REGULAR ARTICLE

blood advances

Gastrointestinal microbiota contributes to the development of murine transfusion-related acute lung injury

Rick Kapur,[1] Michael Kim,[2,3] Johan Rebetz,[1] Björn Hallström,[4] Jonas T. Björkman,[5] Alisa Takabe-French,[2,3] Noel Kim,[2,3] Jonathan Liu,[2,3] Shanjeevan Shanmugabhavananthan,[2,3] Stefan Milosevic,[2,3] Mark J. McVey,[2,3,6-9] Edwin R. Speck,[2,3] and John W. Semple[1-3,6,10-12]

[1]Division of Hematology and Transfusion Medicine, Lund University, Lund, Sweden; [2]Keenan Research Centre for Biomedical Science and [3]The Toronto Platelet Immunobiology Group, St. Michael's Hospital, Toronto, ON, Canada; [4]Center for Translational Genomics, Lund University, Lund, Sweden; [5]Center for Molecular Diagnostics, Skåne University Hospital, Lund, Sweden; [6]Canadian Blood Services, Toronto, ON, Canada; [7]Department of Anesthesia and [8]Department of Physiology, University of Toronto, Toronto, ON, Canada; [9]Department of Anesthesia and Pain Medicine, Hospital for Sick Children, Toronto, ON, Canada; and [10]Department of Pharmacology, [11]Department of Medicine, and [12]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Key Points

- Gastrointestinal flora contributes to development of antibody-mediated murine TRALI.
- Depletion of gastrointestinal flora prevents TRALI by inhibiting MIP-2 secretion and pulmonary neutrophil accumulation.

Transfusion-related acute lung injury (TRALI) is a syndrome of respiratory distress upon blood transfusion and is the leading cause of transfusion-related fatalities. Whether the gut microbiota plays any role in the development of TRALI is currently unknown. We observed that untreated barrier-free (BF) mice suffered from severe antibody-mediated acute lung injury, whereas the more sterile housed specific pathogen-free (SPF) mice and gut flora-depleted BF mice were both protected from lung injury. The prevention of TRALI in the SPF mice and gut flora–depleted BF mice was associated with decreased plasma macrophage inflammatory protein-2 levels as well as decreased pulmonary neutrophil accumulation. DNA sequencing of amplicons of the 16S ribosomal RNA gene revealed a varying gastrointestinal bacterial composition between BF and SPF mice. BF fecal matter transferred into SPF mice significantly restored TRALI susceptibility in SPF mice. These data reveal a link between the gut flora composition and the development of antibody-mediated TRALI in mice. Assessment of gut microbial composition may help in TRALI risk assessment before transfusion.

Introduction

Transfusion-related acute lung injury (TRALI) is a serious complication of blood transfusions and is characterized by the onset of acute respiratory distress resulting from pulmonary edema within 6 hours after transfusion.[1] At present, TRALI is the leading cause of transfusion-related fatalities and, apart from supportive measures such as oxygen or ventilation, no specific therapies are available. The pathogenesis of TRALI is incompletely understood but generally, TRALI is hypothesized to occur because of a 2-hit model.[2] The first hit is recipient-predisposing factors, such as systemic inflammation, which can manifest itself as increased interleukin (IL)-6,[3,4] IL-8[3-5] or C-reactive protein (CRP) levels.[6,7] The second hit is subsequently conveyed by pathogenic antileukocyte antibodies or other biological response modifiers present in the transfused donor blood.[2,8] In the majority of the cases, human neutrophil antigen– or HLA-specific antibodies in the transfused blood are involved.[2] Using animal models of antibody-mediated TRALI, it has become clear that neutrophils (polymorphonuclear cells [PMNs]) are the major effector cells in TRALI.[6,9-13] Moreover, using a novel C57BL/6 knockout (KO) model of antibody-mediated TRALI, we recently demonstrated that PMN-reactive oxygen species are critically essential for inducing the acute lung injury in TRALI.[12] In addition, $CD4^+$ T regulatory cells and dendritic cells were identified as important suppressor cells of antibody-mediated TRALI and this protective response was associated with production of the anti-inflammatory cytokine IL-10.[12] Similarly, we recently found that

Submitted 22 March 2018; accepted 22 May 2018. DOI 10.1182/bloodadvances.2018018903.

Presented at the 59th annual meeting of the American Society of Hematology, Atlanta, GA, 9-12 December 2017.

The full-text version of this article contains a data supplement.

plasma IL-10 levels were significantly lower in patients undergoing TRALI reactions compared with, for example, patients who suffered from septic acute lung injury.[14]

In the current paper, we have further investigated the pathogenesis of antibody-mediated TRALI using the murine C57BL/6 TRALI model based on $CD4^+$ T-cell depletion and followed by infusion of anti-major histocompatibility complex (MHC) class I antibodies.[12] We hypothesized that the gut microbiota composition may be a factor driving the secretion of the PMN-chemoattractant macrophage inflammatory protein-2 (MIP-2) and pulmonary PMN recruitment during antibody-mediated TRALI in mice. To investigate this, we housed mice in a barrier-free (BF) setting compared with a specific pathogen-free (SPF) environment. SPF is a term used for laboratory animals that are guaranteed free of particular pathogens. The population is therefore checked for the presence of antibodies against the specified pathogens. BF housing, on the other hand, also ensures the health of the animals but provides a less stringent pathogen-free environment and consequently reflects a less sterile housing environment compared with SPF housing. This may, for instance, be reflected by the strict adherence to closed, internally ventilated cages and/or wearing more protective clothing in an SPF setting compared with open cages and/or less protective covers in a BF setting. We demonstrate that SPF mice as well as BF mice that were depleted of their gastrointestinal (GI) flora by broad spectrum antibiotics are protected from antibody-mediated TRALI development by impairment of plasma MIP-2 secretion and pulmonary PMN recruitment and have a different GI microbiota composition compared with BF mice, which are susceptible to TRALI. These results suggest that the GI microbiota composition contribute to the susceptibility to murine antibody-mediated TRALI.

Methods

Mice

BF-housed C57BL/6 (H-2b, C57BL/6NCrl) mice were obtained from Charles River Laboratories (Montreal, QC, Canada) and SPF-housed C57BL/6 (H-2b, C57BL/6NCrl) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). IL-10 KO mice (B6.129P2-Il10tm1Cgn/J, background strain C57BL/6) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in an SPF setting. All mice were males, 8 to 10 weeks of age, and housed for at least 1 week in the designated animal facility before initiating experiments. All animal studies were approved by the St. Michael's Hospital Animal Care Committee, Toronto, and the Animal Ethics Committee of Lund University, Lund, Sweden.

