nature microbiology

ARTICLES

PUBLISHED: 18 JULY 2016 | ARTICLE NUMBER: 16113 | DOI: 10.1038/NMICROBIOL.2016.113

Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome

Robert P. Dickson[1]*, Benjamin H. Singer[1], Michael W. Newstead[1], Nicole R. Falkowski[1], John R. Erb-Downward[1], Theodore J. Standiford[1†] and Gary B. Huffnagle[1,2†]

Sepsis and the acute respiratory distress syndrome (ARDS) are major causes of mortality without targeted therapies. Although many experimental and clinical observations have implicated gut microbiota in the pathogenesis of these diseases, culture-based studies have failed to demonstrate translocation of bacteria to the lungs in critically ill patients. Here, we report culture-independent evidence that the lung microbiome is enriched with gut bacteria both in a murine model of sepsis and in humans with established ARDS. Following experimental sepsis, lung communities were dominated by viable gut-associated bacteria. Ecological analysis identified the lower gastrointestinal tract, rather than the upper respiratory tract, as the likely source community of post-sepsis lung bacteria. In bronchoalveolar lavage fluid from humans with ARDS, gut-specific bacteria (*Bacteroides* spp.) were common and abundant, undetected by culture and correlated with the intensity of systemic inflammation. Alveolar TNF-α, a key mediator of alveolar inflammation in ARDS, was significantly correlated with altered lung microbiota. Our results demonstrate that the lung microbiome is enriched with gut-associated bacteria in sepsis and ARDS, potentially representing a shared mechanism of pathogenesis in these common and lethal diseases.

Sepsis and the acute respiratory distress syndrome (ARDS) are common, costly diseases with high mortality and no targeted therapies[1,2]. Sepsis is the most common cause of ARDS[2,3], and sepsis-related ARDS is more severe and lethal than non-sepsis-related ARDS[4]. Numerous experimental and clinical observations over decades of study have demonstrated that the gut microbiome plays a key role in the pathogenesis of sepsis and ARDS[5]: antibiotic-suppressed and germ-free animals are protected from the lung injury and mortality of experimental sepsis[6–8] and numerous clinical trials have demonstrated that prophylactic suppression of gut microbiota with antibiotics is protective against multiorgan failure and mortality in patients with critical illness[5,9]. Yet the mechanism of the gut microbiome's role in sepsis and ARDS is undetermined[5,10,11] and previous studies have been limited by a dependence on culture-based techniques of microbial identification[12].

Here, we report that the lung microbiome is enriched with gut bacteria in experimental sepsis and human ARDS. Enrichment of the lung microbiome by gut-associated bacteria occurs independent of the upper respiratory tract, correlates with severity of systemic inflammation and results in the persistence of viable bacteria within the lung microbiome. Alveolar tumour-necrosis factor-α (TNF-α), a key mediator of alveolar inflammation in ARDS, is significantly correlated with alterations in lung microbiota. Our results suggest a potential common mechanism behind the gut microbiome's established role in these common diseases of critical illness.

Results

Lung microbiota are altered after experimental sepsis. To determine whether sepsis results in altered lung microbiota we used a well-established model of abdominal sepsis—caecal ligation and puncture (CLP). As previously described, this survivable model provokes transient systemic inflammation that mirrors the human septic response[13]. We studied the microbiota of the gastrointestinal and respiratory tracts via sequencing of bacterial 16S ribosomal RNA-encoding genes. Compared to the lungs of untreated mice, lungs of post-sepsis mice had greater community richness (number of detectable species) (Fig. 1a), consistent with immigration of new species. Bacterial communities in post-sepsis lungs were significantly distinct from those of untreated mice and mice that underwent sham surgery ($P = 0.02$ for both). Lung communities of post-sepsis mice were significantly enriched with numerous bacteria found in the murine gut, including members of the Bacteroidales order, *Enterococcus* species (sp.) and *Lachnospiraceae* sp. (Fig. 1b). The two most abundant taxonomic groups, comprising nearly 40% of community members, were members of the Bacteroidales order, an abundant member of the murine gut microbiome. Abundant taxa in lungs of untreated mice are shown in Supplementary Fig. 1. We then directly compared the bacterial community membership of colon and lung communities within each mouse to determine whether post-sepsis lung communities more closely resembled those of the gut. Gut–lung similarity was significantly associated with experimental intervention ($P \leq 0.05$); gut and lung communities were most similar in post-sepsis lungs and most dissimilar in untreated mice (Fig. 1c).

Given this evidence of gut-associated bacteria in the lungs 24 h after experimental sepsis, we performed an experiment with additional experimental controls and extended time points to determine the duration of altered lung microbiota after sepsis. We compared mice exposed to experimental sepsis (CLP and concurrent

[1]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109, USA. [2]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109, USA. †These authors contributed equally to this work. *e-mail: rodickso@med.umich.edu

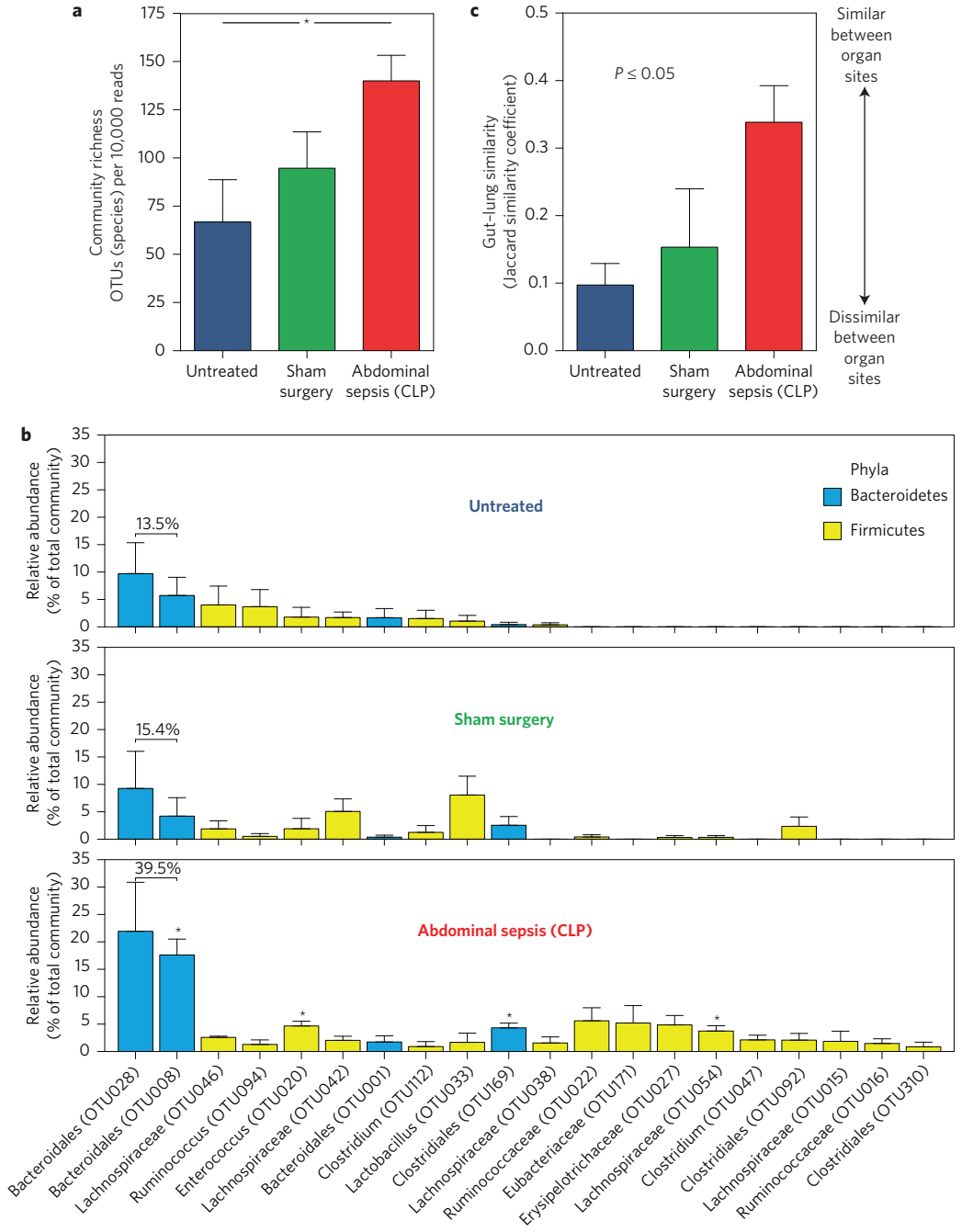

Figure 1 | Altered lung microbiota 24 h after experimental sepsis. Abdominal sepsis was induced in mice via caecal ligation and puncture, and lung bacterial communities were sequenced and analysed after 24 h. **a**, The lungs of post-sepsis mice contained increased species richness compared to untreated mice, consistent with immigration of new species. **b**, Relative abundance of bacteria in the lung microbiome following sepsis. The 20 most abundant OTUs detected in post-sepsis lungs are shown across experimental arms. Asterisks indicate OTUs significantly enriched in post-sepsis lungs compared to control groups. **c**, Gut–lung community similarity after sepsis. For each mouse, the community similarity was calculated for paired lung and colon communities. Four mice were used in each intervention group. Group means and standard errors of the mean are depicted. Statistical significance was determined with two-way analysis of variation (ANOVA) with Tukey's multiple comparisons test. $^*P \leq 0.05$. Values are presented as means ± standard error of the mean. Significance in **c** reflects the overall effect of intervention on community richness. Between-group differences were not significant after controlling for multiple comparisons.

