and provider-dependent variations in gut microbiota of laboratory mice, showing differences in the diversity of the intestinal microbiota as well as in the abundance of operational taxonomic units and higher taxa.[20] Our results reflect the ones of that study. The assay we used is, of course, rather rough, but more easily accessible and cheaper and therefore easy to implement for routine characterization of feces in animal experiments.

When we purchased C57BL/6 mice from the same providers approximately 16 mo after the initial analyses, the gut microbiota had shifted substantially (Fig. 4 A). Variables such as housing conditions, mouse strain, nutrition, or gender are crucial for the development of a characteristic microbial configuration of laboratory mice and are thus potential confounding factors in animal studies.[5,21] Mice being housed together show a homogenization of the intestinal microbiota over time, due to, for example, coprophagy, with significant consequences for the immunologic competence, which is referred to as "cage effect".[22,23] Of note, such phenomena may be of particular significance for the dissemination of *B* spp., as mice that have been given spores orally excrete more spores in their feces than were administered due to germination and resporulation in the gastrointestinal tract, facilitating horizontal transfer.[24] In summary, cage effects contribute to the observed change of gut microbiota over time as well as to the variations between animals sharing the same genetic background but being purchased from different providers.

A marked modulating effect of the indigenous intestinal microbiota on the host's immune system is generally accepted.[4] Mice delivered and housed under sterile conditions (referred to as "germ-free") demonstrate fundamental differences in their susceptibility to experimental infections when compared to animals maintained under ordinary housing conditions.[25,26] Various potentially contributing candidate bacteria have been identified (segmented filamentous bacteria, *Bilophila wadsworthia*, *Lactobacillus,* and *Bifidobacterium spp*., and *Sphingomonas spp*.).[23,27-30] We demonstrate this phenomenon also in mice of different vendor origin, when they were challenged with stool from donors from one same distributor. This elicited significant variations in the clinical symptoms (Fig. 5 B). If *Proteus* spp., which we identified to be of diverging abundance in CR and J animals' feces (Fig. 4 A) and which are described to exert profound stimulating activity on the host's immune system, may contribute to this phenomenon, should be further elucidated.[31] Interestingly, it is reported that elimination of vendor-specific differences in intestinal microbiota by cohousing of animals likewise eliminates the influence on the clinical phenotype.[27,29,30]

Obviously, in addition to shaping the host's immune system, the intestinal microbiota of laboratory animals is of particular importance in experimental models of peritonitis and abdominal sepsis using fecal contamination. In human, the bacterial flora isolated from an abdominal focus was demonstrated to influence the outcome of septic patients.[3] Over decades, various animal models have been developed to study pathogenesis of abdominal sepsis as well as to validate new therapeutic strategies.[2] CLP and CASP are widely used approaches accepted to accurately reflect the typical pathophysiological changes observed in polymicrobial sepsis. However, due to invasiveness, uncertainty about reproducibility and technical difficulties, they may be unsuitable to address specific issues.[32] The IPSI model used in the herein presented observation has been described as a simple as well as reproducible model reflecting many clinical features of sepsis.[33] Being minimal invasive, it produces low background noise and provides stable results even over a long-term period. Due to easy titration of inoculum dose, the lethality can be well controlled, allowing therapeutic dose response experiments. Meanwhile, this model has been widely adopted, and variations such as the use of donors and recipients of different species, the encapsulation of feces in pellets to modulate onset, and development of symptoms or the injection of sterile feces mixed with, for example, *E coli* to mimic gram-negative instead of polymicrobial sepsis have been described.[33-35] However, despite the advantages, we recommend to respect the significant impact of either intervendor or time-dependent variations of the donors' fecal microbial configuration. With our results, we demonstrate a profound influence on the clinical phenotype together with corresponding changes in the expression patterns of sepsis-related genes (Figs. 2B, 3, and 4B). Proinflammatory cytokines such as IL-1β, IL-6, and TNF-α have previously been shown to be associated with the clinical outcome in severe sepsis and septic shock, and we demonstrate significant upregulation of these mediators in those animals displaying a high disease severity after injection of stool from CR and H donors.[36] ANG1 and -2, together with their endothelial receptor TIE2, are particularly dysregulated in sepsis, mediating vascular barrier disruption and being likewise associated with a poor outcome.[8] In our study, animals with pronounced clinical symptoms showed a significant downregulation of the two barrier-protective factors ANG1 and TIE2, whereas in those mice, the barrier-destructive ANG2 was upregulated.

Taken together, our results strongly emphasize the importance of strictly controlled, homogenous baseline conditions when designing experimental peritonitis and sepsis models using fecal contamination (CLP, CASP, and IPSI), particularly when the results are to be compared with one another. We advise respecting the following recommendations:

- Littermate controlled experiments should be preferred.
- Commercially purchased animals should be of the same genetic background and should be obtained from the same provider. The animals should be housed under same conditions for an extended period of time on purchase (e.g., 2 wk).[27]
- Latencies between repeated experiments should be kept as short as possible.
- Adequate *n* numbers should be achieved to minimize the impact of interindividual differences of the intestinal microbial flora.

- The microbial composition of the animals' stool should be analyzed, monitored over time, and published together with the results. Cultivation on selective agar media, denaturing gradient gel electrophoresis, or quantitative PCR analysis may be appropriate.[26,37,38] For defined (e.g., immunologic) issues, the use of specifically designed (so-called gnotobiotic) organisms with exactly known microbiota should be critically evaluated.
- Techniques to preserve and store stool should be further evaluated. Recently, it was demonstrated that, for example, freezing and thawing may dramatically affect the abundance of selected bacteria in the feces.[39]

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Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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