Antibodies and reagents

TRALI induction antibodies: 34-1-2s (monoclonal mouse IgG2a that reacts with murine H-2Kd and H-2Dd MHC class I molecules) and AF6-88.5.5.3 (monoclonal mouse IgG2a that reacts to murine MHC class I H-2Kb), as well as the in vivo $CD4^+$ T cell–depleting antibody: GK1.5 (anti-CD4, rat IgG2b) were all purchased from Bio X Cell (West Lebanon, NH); RM4-5 (anti-mouse CD4-PE, anti-mouse CD4-FITC) was purchased from BD Pharmingen, San Diego, CA. Antibiotic drinking water: sucrose, vancomycin, ampicillin sodium salt, neomycin trisulfate salt, and metronidazole were all purchased from Sigma-Aldrich, Oakville, ON, Canada.

Pretesting and the antibody-mediated murine TRALI model

Mice were randomized into the indicated groups and rectal temperatures were taken directly upon arrival as well as after 1 week of housing in the indicated animal facility. TRALI was induced as previously described.[12] Briefly, 18 hours before TRALI induction, mice were injected intraperitoneally with the monoclonal antibody GK1.5 (4.5 mg/kg) to deplete $CD4^+$ T cells. On the day of experiment, the CD4-depleted mice were injected IV with 600 μL of a mixture of the TRALI-inducing antibodies 34-1-2s (45 mg/kg) and AF6-88.5.5.3 (4.5 mg/kg). Subsequently, rectal temperatures, indicative of systemic shock, were recorded every 30 minutes (up to 90 minutes) using an RET-3 rectal probe for mice (ADInstruments Limited, Oxford, United Kingdom) connected to a traceable digital thermometer (VWR International, Stockholm, Sweden). Ninety minutes after TRALI initiation, mice were anesthetized with a mixture of Ketaminol and Rompun and exsanguinated by cardiac puncture with blood drawn into 100 μL of PBS/citrate phosphate dextrose adenine. The study design, including a schematic overview of the antibody-mediated TRALI model, is described in the supplemental Methods. In indicated experiments, mice were primed 18 hours before TRALI induction with 0.1 mg/kg lipopolysaccharide (LPS) intraperitoneally (from *Escherichia coli* O55:B5, Sigma-Aldrich Sweden AB). In the indicated experiments, SPF mice were primed twice a day, 2 days before TRALI induction, intrarectally with 100 μL of BF fecal matter dissolved in sterile PBS.

Antibiotic treatment and bacterial depletion

Selected mouse groups were depleted of both aerobic and anaerobic bacteria by administration of a combination of broad spectrum antibiotics (vancomycin, ampicillin, neomycin, and metronidazole, all at 1 mg/mL) in drinking water (containing 1.75% weight-to-volume sucrose) that was changed every 48 hours for 1 week. Further details on assessment of bacterial depletion are described in the supplemental Methods.

Tissue processing

See supplemental Methods.

Lung W/D weight ratios

Lung wet/dry (W/D) weight ratios are a measure of pulmonary edema and were determined as previously described.[6,10-13] Briefly, the right lung of each mouse was removed and weighed to determine the wet weight and then dried in an oven at 60°C for 48 hours. The dried samples were reweighed to obtain the dry weight. The lung W/D weight ratio was calculated by the formula: net wet weight/net dry weight.

Pulmonary PMN enumeration

The red blood cell–lysed lung cell suspensions were mounted on microscope slides (VWR Frosted Economy slides) using a Shandon Cytospin 4 (ThermoFisher Scientific, Nepean, ON, Canada) followed by staining with a hematoxylin and eosin kit (Harleco-Hemacolor; EMD Chemicals, Darmstadt, Germany). The slides were examined under Permount (Fisher Scientific Company, Ottawa, ON, Canada) using a Nikon Eclipse E800 microscope equipped with a ×40/0.75 objective lens, in a blinded manner. Pulmonary PMN were evaluated by counting the total number of nucleated cells in 4 randomly selected and nonoverlapping fields and PMN numbers were

enumerated by using ImageJ software in a blinded manner. The pulmonary PMN percentage was determined by the formula: average number of observed PMN (in 4 fields)/average number of nucleated cells (in 4 fields) × 100.

Lung tissue histology

A segment of the left lung was fixed in 10% formalin solution and the fixed sample was embedded into paraffin and sectioned with a microtome. The sections were stained with hematoxylin and eosin using a Leica AutoStainerXL (Leica Biosystems, Nussloch, Germany). The stained slides were examined under Permount (Fisher Scientific Company) using a Nikon Eclipse E800 microscope equipped with a ×20 and a ×40/0.75 objective lens.

Cytokine measurements

Collected blood was centrifuged twice at 2500*g* for 15 minutes (without brake) and plasma was collected, aliquoted, and stored at −80°C for subsequent batch analysis. The thawed plasma was analyzed for murine MIP-2 and IL-10 using solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (Mouse CXCL2/MIP-2 Quantikine ELISA Kit and Mouse IL-10 Quantikine ELISA Kit; R&D Systems, Minneapolis, MN), according to the manufacturer's protocol.

Microbial profiling using 16S ribosomal RNA gene amplicon sequencing

DNA was isolated from fecal matter collected from indicated mouse groups, according to the manufacturer's protocol (QIAamp DNA stool mini kit; Qiagen, GmbH, Hilden, Germany). Sequencing was performed according to the Illumina 16S metagenomic sequencing library preparation guide (Illumina part #15044223 Rev. B), as described in the supplemental Methods.

Statistical analysis

The specific statistical tests used are listed in each of the figure legends. Statistical significance was set at $P < .05$ and statistical analysis was performed using GraphPad Prism 7.03 software for Windows (GraphPad Software, San Diego, CA).

Results

BF mice display elevated body temperatures indicative of a differing GI bacterial composition

It has been described that GI flora can upregulate body temperature in mice[15]; therefore, we assessed temperatures in the indicated mouse groups. Compared with the SPF-housed mice, after 1 week of housing, the temperatures of BF mice significantly increased (Figure 1). Moreover, the rectal temperatures of BF mice were significantly higher compared with the SPF mice after 1 week of housing (39.5 ± 0.23°C vs 38.3 ± 0.36°C, respectively; Figure 1, column 2 vs 5). The rectal temperatures remained elevated in BF mice for multiple weeks after arrival, even up to 28 weeks after arrival (supplemental Figure 1). To further confirm if this increased temperature was related to their GI bacterial composition, we treated the BF mice with antibiotics on the day of their arrival. The 1-week rectal temperatures in the antibiotic-treated BF mice did not elevate compared with the untreated BF mice (Figure 1, column 1 vs 3). The antibiotic-related static temperatures were associated with at least a 75% depletion of both aerobic and anaerobic GI flora (supplemental Figure 2). These data indicate that higher body temperatures in BF mice compared with antibiotic-treated BF mice and SPF mice were due to their GI bacterial composition. We then investigated whether this difference in GI flora could affect TRALI disease-susceptibility.