imipenem) with mice exposed to sham surgery plus imipenem, mice exposed to imipenem only, and mice that received no intervention. We studied their lung communities at five days, two weeks and eight weeks following intervention. Lung communities significantly differed collectively according to intervention at five days ($P = 0.003$). This collective difference in lung microbiota across intervention arms had resolved at two weeks and eight weeks ($P > 0.05$, Supplementary Fig. 2). Five days post-sepsis, bacterial communities from lungs of post-sepsis mice were distinct from those of all control groups: sham surgery plus imipenem ($P = 0.001$), imipenem only ($P = 0.004$) and untreated ($P = 0.02$) (Fig. 2a). By contrast, two weeks and eight weeks following exposure, lung communities from post-sepsis mice were indistinguishable from those of untreated mice ($P > 0.05$, Fig. 2b). The lung microbiome is thus altered following experimental sepsis, remains altered for at least five days, and normalizes by two weeks.

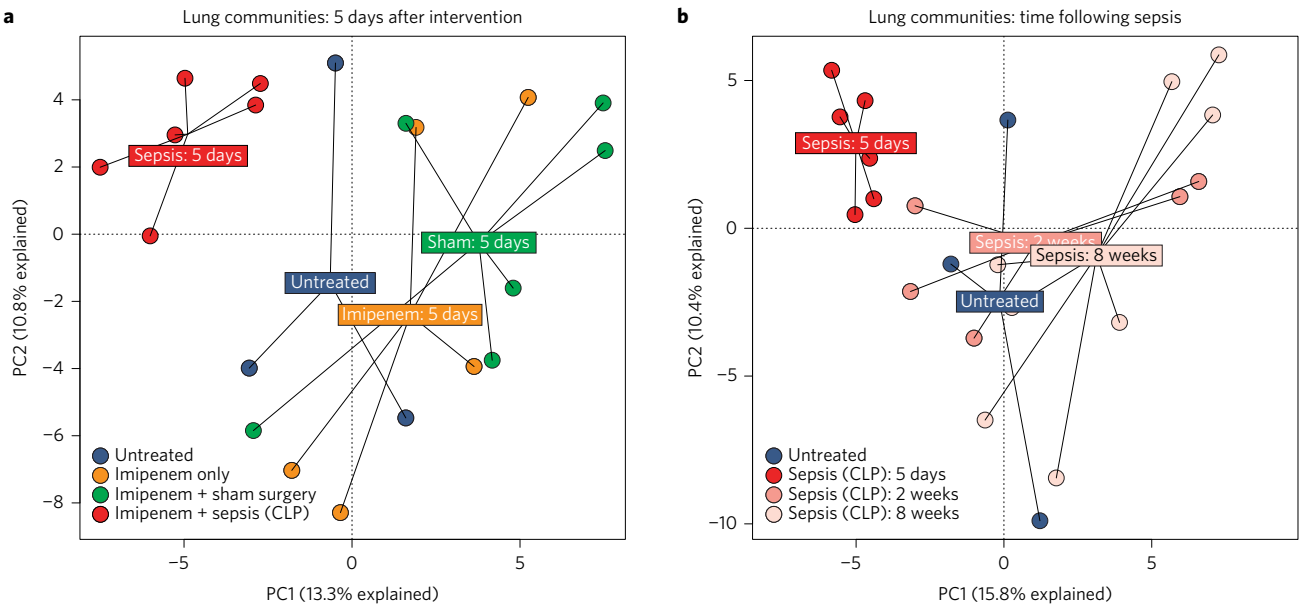

Figure 2 | Transient alteration of the lung microbiome after experimental sepsis. Mice were exposed to abdominal sepsis (caecal ligation and puncture and imipenem) and compared at multiple time points to three experimental control groups: untreated, imipenem only and imipenem with sham surgery. **a**, Five days after exposure, bacterial communities in the lungs of post-sepsis mice were distinct from those of all control groups. **b**, Lung communities of post-sepsis mice were distinct from untreated mice after five days but indistinguishable at two and eight weeks following injury.

The post-sepsis lung microbiome is enriched with gut-associated bacteria. We then performed ecological analysis to identify the source of altered lung microbiota after sepsis. To determine which bacteria were responsible for the altered communities in post-sepsis lungs, we used biplot analysis of principal component analysis (*vegan*[14]) as well as model-based analysis of multivariate abundance data (*mvabund*[15]) to identify key microbial drivers of altered communities. Both techniques identified a member of the Bacteroidales order (OTU008) as enriched within the post-sepsis lung community (Supplementary Fig. 3a). Alignment searches of sequence databases revealed that although this taxonomic group (sequences with 100% coverage and 100% alignment) has been detected frequently in the murine gut[16–18], no cultured specimen has been reported (Supplementary Fig. 4). This Bacteroidales operational taxonomic unit (OTU) comprised one-third (33.1%) of total community membership in post-sepsis lungs at five days and was not enriched in the lungs of any control group (Fig. 3a). Its abundance normalized at two weeks. We analysed oral bacterial communities to determine if enrichment of the lungs with this Bacteroidales OTU was secondary to altered upper respiratory tract microbiota. This Bacteroidales OTU was not abundant in bacterial communities of the tongues of mice following sepsis at any time point (Fig. 3a), indicating that the upper respiratory tract was not the source of altered lung microbiota in the post-sepsis period.

We then compared other potential source communities to determine the origin of this Bacteroidales OTU. This sequence was detected in only one of 14 procedural and reagent control specimens and comprised only 0.11% of bacterial sequences in these controls (Fig. 3b). By contrast, this Bacteroidales OTU was the most abundant taxonomic group detected in specimens from the gut (caecum, colon and faeces) of untreated mice (Fig. 3b), comprising 18% of all sequences. The time course of gut–lung similarity in paired specimens from mice following experimental sepsis is shown in Supplementary Fig. 5. This Bacteroidales OTU was detected inconsistently in blood specimens taken from post-sepsis mice (three of six mice at five days post-sepsis). By contrast, it was uniformly present in the lungs of post-sepsis mice (six of six mice). Supplementary Fig. 6 shows the relative abundance of this Bacteroidales OTU in gut specimens from post-sepsis mice following injury. Taken together, these results indicate that the lower gastrointestinal tract is the most likely source community of bacteria in the post-sepsis lung microbiome. The route of translocation (via portal circulation, systemic circulation or mesenteric lymphatics), if present, remains undetermined.

Unlike bacterial culture, sequencing-based approaches depend only on the presence of bacterial DNA and do not reflect bacterial viability. To determine if the post-sepsis lung microbiome contains viable gut-associated bacteria, we cultured homogenized lung specimens of post-sepsis mice. *Enterococcus faecalis* was cultured from the lungs of all post-sepsis mice five days after exposure and was not cultured from the lungs of mice in any control group (Fig. 3c). No bacterial growth, *E. faecalis* or otherwise, was observed from concurrent blood cultures. *E. faecalis* corresponded genetically to the second most abundant OTU in the lungs of post-sepsis mice (Fig. 3c) and was identified by *mvabund* as relatively enriched within the lungs of post-sepsis mice. As it has previously been reported that lung injury alone, in the absence of sepsis, is sufficient to alter the lung microbiome of mice[19], we used a model of direct lung injury (intratracheal lipopolysaccharide (LPS) instillation) to test whether direct lung injury could provoke the outgrowth of gut-associated bacteria. No bacterial growth, *E. faecalis* or otherwise, was observed from the lungs of either the LPS-instilled mice or those of controls (Fig. 3c). These data demonstrate that the gut-associated bacteria present in the post-sepsis lung microbiome are viable and are not attributable to local inflammation alone.

To determine if enrichment of lung microbiota with gut-associated bacteria was specific to the CLP model, we employed a systemic LPS model of sepsis for validation. One day after LPS-induced shock, lung communities were significantly distinct from those of untreated mice ($P = 0.004$, Supplementary Fig. 7a), a difference that resolved by four days. This was driven by an *Enterobacteriaceae*-classified OTU that was enriched in post-sepsis lungs compared to those of untreated mice ($P < 0.01$, Supplementary Fig. 7b,c). A database alignment search determined that this OTU shares 100% genomic identity with gut-associated members

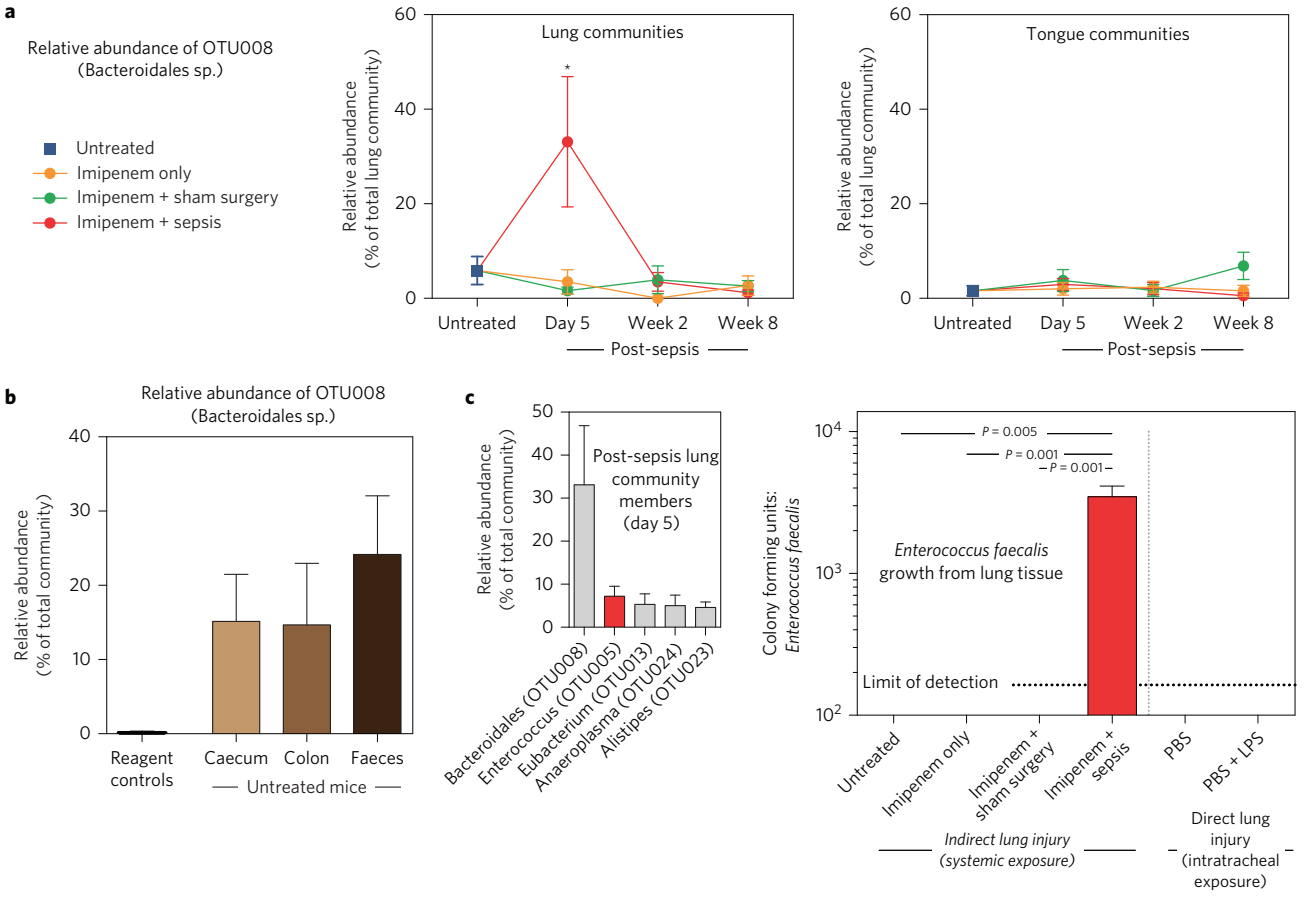

Figure 3 | Evidence suggesting gut–lung translocation after experimental sepsis. a, The bacterial communities of post-sepsis lungs were dominated by an uncultured bacterium, OTU008 (Bacteroidales sp.), that normalized after two weeks. This bacterium did not dominate the bacterial communities detected in simultaneously collected tongue specimens. **b**, This Bacteroidales sp. was of minimal abundance in reagent control specimens, but was the most abundant community member in all lower gastrointestinal sites of mice before injury. **c**, This Bacteroidales sp. was not detected by culture, but *Enterococcus faecalis* (the second most abundant species detected in the lungs of mice five days after sepsis) was isolated via aerobic culture from the lungs of all post-sepsis mice five days after injury. It was not isolated from the lungs of any control group, including mice exposed to direct lung injury via intratracheal LPS instillation. Statistical significance was determined by Kruskal–Wallis one-way analysis of variance with Dunn's multiple comparisons test (**a**,**c**). Values are presented as means ± standard error of the mean. Six mice were used in each intervention arm and time point. PBS, phosphate-buffered saline; LPS, lipopolysaccharide.

of the *Enterobacteriaceae* family such as *Enterobacter cloacae* and *Klebsiella pneumoniae* (Supplementary Fig. 7d). We concluded that enrichment of the lung microbiome with gut-associated bacteria is not specific to the CLP model of sepsis.

The lung microbiome of humans with ARDS is enriched with uncultured gut bacteria. Given this experimental evidence suggesting translocation of gut bacteria to the lungs in sepsis, we asked whether gut-associated bacteria are present in the lung microbiota of humans with ARDS. We sequenced bacterial communities in 100 specimens of bronchoalveolar lavage (BAL) fluid collected from 68 patients with ARDS and compared these communities to those of seven healthy volunteers. The demographics and clinical features of ARDS patients are presented in Supplementary Table 1. We employed analysis of multivariate abundance (*mvabund*) to identify taxa enriched in BAL specimens from patients with ARDS relative to healthy subjects (Supplementary Fig. 3b); of these, we focused on a prominent member of the *Bacteroides* genus (OTU009) that was classified to the same order (Bacteroidales) as the OTUs enriched in the post-sepsis lungs in our murine experiments. Alignment searches of databases revealed perfect alignment (100% coverage, 100% alignment) between this *Bacteroides* sp. and four anaerobic species abundant in the human gut[20–22]: *B. fragilis*, *B. thetaiotaomicron*, *B. faecichinchillae* and *B. salyersiae* (Supplementary Fig. 4). Of the 24 BAL specimens in this cohort studied by aerobic culture at the University of Michigan Clinical Microbiology Laboratory, none grew *Bacteroides* sp., despite culture-independent identification of this organism in eight (33%), confirming the limited sensitivity of aerobic culture-based techniques in identifying gut-associated anaerobes in respiratory specimens.

This gut-associated *Bacteroides* OTU was common and abundant in the BAL fluid of patients with ARDS and was not detected in reagent control specimens nor in the BAL fluid of healthy subjects (Fig. 4a). It was detected in 33% (33) of ARDS BAL specimens, representing 41% (28) of all unique ARDS patients. This *Bacteroides* OTU comprised 5.7% of all sequences detected in ARDS BAL specimens. Supplementary Fig. 8 shows the relative stability of this OTU in BAL communities of patients for whom serial BAL specimens were available. No association was detected between the relative abundance of this Bacteroidales OTU and time since onset of ARDS, predisposing factor for ARDS, or severity of illness at diagnosis ($P > 0.05$ for all). In a validation analysis using a previously published data set of bacterial communities sequenced from BAL from healthy subjects[23], we identified a representative sequence corresponding to this *Bacteroides* OTU (100% alignment).