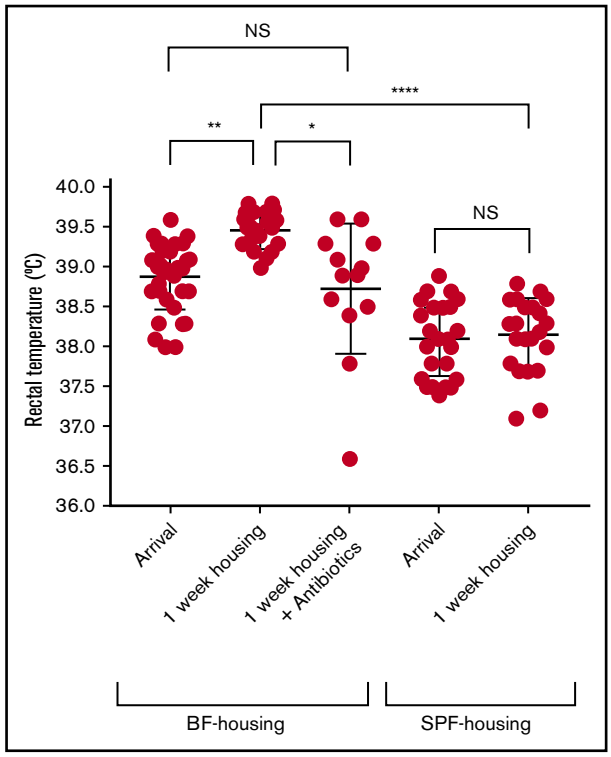
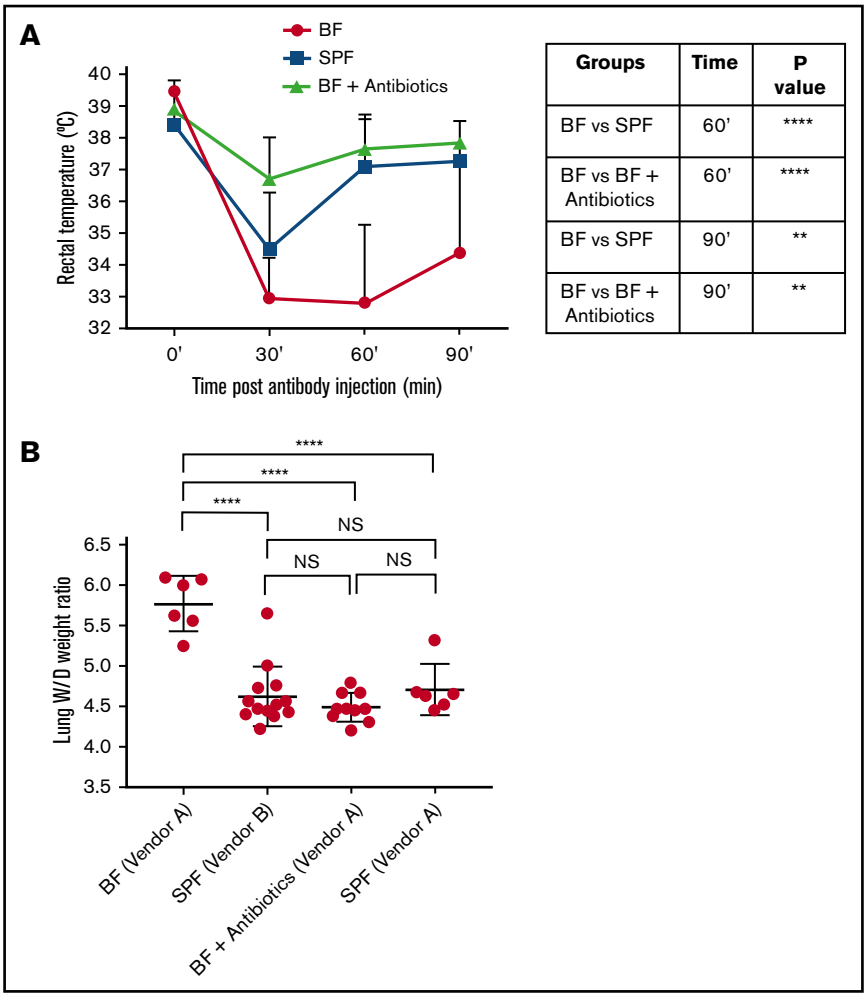


Figure 1. Rectal temperatures are increased in BF mice compared with SPF mice and BF mice with antibiotics-induced gut flora depletion. Rectal temperatures of C57BL/6 mice upon arrival and after 1 week of housing in a BF or SPF environment. BF-housed mice that were housed for a week were also subsequently treated with broad spectrum antibiotics for a week. For the statistical analyses, only significant comparisons of interest are shown. Statistical analysis was performed with a 1-way analysis of variance (ANOVA) with Dunn multiple comparison's test. Each dot represents 1 mouse; error bars represent standard deviation (SD). Figure shows combinatorial data from 3 experiments. *$P < .05$, **$P < .01$, ****$P < .0001$. NS, nonsignificant.

GI bacterial composition determines TRALI initiation

Based on a previously established murine TRALI model,[12] SPF mice and BF mice (either treated or not with antibiotics) were $CD4^+$ T cell–depleted and exposed to the TRALI inducing antibodies. In a time course experiment, at both 60 and 90 minutes after antibody infusion, the rectal temperatures of untreated BF mice were significantly lower compared with SPF mice or BF mice treated with antibiotics (Figure 2A). The low body temperatures in the untreated BF mice correlated with a significant increase in lung W/D ratios, indicating acute lung injury (Figure 2B; lung W/D, 5.77) compared with SPF mice (lung W/D, 4.62) or BF mice treated with antibiotics (lung W/D, 4.49) or mice from the same supplier as used in the BF setting (vendor A) housed in an SPF setting (lung W/D, 4.71). Histological analyses confirmed significant acute lung injury in BF-housed mice including increased alveolar septal thickening, acute inflammatory infiltrates, and alveolar exudates indicative of pulmonary edema (Figure 3Bi-ii). Furthermore, IL-10 KO mice that



Groups	Time	P value
BF vs SPF	60'	****
BF vs BF + Antibiotics	60'	****
BF vs SPF	90'	**
BF vs BF + Antibiotics	90'	**

Figure 2. SPF and BF mice with antibiotics-induced gut flora depletion are protected from antibody-mediated TRALI. (A) Rectal temperatures, (B) lung W/D weight ratios upon $CD4^+$ T-cell depletion, and anti-MHC class I antibody (clones 34-1-2s and AF6-88.5.5.3) infusion into indicated mouse groups. Vendor A and B indicate 2 vendors for laboratory mice. For the statistical analyses, only significant comparisons of interest are shown. Statistical analysis was performed with 1-way ANOVA with a Tukey post hoc test at 60 and at 90 minutes, as depicted in the accompanying table in panel A and 1-way ANOVA with a Tukey's post hoc test (B). Data are based on 5, 13, and 10 mice per time point for BF, SPF, and BF antibiotic mice, respectively. (B) Each dot represents 1 mouse; error bars represent SD. Figure represents combinatorial data from 3 experiments. $^{**}P < .01$, $^{****}P < .0001$.

were previously found to be hypersensitive to antibody-mediated TRALI induction in a BF setting[12] were resistant to TRALI induction when housed in an SPF setting (Figures 3Fi-ii and 4).