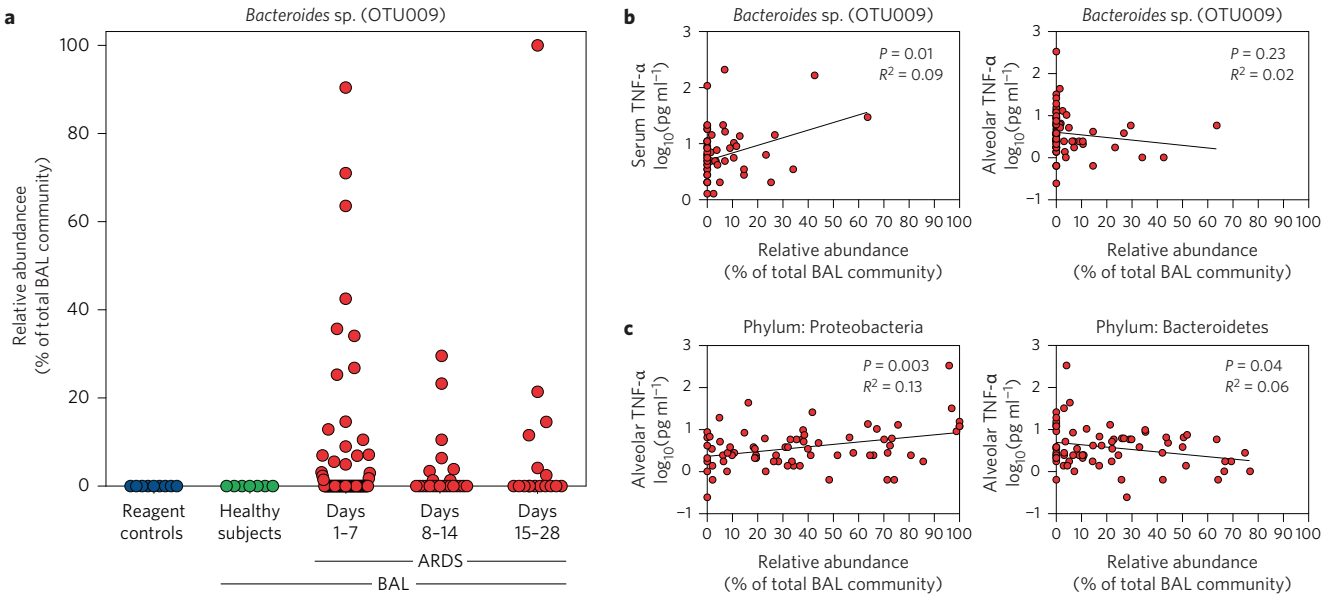

Figure 4 | Evidence suggesting gut–lung translocation of bacteria in humans with ARDS. a, *Bacteroides* sp. (OTU009), a representative of the most abundant bacterial genus in the human gut microbiome, was absent from reagent control specimens and the lungs of healthy subjects, but common (33% of specimens) in the lungs of patients with ARDS. **b**, The relative abundance of this *Bacteroides* sp. was positively correlated with serum TNF-α but not associated with alveolar TNF-α. **c**, By contrast, alveolar TNF-α was positively correlated with the relative abundance of the Proteobacteria phylum and negatively correlated with the Bacteroidetes phylum. A total of 100 bronchoalveolar lavage specimens from 68 unique patients were studied. Statistical significance was determined by linear regression using logarithmically transformed cytokine data (**b**,**c**).

This *Bacteroides* OTU was detected in the BAL of only one of twenty-six healthy subjects. Thus, collectively, this gut-associated *Bacteroides* sp. was detected in the lungs of only one of thirty-three (3.0%) healthy subjects, confirming it is not a normal community member in the healthy lung microbiome.

We then asked if enrichment of lung microbiota with gut bacteria was associated with systemic and alveolar inflammation, both as measured on the day of bronchoscopy. We compared alveolar microbiota with concentrations of TNF-α in serum (which is correlated with the diagnosis and severity of sepsis[24,25]) and BAL fluid (which is correlated with the diagnosis, severity and mortality of ARDS[26,27]). Among patients with ARDS, we observed a significant association between relative abundance of this gut-associated *Bacteroides* OTU and patients' concurrent serum TNF-α concentration ($P = 0.01$, Fig. 4b). This finding indicates that enrichment of gut bacteria in the lung microbiome is correlated with severity of acute systemic inflammation. By contrast, no association was found between relative abundance of this *Bacteroides* OTU and alveolar TNF-α concentration ($P > 0.05$, Fig. 4b), suggesting that gut–lung bacterial translocation, if present, correlates with systemic, but not alveolar, inflammation.

Relative enrichment of lung microbiota with the Proteobacteria phylum has been repeatedly associated with inflammatory lung disease[28–30], as compared to enrichment with Bacteroidetes (for example, *Prevotella* genus), the most abundant phylum in the healthy respiratory tract[27,28]. We hypothesized that the relative abundance of Proteobacteria in the lungs of patients with ARDS would be correlated with alveolar inflammation. A significant positive correlation was observed between relative abundance of Proteobacteria and alveolar TNF-α ($P = 0.003$, Fig. 4c). This relationship remained significant when we excluded specimens from subjects with a predisposing condition of pneumonia ($P = 0.02$). By contrast, relative abundance of the usually prominent Bacteroidetes phylum was negatively correlated with alveolar TNF-α ($P = 0.04$, Fig. 4c). These data confirm that key features of alveolar inflammation in ARDS are associated with alterations in lung microbiota.

Discussion

The core finding of our study is that gut-associated bacteria, undetectable via conventional culture techniques, enrich the lung microbiome in experimental sepsis and human ARDS. Microbial evidence suggesting gut–lung translocation and disorder of the lung microbiome are respectively correlated with indices of systemic and alveolar inflammation. Our results suggest a potential common mechanism behind the gut microbiome's established role in these common and lethal diseases.

The importance of the gut microbiome in the pathogenesis of critical illness has been established for more than 60 years[6], though to date the mechanism of its role has been undetermined[5,10,11]. The suppression of gut bacteria, either via enteric antibiotics or the use of germ-free animals, is protective against the lung injury and mortality of critical illness, a robust observation described widely across species (mice[8,31], rats[32], rabbits[7] and dogs[6]) and models of shock (haemorrhage[6], sepsis[7] and ischaemia/reperfusion[8]). Dozens of clinical trials have demonstrated that suppression of the gut microbiome (selective decontamination of the digestive tract) is protective against multiorgan failure and mortality in patients with critical illness[5,9]. The permeability of the intestinal wall is increased in critical illness and is predictive of clinical outcome[33], and culture-identified translocation of gut bacteria to mesenteric lymphatics is observed in experimental shock[34] and is predictive of subsequent sepsis in postoperative patients[35]. In a recent culture-independent study of patients undergoing haematopoietic stem cell transplant, features of the gut microbiome were predictive of pulmonary infiltrates (as observed in ARDS) and mortality[36]. However, prior studies have failed to find evidence of bacterial translocation via sampling of the systemic, portal and lymphatic circulations[12,37]. Two potential explanations may explain the discrepancy between our findings and those of previous studies. First, our culture-independent methodology is far more sensitive than previous culture-based approaches, as many bacteria in the human gut are undetectable via conventional culture[38]. The dominant lung bacteria in our experimental sepsis model is an as-of-yet uncultured Bacteroidales sp. and the most prominent gut-associated bacteria

in our human ARDS specimens were of the *Bacteroides* genus; neither grow in the standard conditions of clinical microbiology laboratories. Second, our results demonstrate the persistence of viable gut bacteria in the lungs days after sepsis, even in the absence of persistent bacteraemia. This indicates that even a brief, transient immigration of gut bacteria may alter the lung microbiome. Gut permeability and the toxicity of mesenteric lymph peak in the immediate hours following experimental shock[39,40], so subsequent assessment of blood or mesenteric lymph may fail to detect the presence of bacteria.

Our study is the first culture-independent analysis of the human lung microbiome in ARDS. Previous culture-dependent studies have suggested a relationship between bacteria of the respiratory tract and the pathophysiology of ARDS[5]. Pneumonia occurs far more frequently in patients with ARDS than in other mechanically ventilated patients[41], and pulmonary exposure to bacterial factors (for example, LPS and flagellin) provokes inflammation and injury characteristic of ARDS. Culture-independent studies of mechanically ventilated patients have demonstrated a previously unappreciated complexity to the respiratory microbiome[5,42] and animal experimentation has recently shown that direct lung injury alters the lung microbiome[19]. In our study, disorder of the lung microbiome, characterized by enrichment with the Proteobacteria phylum (commonly enriched in the lung microbiota of patients with inflammatory lung conditions[28–30]), was significantly associated with elevated alveolar concentrations of TNF-α, a key mediator of pulmonary inflammation in ARDS that is independently predictive of mortality[27]. By contrast, decreased alveolar concentrations of TNF-α were associated with enrichment of the lung microbiome with Bacteroidetes, the most abundant phylum in the lung microbiome of healthy subjects[23,28]. Our results do not imply that bacterial translocation and dysbiosis are the sole or primary cause of ARDS, as numerous non-bacterial exposures (for example, oleic acid, hydrochloric acid and gut ischaemia factors[40]) can initiate alveolar injury, and the complex clinical syndromes of sepsis and ARDS each encompass multiple pathophysiological pathways. Sepsis and ARDS both have dynamic and temporally heterogeneous clinical and biological courses. Our study of BAL specimens from patients with established ARDS may not reflect the microbiology of early and developing ARDS. Although we could control for antibiotic exposure in our murine model of sepsis, the effects of ubiquitous antibiotic exposure on lung microbiota in humans with ARDS is unknown. Additionally, although TNF-α is a key mediator in these conditions (systemically in sepsis[28,29], in the alveolar space in ARDS[30,31]), it alone cannot represent the complexity of the dysregulated host response of critical illness. Further study will be needed to determine the role of lung microbiota in the initiation, augmentation and perpetuation of ARDS.

In both our animal model of sepsis and our human study of ARDS, we identified prominent anaerobic gut-associated bacteria not previously implicated in sepsis or ARDS. The Bacteroidales OTU that dominates post-sepsis lungs has previously been identified molecularly, but not cultured, in the murine gut[16–18]. Separately, a distinct OTU of the *Bacteroides* genus was common and abundant in the lungs of humans with ARDS but absent from those of healthy subjects. This genus is the most abundant taxonomic group in the human gut microbiome[43] and this OTU was absent from lung specimens both in our study's healthy subjects and from previously published reports of the normal lung microbiome[23,44,45]. Within the field of environmental microbiology, molecular detection of the *Bacteroides* genus is used as an indicator of human faecal contamination in watersheds[46]. Our results suggest it may serve a similar role within the respiratory ecosystem. The relative abundance of this *Bacteroides* OTU in the lung microbiome, plausibly reflecting the degree of gut–lung translocation, was significantly associated with serum concentrations of TNF-α, a key mediator of the septic stress response that is predictive of patient mortality[24,25]. In an innovative recent study of patients with established ARDS, latent class analysis identified a subphenotype of ARDS patients characterized by high systemic inflammation, associated with worse prognosis and a distinct response to therapy[47]. Our finding that systemic TNF-α correlates with enrichment of gut bacteria in BAL fluid suggests that this subphenotype may be characterized by differences in lung microbiota. Importantly, the *Bacteroides* genus is highly variable in its abundance, is dominant in only a subset of healthy adults[43] and is diminished in patients with established critical illness[48]. It is thus not a universal marker of human gut microbiota. Further longitudinal study of paired stool and lung specimens will be needed to determine the true prevalence of gut–lung translocation in sepsis and ARDS.

The lung microbiome is determined by the balance of three ecological factors: immigration, elimination and the relative growth rates of the resident microbiota[49]. Our experimental results demonstrate that the upper respiratory tract, the lung microbiome's primary source community in health, is not the source of altered lung microbiota after sepsis. Although our findings suggest a mechanism of immigration via translocation of gut microbiota, a competing explanation is the relative outgrowth of bacteria present in low abundance before injury. Indeed, the Bacteroidales OTU enriched after CLP-induced sepsis was detectable in low levels in lung communities of untreated mice. Yet *E. faecalis*, detected via culture in all post-sepsis lung specimens, was not cultured from the lungs of any control animal. Thus, if this gut-associated bacteria is present in lung communities before injury, its abundance is less than our assay's very low limit of detection (100 c.f.u. per lung). Analogously, if the *Bacteroides* OTU that was abundant in our human ARDS specimens is present in the healthy lung microbiome, it is present but below the highly sensitive limit of detection of 16S sequencing. Furthermore, our model of direct lung injury (intratracheal LPS) resulted in no detectable outgrowth of gut-associated bacteria, a finding consistent with previously published studies[19]. Definitive proof of gut–lung translocation will require additional study, including techniques such as paired metagenomic comparisons of gut and lung microbiota and the use of labelled 'tracer' bacteria in gnotobiotic animals. We have recently posited a conceptual model of how alveolar inflammation and injury can perpetuate—and be perpetuated by—disordered bacterial communities in the respiratory tract[5,50]. The significant positive correlation we report between alveolar TNF-α and enrichment with the Proteobacteria phylum is evidence that even in the absence of gut–lung translocation, the microbiome may play an important role in ARDS.

In summary, these data demonstrate that the lung microbiome is enriched with gut bacteria in experimental sepsis and human ARDS. Enrichment with gut bacteria is significantly associated with key features of systemic inflammation. Alveolar TNF-α, a key mediator of the alveolar inflammation of ARDS, is significantly correlated with disorder of the lung microbiome. Our findings suggest that gut–lung translocation and alteration of the lung microbiome may represent a common mechanism of pathogenesis in sepsis and ARDS, and suggest potential therapeutic targets for the prevention and treatment of these common and lethal diseases.

Methods

Ethics statement. The animal studies contained in this manuscript were approved by the University Committee on the Care and Use of Animals (UCUCA) at the University of Michigan. Laboratory animal care policies at the University of Michigan follow the Public Health Service policy on Humane Care and Use of Laboratory Animals. Animals were assessed twice daily for physical condition and behaviour. Animals assessed as moribund were humanely euthanized by CO_2 asphyxiation.

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The human study protocol was approved by the institutional review board of the University of Michigan Healthcare System. All patients or their legal surrogates provided written informed consent. The

institutional review boards have examined the protocols and certified that 'The risks are reasonable in relation to benefits to subjects and the knowledge to be gained. The risks of the study have been minimized to the extent possible.'

Mice. Eight- to 12-week-old male C57Bl/6 mice were purchased from Jackson Laboratories and housed under specific pathogen-free conditions. For safety, post-surgical (sham and CLP) mice were housed separately from untreated and antibiotic-only mice. Apart from this isolation of post-surgical mice, mice were not separately housed according to intervention arm or time point. Given the copraphagic behaviour of co-housed mice, cage number was recorded for each mouse and incorporated as a potential confounder into multivariable microbiome analysis as reported in the 'Results' section. The intervention group was determined for each mouse by random selection by investigators. In the initial (24 h CLP) experiment, four mice were used per intervention arm. In the subsequent experiment (extended time point CLP), six mice were used per intervention arm per time point. These numbers were used based on prior experiments on antibiotic-treated mice that demonstrated appreciable microbial community effects when comparing comparably sized groups. Except where reported otherwise, all reported replicates were biological (distinct mice separately exposed to the same intervention) rather than technical (repeated measurement or analysis of the same sample).

Caecal ligation and puncture (CLP). CLP was used as a model of systemic sepsis syndrome, as described previously[13,51]. Mice were anaesthetized with a mixture of intraperitoneal xylazine and ketamine. A 1 cm longitudinal incision was made to the lower-right quadrant of the abdomen and the caecum exposed. The distal one-third of the cecum was ligated with a 3–0 silk suture and punctured through with a 19-gauge needle. The caecum was then placed back into the peritoneal cavity and the incision was closed with surgical staples. In sham surgical controls, the caecum was exposed but not ligated or punctured, then returned to the abdominal cavity. All mice were administered with 1 ml of sterile saline subcutaneously for fluid resuscitation and 0.5 mg of subcutaneous imipenem during the immediate postoperative period. As described in the Results, various control arms received no intervention, imipenem only, imipenem plus sham surgery and imipenem plus CLP. Each experiment, as detailed in the Results, was performed once. No unreported experiments have been omitted from the Results.

Lipopolysaccharide models. Lipopolysaccharide (LPS) (derived from *Escherichia coli* 0111:B4, Sigma-Aldrich) was given systemically as a model of systemic shock and intratracheally as a model of direct lung injury. Systemic shock was induced using a single intraperitoneal injection of 5 mg kg^{-1}. Control mice received an intraperitoneal injection of phosphate buffer solution (PBS). Mice were harvested at 1 and 4 days following exposure. Direct lung injury was induced via intratracheal instillation of 10 μg LPS; control mice received an intratracheal instillation of PBS. Mice were harvested 3 days following exposure.

Mouse tissue collection and processing. Mice were euthanized via CO_2 asphyxiation. Low biomass specimens (tongue, lung) were removed prior to high biomass specimens (caecum, colon, faeces). Blood was collected from mice during harvest using needle aspiration via the right ventricle. Instruments were rinsed with ethanol and flamed between each organ. Murine lungs were excised, placed in tubes containing 1 ml of sterile water and homogenized mechanically using a Tissue-Tearor (Biospec Products). The tissue homogenizer was cleaned and rinsed in ethanol and water between tissues. Water control specimens from homogenization, exposed to cleaned instruments, were included in sequencing as procedural controls.