TRALI protection from decreased GI flora is associated with reduced MIP-2 chemokine production and pulmonary PMN accumulation

Plasma levels of MIP-2, a potent PMN chemoattractant (murine equivalent of human IL-8), were analyzed because it was been previously shown that MIP-2 is essential for antibody-mediated TRALI induction.[11] Compared with TRALI-protected SPF mice and antibiotic-treated BF mice, only the untreated BF mice undergoing TRALI reactions had significantly higher plasma levels of MIP-2 (Figure 5A; 82.94 ± 67.93 pg/mL). Similarly, low levels of MIP-2 were also found in the plasma of the TRALI-protected SPF-housed IL-10 KO mice (Figure 5A). When the numbers of pulmonary PMN were enumerated, the baseline pulmonary PMN were found to be 23% on average in BF-housed mice vs 6% in SPF-housed mice. Subsequently, significant increases in pulmonary PMN were observed only in the TRALI-responsive BF mice (46 ± 10%) compared with TRALI-protected SPF mice (28 ± 9%) and antibiotic-treated BF mice (25 ± 5%) (Figure 5B). Similar findings were observed in TRALI-protected SPF IL-KO mice (27 ± 12%) (Figure 5B).

Pathogenic TRALI response can be significantly restored by priming SPF mice with LPS before inducing TRALI

To investigate if we could restore the pathogenic TRALI response in SPF-housed mice, we first primed the mice with a low dose of LPS (0.1 mg/kg), a gram-negative bacterial endotoxin that has been used in other TRALI mouse models as a priming factor.[16] When the SPF mice were primed with low-dose LPS, depleted of their $CD4^+$ cells, and infused with TRALI antibodies, the pathogenic TRALI reaction was significantly restored, as is demonstrated by increased lung W/D weight ratios, increased MIP-2 levels (supplemental Figure 3), increased pulmonary PMN, and signs of acute lung injury on histology (Figure 6).

TRALI-susceptible BF mice display a different GI microbiome composition than TRALI-resistant SPF mice

To further confirm if the differences in TRALI susceptibility between BF and SPF mice may indeed be due to the GI flora, we characterized the gut microbiome by isolating DNA from fecal samples from BF as well as SPF mice and performed 16S ribosomal RNA gene amplicon sequencing The phylogenetic tree of the gut

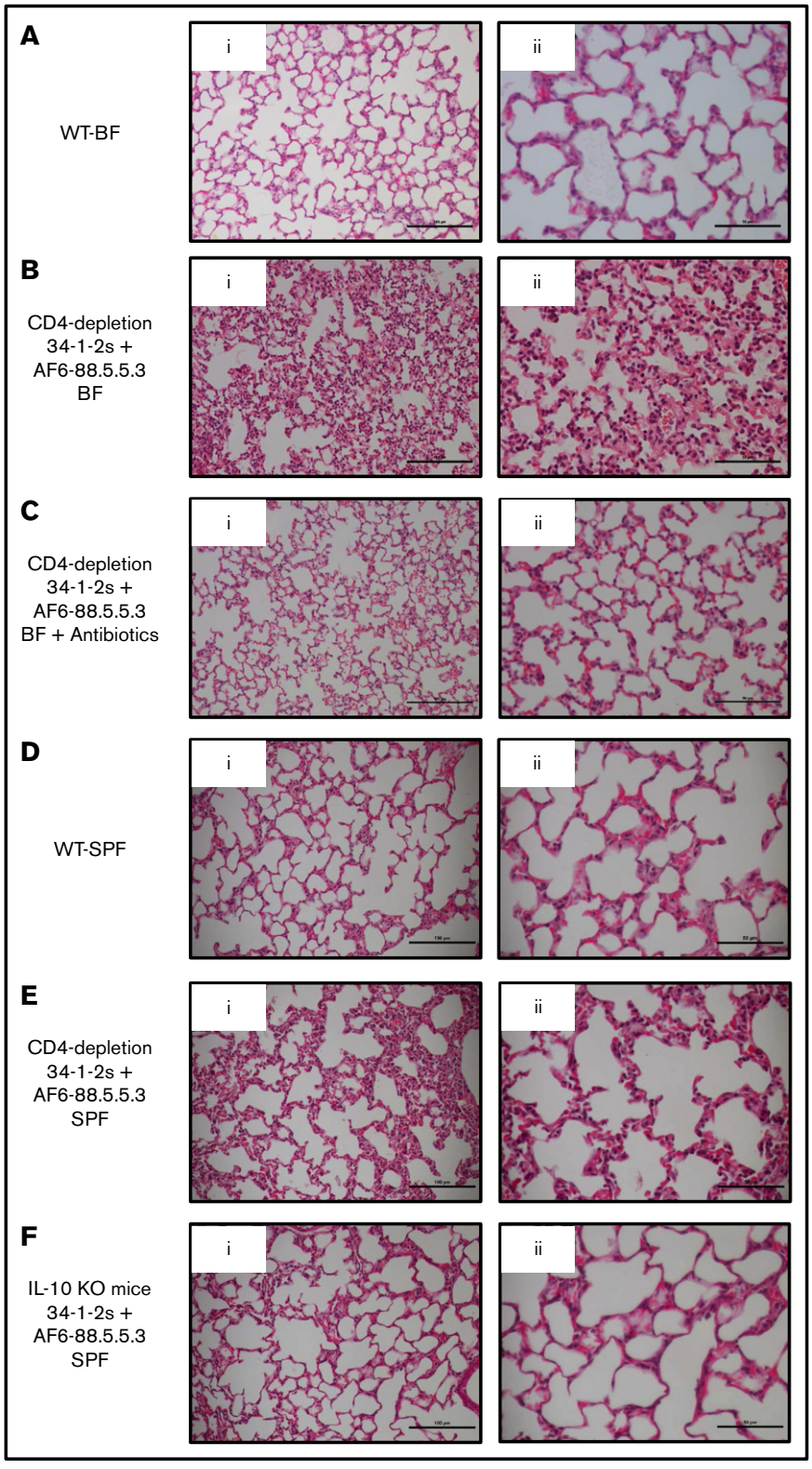


Figure 3. SPF and BF mice with antibiotic-induced gut flora depletion do not display signs of severe antibody-mediated acute lung injury on lung tissue histology. Lung histology from indicated mouse groups. Panels Ai-Fi and Aii-Fii represent lung tissue images taken at original magnification ×20 and ×40, respectively (hematoxylin and eosin stain). Representative images of each indicated group are shown. Scale bars represent 100 μM (Ai-Fi) and 50 μM (Aii-Fii). WT, wild type.

microbiota shows a different clustering pattern of fecal bacteria in BF mice that are susceptible to TRALI compared with SPF mice (and BF antibiotic-treated mice), which were resistant to TRALI development (Figure 7A). Heat maps and pie charts of family (Figure 7B-C), genus (Figure 7D), and species levels (Figure 7E) illustrate the presence of a different bacterial environment in the gut of BF vs SPF mice. Further analyses at the level of bacterial species indicated that the anaerobic gram-negative bacterium

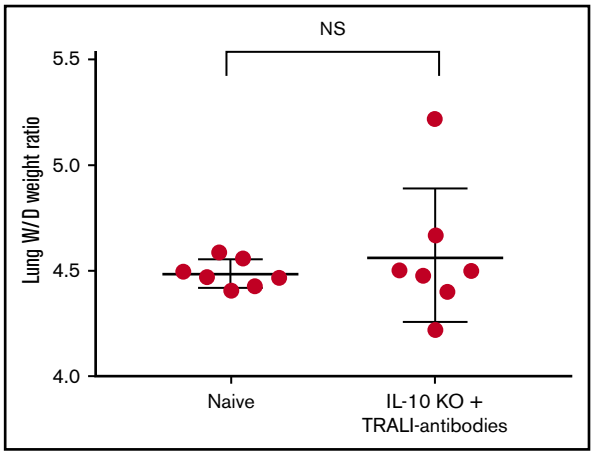


Figure 4. SPF IL-10 KO mice are protected from antibody-mediated TRALI. Lung W/D weight ratios upon anti-MHC class I antibody (clones 34-1-2s and AF6-88.5.5.3) infusion into IL-10 KO mice. IL-10 KO status was confirmed by performing an IL-10 ELISA using collected plasma, as described in the "Methods." Statistical analysis was performed using an unpaired Student *t* test. Each dot represents 1 mouse. Figure shows combinatorial data from 2 experiments.

Bacteroides caccae is significantly increased in the TRALI-susceptible BF-housed mice whereas, in contrast, this bacterium was not present in TRALI-resistant SPF-housed mice (supplemental Figure 4).

Fecal transfer from BF mice to SPF mice significantly restores TRALI susceptibility

To directly investigate if BF fecal transfer could restore TRALI susceptibility in SPF mice, SPF mice were primed with fecal matter from BF mice. When the primed SPF mice where depleted of their $CD4^+$ T cells and infused with TRALI antibodies, the pathogenic TRALI reaction was significantly restored, as is demonstrated by increased lung W/D weight ratios (Figure 7F) and increased pulmonary PMN (Figure 7G).

Discussion

The GI flora has been shown to be altered in many disease states, such as cardiovascular disease,[17] colitis,[18] obesity,[19] malignancy,[20] type 2 diabetes,[21] psychiatric disorders (anxiety, depression, schizophrenia),[22,23] neurodegeneration (murine model of Parkinson disease),[24] neurodevelopmental abnormalities in murine offspring,[25,26] and asthma.[27] In the current study, we therefore specifically investigated if the GI flora may also be of importance for the pathogenic responses in antibody-mediated TRALI. We describe an association between the GI microbiota and the onset of TRALI using our previously established TRALI model in C57BL/6 mice.[12]

We directly compared mice housed in an SPF setting (restricted pathogen environment) vs those housed in a BF setting (less restricted pathogen environment). Strikingly, mice housed in a BF setting displayed higher baseline rectal temperatures and higher baseline pulmonary PMN levels compared with mice housed in the SPF environment (Figures 1 and 5B, respectively). This difference in temperature was not related to the temperature in the animal rooms (all housed at 21°C room temperature) but was found to be related to the GI microbiota in BF mice, which was in accordance with a previous study.[15] In that study, Kluger and colleagues demonstrated

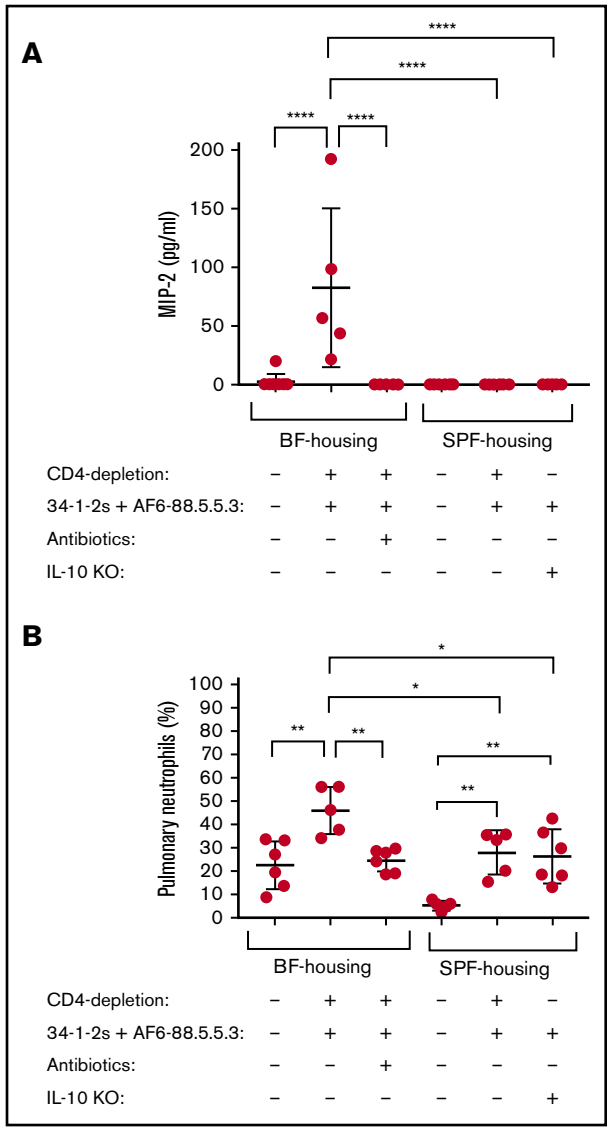


Figure 5. Protection from TRALI is associated with decreased levels of plasma MIP-2 and decreased pulmonary PMN in both SPF and BF mice with antibiotic-induced gut flora depletion. (A) Plasma MIP-2 and (B) pulmonary PMN levels in indicated mouse groups. For the statistical analyses, only significant comparisons of interest are shown. Statistical analysis was performed with 1-way ANOVA with a Tukey post hoc test. Each dot represents 1 mouse; error bars represent SD. Figure panels show combinatorial data from 3 experiments. $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$.

that GI flora in BF-raised mice had a stimulatory effect on body temperature (unrelated to physical activity) compared with germ-free mice.[15] In addition to the untreated SPF mice, we examined BF mice that were first treated with broad spectrum antibiotics, a widely established technique to significantly deplete the gut flora (supplemental Figure 2).[28-31] We observed that after depletion of the GI flora in BF mice (supplemental Figure 2), their rectal temperatures remained static after housing for 1 week (Figure 1). Antibiotics are known to have a significant impact on GI flora by decreasing its density and modifying its composition in a long-lasting fashion.[32] This can cause reduced signaling to the GI mucosa and peripheral organs and impair the function of the immune system.