Lung and blood c.f.u. assay. Aliquots of lung homogenates and blood were plated onto LB media in tenfold dilutions and incubated aerobically at 37 °C. Colonies were counted after 24 h of growth. In addition to the *Enterococcus* growth depicted in Fig. 3, the lungs of two sham surgery animals and one post-sepsis animal grew *Lactobacillus* spp., and the lungs of one CLP mouse grew *Staphylococcus* sp. Overall c.f.u. counts were significantly greater in post-sepsis lungs ($P \leq 0.01$), and the specific growth of *Lactobacillus* spp. and *Staphylococcus* sp. was not significantly different across intervention arms ($P > 0.05$). No growth was observed three days after intratracheal instillation of PBS or PBS and LPS. The c.f.u.s were plated and measured in duplicate for each mouse, and the values reported are means for each animal.

Subject enrolment. All human ARDS subjects were enrolled in a multicentre randomized trial of recombinant human granulocyte-macrophage colony stimulating factor for patients with ARDS (ClinicalTrials.gov no. NCT00201409). Details of study sites and enrolment have been described previously[52]. ARDS was defined clinically according to the American–European Consensus Conference definition[53]. Patients were excluded if younger than 18 years, if seven or more days had elapsed since the onset of ARDS, if there was evidence of pre-existing chronic respiratory failure, if the patient was neutropenic, if the patient had a history of haematological malignancy or bone marrow transplantation, if the patient was already enrolled in other therapeutic trials or if the patient or his/her authorized decision maker or attending physician had decided to forego aggressive care. Patients were enrolled within 7 days of first meeting the criteria for ARDS. Reported time points are relative to the time of randomization. Both pre-intervention and post-intervention BAL specimens were used from patients in both study arms. Randomization to the granulocyte-macrophage colony stimulating factor arm had no detectable effect on the BAL microbiota ($P > 0.05$) or the abundance of the *Bacteroidetes*-classified OTU009, using either initial or post-intervention BAL specimens. Healthy control subjects were recruited from the southeast Michigan community. All control subjects were less than 55 years of age, were taking no medications and were life-long non-smokers. The validation cohort of BAL specimens from healthy subjects has been published and described in detail previously[23]. Specimens in this cohort were similarly sequenced using the 454 Pyrosequencing platform, although whole BAL specimens were used; the cell removal step results in the detection of a related but taxonomically distinct community in BAL fluid[54].

Study population. We sequenced 100 specimens of BAL fluid collected from 68 unique patients with ARDS admitted to intensive care units in three academic medical centres: the University of Michigan Medical Center, Emory University Hospitals and the University of Colorado Denver. The mean time since study enrolment (which was within 7 days of meeting the ARDS criteria) was 8.7 ± 6.2 days. A total of 25 specimens were obtained within 3 days of enrolment, 61 within seven days, 84 within two weeks and 99 within 3 weeks; one specimen was obtained 28 days after study enrolment. Patients had a mean age of 47.4 ± 14.5 years and were predominantly male (67%) and Caucasian (66%). The most common ARDS risk factors were pneumonia (29.4%), sepsis (26.4%) and aspiration (20.6%). Other aetiologies included pancreatitis, trauma and transfusion-related lung injury. Additional information including clinical microbiology results and antibiotic exposure was obtained from the electronic medical records of 18 subjects enrolled at the University of Michigan Medical Center, representing 24 total BAL fluid specimens. Of these, at the time of bronchoscopy, all specimens (100%) were obtained while the subject was receiving antibiotics and most (83.3%) were obtained while subjects were receiving multiple antibiotics. The most common antibiotics used were vancomycin (50%), piperacillin-tazobactam (41.7%), levofloxacin (41.7%) and cefepime (16.7%). Relatively few specimens were obtained from subjects receiving antifungal therapy (12.5%) or macrolide antibiotics (8.3%). The most common clinical microbiology results were no growth (37.5%), 'oral flora' (33.3%), *Pseudomonas aeruginosa* (8.3%), *Burkholderia cepacia* (8.3%) and *Staphylococcus* spp. (8.3%).

BAL collection and processing. BAL was performed using a standard technique before randomization and at designated time points following enrolment (3 days, 7 days, 14 days and 21 days). Sterile isotonic saline (160 ml) was instilled in 20 ml aliquots, with gentle suctioning after each aliquot. Bronchoscopy was not performed for research purposes if the PaO_2/FiO_2 ratio was less than 100, the patient was deemed to be too unstable by the attending clinician, or the surrogate declined participation in this portion of the study. Cells were separated via centrifugation (11*g* for 10 min) and the cell-free supernatant was frozen at −80 °C for subsequent assays. Cell-free supernatants were subsequently centrifuged (22,500*g* for 30 min), and the resulting pellet was used for DNA isolation.

Bacterial DNA isolation. Genomic DNA was extracted from mouse tissue and human acellular BAL pellets resuspended in 360 μl ATL buffer (Qiagen DNeasy Blood & Tissue kit) and homogenized in UltraClean fecal DNA bead tubes (MO-BIO) using a modified protocol previously demonstrated to isolate bacterial DNA[55]. Sterile laboratory water and AE buffer used in DNA isolation were collected and analysed as potential sources of contamination.

16S DNA sequencing. *Mouse tissue specimens.* The V4 region of the 16S rRNA gene was amplified using published primers[56] and the dual-indexing sequencing strategy developed by the laboratory of Patrick D. Schloss[57]. Sequencing was performed using the Illumina MiSeq platform, using a MiSeq Reagent Kit V2 (500 cycles), according to the manufacturer's instructions with modifications found in the Schloss standard operating procedure[58]. Accuprime High Fidelity Taq was used in place of Accuprime Pfx SuperMix. Primary PCR cycling conditions were 95 °C for 2 min, followed by 20 cycles of touchdown PCR (95 °C 20 s, 60 °C 20 s and decreasing by 0.3 °C each cycle, 72 °C 5 min), then 20 cycles of standard PCR (95 °C for 20 s, 55 °C for 15 s and 72 °C for 5 min) and finished with 72 °C for 10 min.

Human BAL specimens. The V3–V5 hypervariable regions of the bacterial 16S rRNA gene were sequenced in the V5–V3 direction using barcoded primer sets corresponding to 357F and 926R[59]. These barcoded primers were originally developed by the Broad Institute. Primary PCR cycling conditions were 95 °C for 2 min, followed by 20 cycles of touchdown PCR (95 °C for 20 s, followed by annealing for 30 s beginning at 60 °C and decreasing by 1 °C every two cycles until 50 °C and an elongation of 72 °C for 45 s), then 20 cycles of standard PCR (95 °C for 20 s, 50 °C for 30 s and 72 °C for 45 s) and finishing with 72 °C for 5 min. Quality control and sequencing was carried out at the University of Michigan using the Roche 454 GS Junior according to established protocols[60].

Cytokine measurement in ARDS patients. TNF-α was measured in the 74 human BAL fluid specimens for which adequate fluid was available (acquired from

55 unique patients). TNF-α was also measured in 58 serum specimens collected from 46 unique patients. These serum specimens were collected on the same day as the bronchoscopy used for comparison. TNF-α was measured in serum and BAL fluid using enzyme-linked immunosorbent assay (R & D Systems) according to the manufacturer's protocol. Samples were assayed in duplicate, and individual data points were analysed and presented as mean values. Cytokine values were log-transformed to obtain a normal distribution before regression analysis.

Statistical analyses. Sequence data were processed and analysed using the software mothur v.1.27.0 according to the standard operating procedure for 454 and MiSeq sequence data using a minimum sequence length of 250 basepairs[58,61,62]. For each experiment and sequencing run, a shared community file and a phylotyped (genus-level grouping) file were generated using operational taxonomic units (OTUs) binned at 97% identity generated using the dist.seqs, cluster, make.shared and classify.otu commands in mothur. OTU numbers were arbitrarily assigned in the binning process and are referred to throughout the manuscript in association with their most specified level of taxonomy. Classification of OTUs was carried out using the mothur implementation of the Ribosomal Database Project (RDP) Classifier and the RDP taxonomy training set 9 (fasta reference = trainset9_032012.pds.fasta, taxonomy reference = trainset9_032012.pds.tax), available on the mothur website.