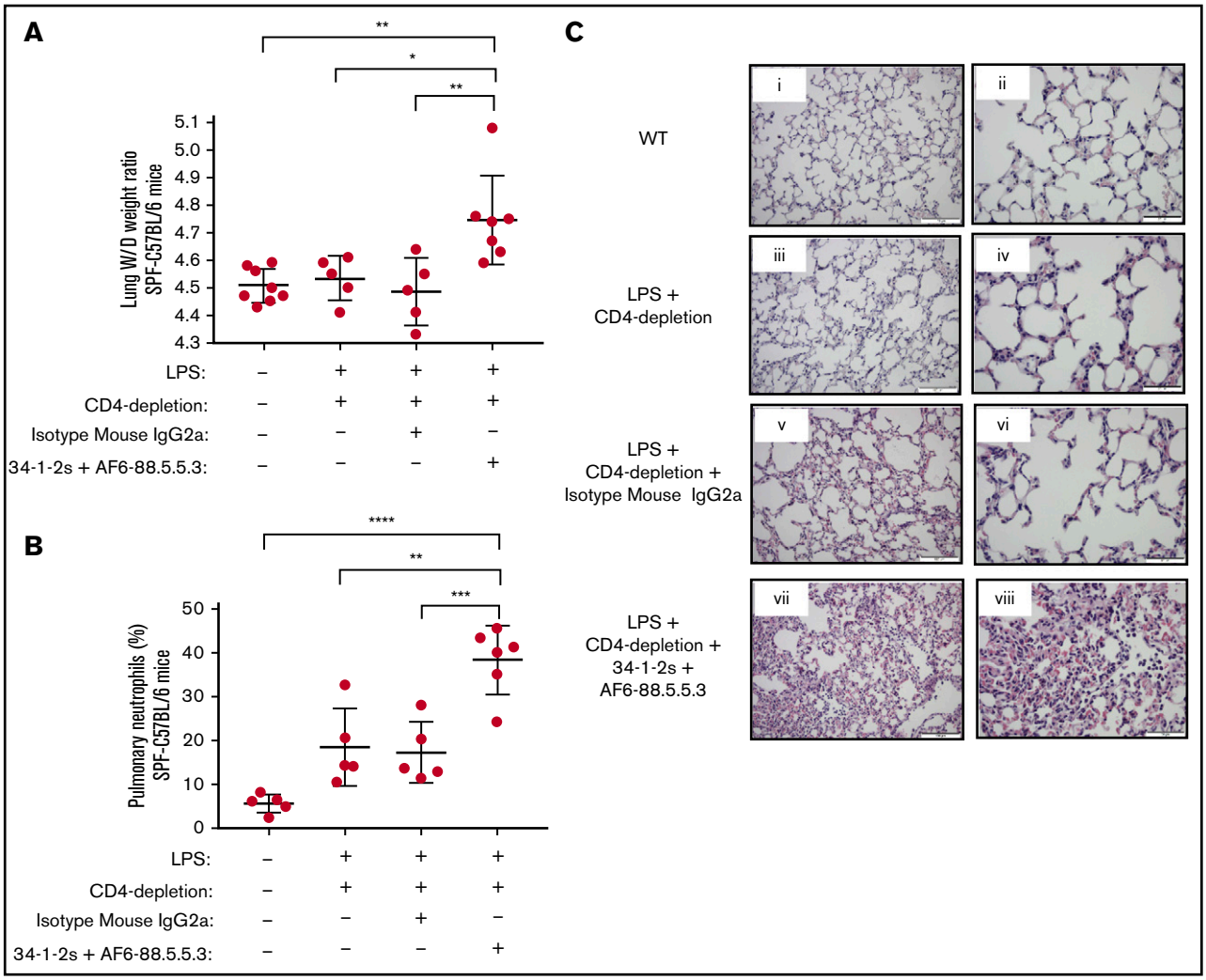


Figure 6. Priming with low-dose LPS restores TRALI in SPF mice. (A) Lung W/D weight ratios, (B) pulmonary PMN numbers, and (C) lung tissue histology analyses in LPS-primed mice (0.1 mg/kg) upon $CD4^+$ T-cell depletion and anti-MHC class I antibody (clones 34-1-2s and AF6-88.5.5.3) infusion into indicated mouse groups. (C) Lung tissue images taken at original magnification ×20 (i,iii,v,vii) and ×40 (ii,iv,vi,viii) (hematoxylin and eosin stain). Representative images of each indicated group are shown. Scale bars represent 100 μM (i,iii,v,vii) and 50 μM (ii,iv,vi,viii). For the statistical analyses, only significant comparisons of interest are shown. Statistical analysis was performed with 1-way ANOVA with a Tukey post hoc test. (A-B) Each dot represents 1 mouse; all error bars represent SD. Figure panels show combinatorial data from 3 experiments.
*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

With regard to how gut flora affects the lungs, little is known about underlying inflammatory disorders of the gut that coexist with inflammatory pulmonary disorders. For instance, it was reported that 33% of 133 patients suffering from irritable bowel syndrome had respiratory problems and that 16% of these patients suffered from asthma.[33] In addition, patients with chronic obstructive pulmonary disorder were found to have a 3 times higher risk of developing Crohn disease and ulcerative colitis compared with healthy controls.[34] Because of the heterogeneous composition of the GI flora, its general depletion may cause either beneficial or adverse effects depending on the specific clinical scenario. For example, it was reported that compared with untreated mice, 20% of gut flora–depleted mice died of pneumonia infection within 50 hours.[35] This suggests that the gut microbiota elicits a protective effect for pneumonia and that perhaps specific antibiotic therapy may be able to treat specific lung infections while leaving commensal GI flora intact. On the other hand, it was recently reported that the lung microbiota in both sepsis and acute respiratory distress syndrome (ARDS) is enriched for bacteria that are usually found in the lower GI tract.[36] Moreover, the abundance of gut bacteria in human ARDS samples correlated with disease severity, indicating the gut's microbiota plays a pathogenic role.[36] We found that in contrast to the BF mice, untreated SPF mice or BF mice treated with antibiotics (gut flora depleted) were protected from antibody-mediated TRALI (Figures 2 and 3). This pathogenic TRALI response was not mouse-vendor dependent, but was determined by the housing conditions (Figure 2B). Interestingly, IL-10 KO mice housed in a BF environment were previously found to be hypersensitive to antibody-mediated TRALI.[12] In contrast, we found that if the IL-10 KO mice were housed in an SPF environment, they became resistant to TRALI development (Figure 4). This suggests that the gut flora, more importantly than IL-10 levels, contribute to the pathogenic