Microbial ecology analysis was performed using the *vegan* package 2.0-4 and *mvabund* in R[14,15,63]. For relative abundance and ordination analysis, samples were normalized to the percentage of total reads and we restricted analysis to OTUs that were present at greater than 1% of the sample population; all OTUs were included in diversity analysis. Direct community similarity comparisons were performed using the Jaccard similarity index. We performed ordinations using principal component analysis on Hellinger-transformed normalized OTU tables generated using Euclidean distances[64]. The significance of differences in community composition was determined using permutational multivariate analysis of variance using distance matrices (PERMANOVA, *adonis*) with 1,000 permutations using the Euclidean distance matrix. We performed all analyses in R and GraphPad Prism 6. Means were compared using the paired *t*-test and paired ANOVA with Tukey's multiple comparisons test as appropriate. Linear regressions between relative abundances and cytokine concentrations were performed using log-transformed cytokine concentrations and adjusted for intrasubject correlations. Permutation analysis using the serial exclusion of subjects was performed to confirm that significance did not depend on the inclusion of outliers. Investigators were not blinded to the intervention status of specimens during analysis.

In the first sequencing run (454, ARDS BALs), we obtained 1,587 ± 1,085 reads per specimen and no specimens were excluded from analysis. In the second sequencing run (MiSeq, CLP, 24 h time point), we obtained 26,440 ± 38,804 reads per specimen. Four specimens, all blood, had too few reads to interpret (range 5–19 reads) and were excluded from analysis. In the third sequencing run (MiSeq, CLP, extended time points), we obtained 23,725 ± 33,792 reads per specimen. Eleven specimens were excluded from analysis because they had too few reads to interpret (range 16–237 reads): four blood specimens, two caecum specimens, two reagent control specimens, one faeces specimen and two lung specimens.

Representative sequences of key microbiota. The representative sequence of the Bacteroidales OTU (OTU008) depicted in Fig. 3 is TACGGAGGATGCGAGCGTTA TCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCTGCCAAGTCA GCGGTAAAATTGCGGGGCTCAACCCCGTACAGCCGTTGAAA CTGCCGGGCTCGAGTGGGCGAGAAGTATGCGGAATGCGTGGTGTA GCGGTGAAATGCATAGATATCACGCAGAACCCCGATTGCGAAGGCAGCA TACCGGCGCCCGACTGACGCTGAGGCACGAAAGTGCGGGGATCAAACAGG.

The representative sequence of the *Bacteroidetes* OTU (OTU009) depicted in Fig. 4 is TCTTGAGTACAGTAGAGGTGGGCGGAATTCGTGGTGTA GCGGTGAAATGCTTAGATATCACGAAGAACTCCGATTGCGAAGGCA GCTCACTGGACTGCAACTGACACTGATGCTCGAAAGTGTGGGTATCAAA CAGGATTAGATACCCTGGTAGTCCACACAGTAAACGATGAATA CTCGCTGTTTGCGATATACAGTAAGCGGCCAAGCGAAAGCATTAAGTA TTCCACCTGGGGAGTACGCCGGCAACGGTGAA.

Identification of procedural contaminants. The reagents used in DNA isolation and library preparation contain bacterial DNA that can contaminate sequence-based studies of microbial communities[65]. To identify potential sources of contamination in sequencing, we collected multiple procedural controls, including sterile water used in tissue collection (exposed to instruments used in harvesting and tissue homogenization), sterile water used in library preparation and AE buffer used in DNA isolation. Procedural and environmental control specimens from the collection of human BAL specimens were not collected at the time of sampling and were not analysed, including bronchoscope rinse specimens, endotracheal tube specimens and sterile (pre-lavage) saline. These procedural controls and mock community standards were analysed as quality controls in each sequencing run. Specimens were processed in a randomized order to minimize the risk of false pattern formation due to reagent contamination[65]. The presence of bacterial DNA in all procedural controls was confirmed in all three sequencing runs. In the first sequencing run (human ARDS BAL specimens), both sterile water used in specimen processing and AE buffer from the DNA isolation kit were sequenced. Sequences detected in procedural control specimens were significantly distinct from sequences detected in both ARDS BAL specimens and healthy subject BALs ($P \leq 0.001$ for both). The dominant taxa in procedural control specimens were a *Lachnospiraceae* sp. (OTU036) and an unclassified OTU (OTU016), which comprised a mean of 22.1% of sequences in procedural controls but only 0.44% of sequences in BAL specimens. The *Bacteroidetes* sp. discussed in the Results (OTU009) was not detected in any of the eight procedural control specimens (0% of total sequences). In the second experiment (CLP, 24 h time point), procedural control specimen communities were distinct from all tissue communities ($P < 0.05$ for blood, $P < 0.004$ for all other organ sites). The most abundant OTUs in procedural controls (comprising a mean of 45.1% of sequences) were an *Acinetobacter* sp. (OTU030), a *Pelomonas* sp. (OTU023), an unclassified OTU (OTU100) and a *Clostridium* sp. (OTU009). These were common and abundant in blood specimens (comprising a mean of 19.0% of sequences) but infrequent and in low abundance in colon, tongue and lung specimens (comprising a mean of 1.6% of sequences). In the third experiment (CLP, extended time points), all types of procedural control specimen communities were distinct from the communities of all tissue types ($P < 0.001$ for all comparisons). All procedural control specimens were dominated by a single *Pseudomonas* sp. (OTU004) that comprised a mean of 46.7% of reads; its presence both in specimen collection water and in kit reagent specimens confirmed that it was introduced in sequencing preparation rather than upstream in the tissue collection step. It was excluded from analysis; this was the only OTU excluded from any analysis in this study. The Bacteroidales sp. (OTU008) discussed in the Results was detected in only one of fourteen procedural control specimens and comprised a mean of only 0.11% of all sequences.

Accession codes. Sequences are available from the NCBI Sequence Read Archive (accession numbers SRP067346, SRP067513 and SRP076183).

Received 6 January 2016; accepted 3 June 2016; published 18 July 2016

References

1. Angus, D. C. *et al.* Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* **29,** 1303–1310 (2001).
2. Rubenfeld, G. D. *et al.* Incidence and outcomes of acute lung injury. *N. Engl. J. Med.* **353,** 1685–1693 (2005).
3. Hudson, L. D., Milberg, J. A., Anardi, D. & Maunder, R. J. Clinical risks for development of the acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* **151,** 293–301 (1995).
4. Sheu, C. C. *et al.* Clinical characteristics and outcomes of sepsis-related vs non-sepsis-related ARDS. *Chest* **138,** 559–567 (2010).
5. Dickson, R. P. The microbiome and critical illness. *Lancet Respir. Med.* **4,** 59–72 (2016).
6. Fine, J., Frank, H., Schweinburg, F., Jacob, S. & Gordon, T. The bacterial factor in traumatic shock. *Ann. NY Acad. Sci.* **55,** 429–445 (1952).
7. Cuevas, P., De la Maza, L. M., Gilbert, J. & Fine, J. The lung lesion in four different types of shock in rabbits. *Arch. Surg.* **104,** 319–322 (1972).
8. Souza, D. G. *et al.* The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J. Immunol.* **173,** 4137–4146 (2004).
9. Silvestri, L., de la Cal, M. A. & van Saene, H. K. Selective decontamination of the digestive tract: the mechanism of action is control of gut overgrowth. *Intens. Care Med.* **38,** 1738–1750 (2012).
10. Alverdy, J. C., Laughlin, R. S. & Wu, L. Influence of the critically ill state on host–pathogen interactions within the intestine: gut-derived sepsis redefined. *Crit. Care Med.* **31,** 598–607 (2003).
11. Deitch, E. A. Gut-origin sepsis: evolution of a concept. *Surgeon* **10,** 350–356 (2012).
12. Moore, F. A. *et al.* Gut bacterial translocation via the portal vein: a clinical perspective with major torso trauma. *J. Trauma* **31,** 629–636; 636–628 (1991).
13. Walley, K. R., Lukacs, N. W., Standiford, T. J., Strieter, R. M. & Kunkel, S. L. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. *Infect. Immun.* **64,** 4733–4738 (1996).
14. Oksanen, J. F. *et al. Vegan: Community Ecology Package. R package version 2.0-4* (2012); http://CRAN.R-project.org/package=vegan
15. Wang, Y., Naumann, U., Wright, S. T. & Warton, D. I. mvabund—an R package for model-based analysis of multivariate abundance data. *Methods Ecol. Evol.* **3,** 471–474 (2012).
16. Evans, C. C. *et al.* Exercise prevents weight gain and alters the gut microbiota in a mouse model of high fat diet-induced obesity. *PLoS ONE* **9,** e92193 (2014).
17. Devkota, S. *et al.* Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10−/− mice. *Nature* **487,** 104–108 (2012).
18. Musch, M. W., Wang, Y., Claud, E. C. & Chang, E. B. Lubiprostone decreases mouse colonic inner mucus layer thickness and alters intestinal microbiota. *Dig. Dis. Sci.* **58,** 668–677 (2013).
19. Poroyko, V. *et al.* Alterations of lung microbiota in a mouse model of LPS-induced lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* **309,** L76–L83 (2015).