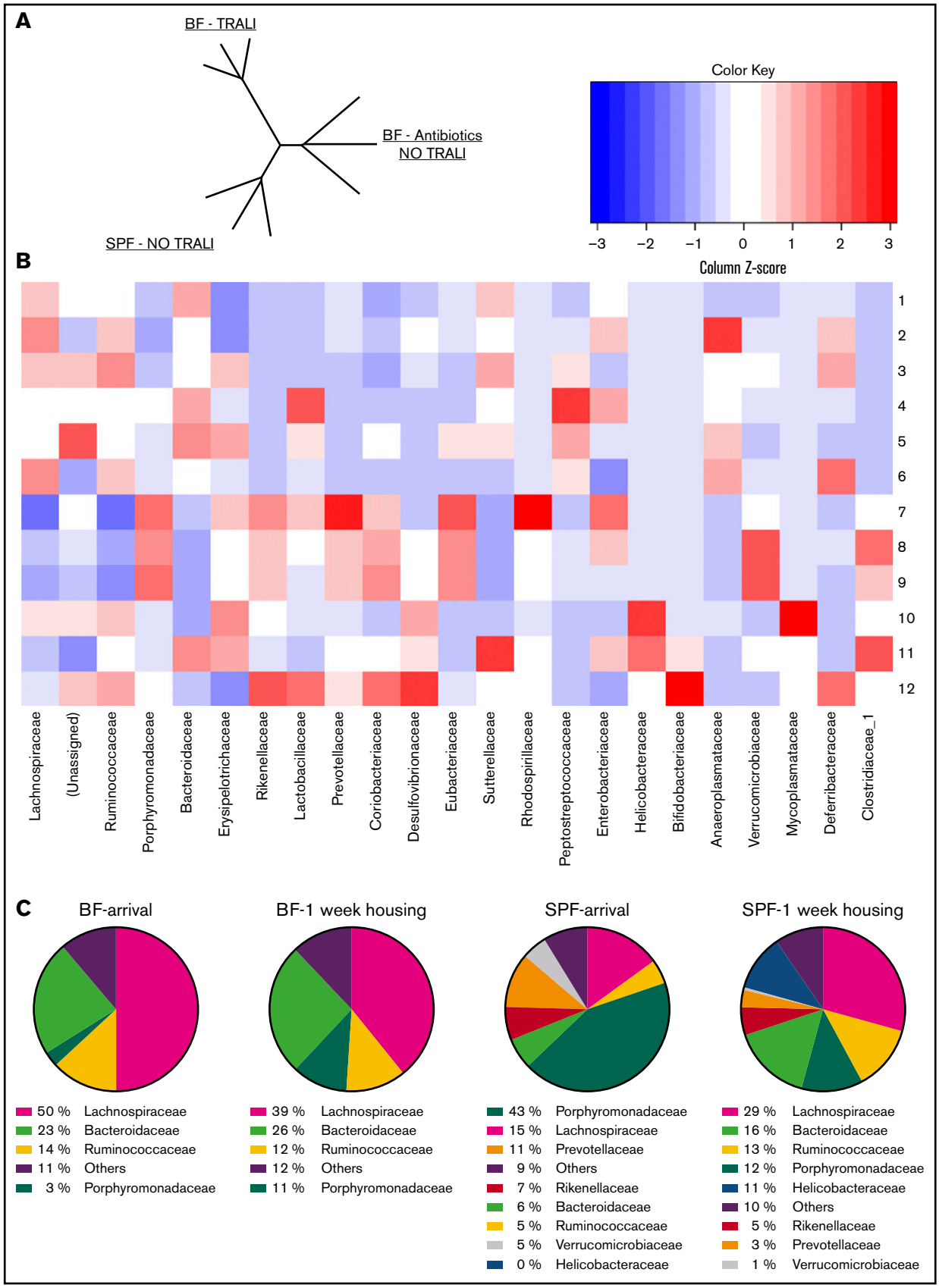
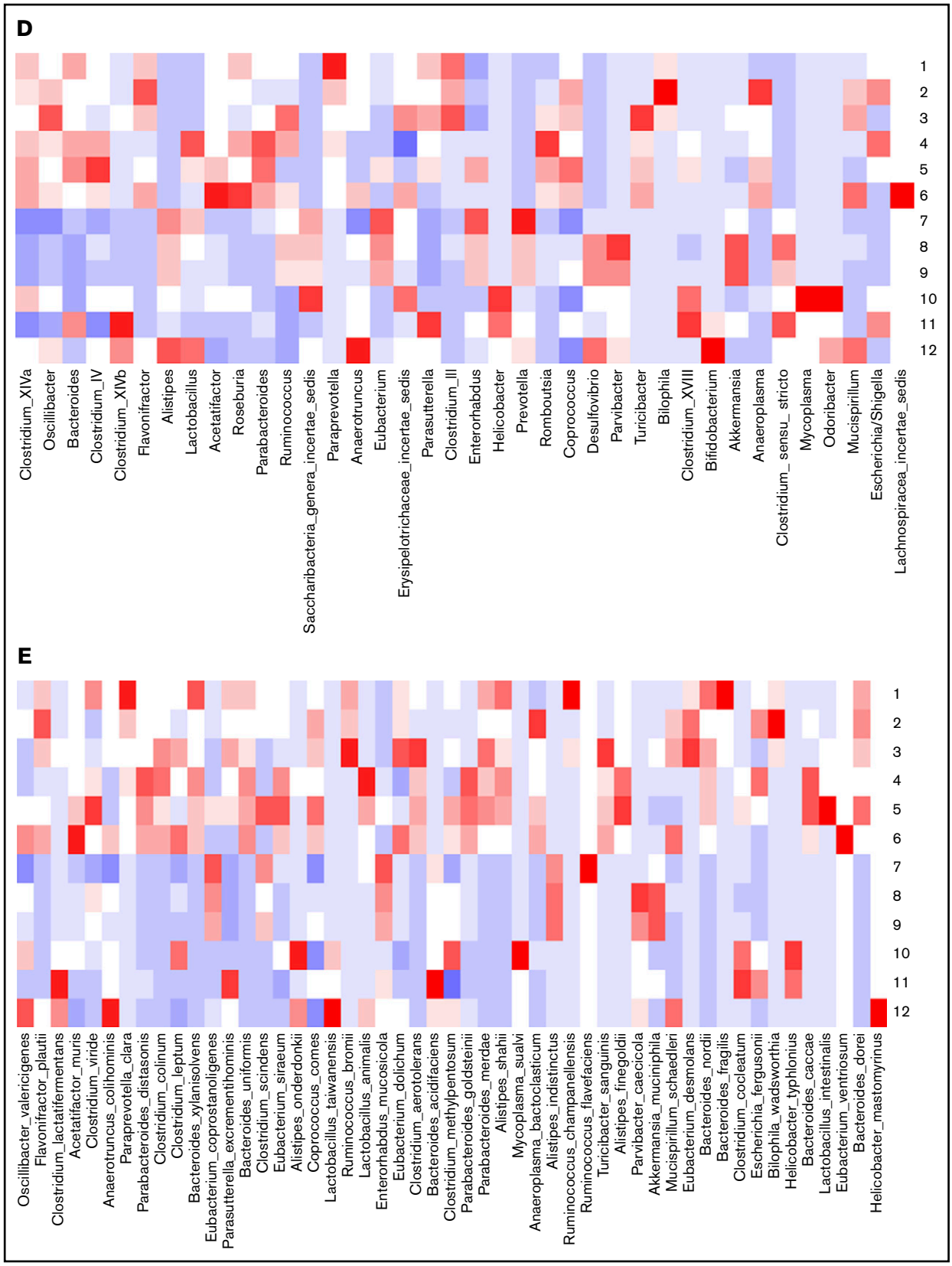