20. Xu, J. *et al.* A genomic view of the human–Bacteroides thetaiotaomicron symbiosis. *Science* **299,** 2074–2076 (2003).
21. Kasai, C. *et al.* Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. *BMC Gastroenterol.* **15,** 100 (2015).
22. Eckburg, P. B. *et al.* Diversity of the human intestinal microbial flora. *Science* **308,** 1635–1638 (2005).
23. Dickson, R. P. *et al.* Changes in the lung microbiome following lung transplantation include the emergence of two distinct Pseudomonas species with distinct clinical associations. *PLoS ONE* **9,** e97214 (2014).
24. Osuchowski, M. F., Welch, K., Siddiqui, J. & Remick, D. G. Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. *J. Immunol.* **177,** 1967–1974 (2006).
25. Damas, P. *et al.* Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. *Crit. Care Med.* **17,** 975–978 (1989).
26. Suter, P. M. *et al.* High bronchoalveolar levels of tumor necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock, or sepsis. *Am. Rev. Respir. Dis.* **145,** 1016–1022 (1992).
27. Meduri, G. U. *et al.* Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* **108,** 1303–1314 (1995).
28. Hilty, M. *et al.* Disordered microbial communities in asthmatic airways. *PLoS ONE* **5,** e8578 (2010).
29. Huang, Y. J. *et al.* Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J. Allergy Clin. Immunol.* **127,** 372–381 (2011).
30. Sze, M. A. *et al.* Host response to the lung microbiome in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **192,** 438–445 (2015).
31. Zhang, D. *et al.* Neutrophil ageing is regulated by the microbiome. *Nature 525,* 528–532 (2015).
32. Rush, B. F. Jr *et al.* Does the bacteremia observed in hemorrhagic shock have clinical significance? A study in germ-free animals. *Ann. Surg.* **210,** 342–345; 346–347 (1989).
33. Doig, C. J. *et al.* Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *Am. J. Respir. Crit. Care Med.* **158,** 444–451 (1998).
34. Baker, J. W., Deitch, E. A., Li, M., Berg, R. D. & Specian, R. D. Hemorrhagic shock induces bacterial translocation from the gut. *J. Trauma* **28,** 896–906 (1988).
35. O'Boyle, C. J. *et al.* Microbiology of bacterial translocation in humans. *Gut* **42,** 29–35 (1998).
36. Harris, B. *et al.* Gut microbiota predict pulmonary infiltrates after allogeneic hematopoietic cell transplantation. *Am. J. Respir. Crit. Care Med.* http://dx.doi.org/10.1164/rccm.201507-1491OC (2016).
37. Lemaire, L. C. *et al.* Thoracic duct in patients with multiple organ failure: no major route of bacterial translocation. *Ann. Surg.* **229,** 128–136 (1999).
38. Suau, A. *et al.* Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.* **65,** 4799–4807 (1999).
39. Guo, S., Al-Sadi, R., Said, H. M. & Ma, T. Y. Lipopolysaccharide causes an increase in intestinal tight junction permeability *in vitro* and *in vivo* by inducing enterocyte membrane expression and localization of TLR-4 and CD14. *Am. J. Pathol.* **182,** 375–387 (2013).
40. Adams, C. A. Jr, Xu, D. Z., Lu, Q. & Deitch, E. A. Factors larger than 100 kD in post-hemorrhagic shock mesenteric lymph are toxic for endothelial cells. *Surgery* **129,** 351–363 (2001).
41. Chastre, J. *et al.* Nosocomial pneumonia in patients with acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* **157,** 1165–1172 (1998).
42. Kelly, B. J. *et al.* Composition and dynamics of the respiratory tract microbiome in intubated patients. *Microbiome* **4,** 7 (2016).
43. Arumugam, M. *et al.* Enterotypes of the human gut microbiome. *Nature* **473,** 174–180 (2011).
44. Morris, A. *et al.* Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am. J. Respir. Crit. Care Med.* **187,** 1067–1075 (2013).
45. Segal, L. N. *et al.* Enrichment of the lung microbiome with oral taxa is associated with lung inflammation of a Th17 phenotype. *Nature Microbiol.* **1,** 16031 (2016).
46. Santoro, A. E. & Boehm, A. B. Frequent occurrence of the human-specific Bacteroides fecal marker at an open coast marine beach: relationship to waves, tides and traditional indicators. *Environ. Microbiol.* **9,** 2038–2049 (2007).
47. Calfee, C. S. *et al.* Subphenotypes in acute respiratory distress syndrome: latent class analysis of data from two randomised controlled trials. *Lancet Respir. Med.* **2,** 611–620 (2014).
48. Zaborin, A. *et al.* Membership and behavior of ultra-low-diversity pathogen communities present in the gut of humans during prolonged critical illness. *mBio* **5,** e01361–e01314 (2014).
49. Dickson, R. P., Martinez, F. J. & Huffnagle, G. B. The role of the microbiome in exacerbations of chronic lung diseases. *Lancet* **384,** 691–702 (2014).
50. Dickson, R. P., Erb-Downward, J. R. & Huffnagle, G. B. Homeostasis and its disruption in the lung microbiome. *Am. J. Physiol. Lung Cell Mol. Physiol.* **309,** L1047–L1055 (2015).
51. Steinhauser, M. L. *et al.* IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense. *J. Immunol.* **162,** 392–399 (1999).
52. Paine, R. 3rd *et al.* A randomized trial of recombinant human granulocyte-macrophage colony stimulating factor for patients with acute lung injury. *Crit. Care Med.* **40,** 90–97 (2012).
53. Bernard, G. R. *et al.* The American–European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am. J. Respir. Crit. Care Med.* **149,** 818–824 (1994).
54. Dickson, R. P. *et al.* Cell-associated bacteria in the human lung microbiome. *Microbiome* **2,** 28 (2014).
55. Mason, K. L. *et al. Candida albicans* and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. *Infect. Immun.* **80,** 3371–3380 (2012).
56. Caporaso, J. G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl Acad. Sci. USA* **108**(Suppl 1), 4516–4522 (2011).
57. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* **79,** 5112–5120 (2013).
58. Schloss, P. D. *454 SOP—mothur* (2015); http://www.mothur.org/wiki/454_SOP
59. Jumpstart Consortium Human Microbiome Project Data Generation Working Group *Human Microbiome Consortium 16S 454 Sequencing Protocol* (2010); http://hmpdacc.org/doc/16S_Sequencing_SOP_4.2.2.pdf
60. Daigle, D., Simen, B. B. & Pochart, P. High-throughput sequencing of PCR products tagged with universal primers using 454 life sciences systems. *Curr. Protoc. Mol. Biol.* Chapter 7, Unit 7.5 (2011).
61. Schloss, P. D. *MiSeq SOP—mothur* (2015); http://www.mothur.org/wiki/MiSeq_SOP
62. Schloss, P. D. *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75,** 7537–7541 (2009).
63. R Core Team *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, 2013); http://www.R-project.org/
64. Legendre, P. & Gallagher, E. D. Ecologically meaningful transformations for ordination of species data. *Oecologia* **129,** 271–280.
65. Salter, S. J. *et al.* Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* **12,** 87 (2014).

Acknowledgements

Funding was provided by the National Institutes for Health: UL1TR000433 (to R.P.D.), K23HL130641 (to R.P.D.), T32HL00774921 (to B.H.S.), R01HL123515 (to T.J.S.), UO1HL123031 (to T.J.S.), U01HL098961 (to G.B.H.) and R01HL114447 (to G.B.H.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Support was provided by the Michigan Institute for Clinical & Health Research (to R.P.D.), the Host Microbiome Initiative of the University of Michigan (to R.P.D. and B.H.S.) and the University of Michigan Center for Integrative Research in Critical Care (to R.P.D.). The authors acknowledge the University of Michigan Multidisciplinary Intensive Care Research Workgroup for discussions, and thank A. Bredenkamp for bioinformatic assistance.

Author contributions

R.P.D. and B.H.S. conceived the experiment. R.P.D., B.H.S., T.J.S. and G.B.H. designed the study. R.P.D., B.H.S., M.W.N. and N.R.F. performed experiments. R.P.D. analysed data. R.P.D., B.H.S., J.R.E.-D., T.J.S. and G.B.H. provided critical analysis and discussions. R.P.D. wrote the first draft and all authors participated in revision.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to R.P.D.

Competing interests

The authors declare no competing financial interests.