Figure 7. (Continued).



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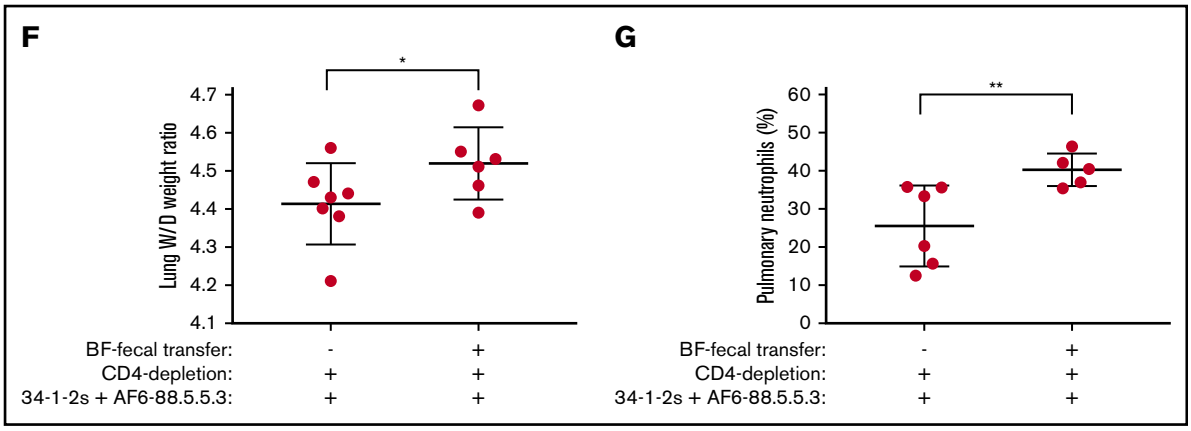

Figure 7. BF and SPF mice demonstrate a different gastrointestinal microbiome composition and priming with BF fecal matter restores TRALI-susceptibility in SPF mice. (A) Phylogenetic tree of BF mice, which are susceptible to TRALI (BF – TRALI); SPF mice, which do not suffer from TRALI (SPF – NO TRALI); and BF mice treated with antibiotics, which are also resistant to TRALI (BF – antibiotics NO TRALI). (B-C) Gastrointestinal bacterial heat maps and pie charts on family level, respectively. (D-E) Gastrointestinal bacterial heat maps on genus and species levels, respectively. (F-G) Data from BF fecal transfer into SPF mice: (A) lung W/D weight ratios, (B) pulmonary PMN numbers upon $CD4^+$ T-cell depletion and anti-MHC class I antibody (clones 34-1-2s and AF6-88.5.5.3) infusion into indicated mouse groups. Heat map row numbers 1-3, BF mice upon arrival; 4-6, BF mice after 1 week of housing; 7-9, SPF mice upon arrival; and 10-12, SPF mice after 1 week of housing. (F-G) Analyzed by a 1-tailed unpaired Student *t* test and represent combinatorial data from 2 experiments. Each dot represents 1 mouse; error bars represent SD. $*P < .05$, $**P < .01$.

TRALI response. It was previously shown that increased levels of MIP-2 and pulmonary PMN accumulation in mice are key early processes that are responsible for initiating antibody-mediated TRALI reactions.[6,10-13] This was underlined by experiments illustrating that mice were fully protected from antibody-mediated TRALI when their PMN were depleted in vivo.[12] We previously found that the anti-MHC class I antibody binding to its cognate antigen on blood monocytes is a required trigger for MIP-2 chemokine production during murine TRALI.[11] In line with this, we observed that GI flora depletion or SPF housing appears to block the secretion of the PMN-chemoattractant MIP-2 and subsequently inhibits PMN influx into the lungs (Figure 5), thereby preventing TRALI induction (Figures 2 and 3). Priming of the SPF mice with LPS, however, was able to significantly restore antibody-mediated TRALI (Figure 6). This may reflect that stimulatory signaling pathways may be different in an LPS setting vs a more complex gut microbial setting. LPS, for instance, stimulates Toll-like receptor-4 and induces a pathogenic release of proinflammatory cytokines activating immune responses.[37] In contrast, interaction of commensal bacteria with Toll-like receptors occurs under normal steady-state conditions, which plays a role in maintaining intestinal homeostasis.[29]

This suggests that the GI flora in untreated BF mice somehow enables pathogenic antibody-mediated TRALI responses; indeed, we observed a different GI microbiome composition in BF vs SPF mice when performing 16S ribosomal RNA gene amplicon sequencing (Figure 7A-E). Although a combination of specific gut bacteria is most likely to form the basis for TRALI susceptibility, we found *B caccae* to be at least 1 of the promising candidates because it was significantly increased in the TRALI-susceptible BF-housed mice but not present in TRALI-resistant SPF-housed mice (supplemental Figure 4). This bacterium has been suggested to have a pathogenic role in inflammatory bowel disease[38]; this may potentially also be the case in antibody-mediated TRALI, although further studies will be required to more directly investigate this. We did, however, prime TRALI-resistant SPF mice with fecal matter from TRALI-susceptible BF mice. The fecal transfer significantly restored the TRALI reaction, with increased levels of pulmonary edema and pulmonary PMN accumulation in SPF mice (Figure 7F-G). Notably, the degree of antibody-mediated acute lung injury was significant, but was not as severe as observed in the BF mice. This may be due to the already present gut microbes in the SPF mice, indicating a complex interplay between different gut microbiota.

The intestine is the most densely bacterially colonized surface in the human body (10^{14} bacteria),[39] and the lower respiratory tract is 1 of the least colonized surfaces in the human body (with 10-100 bacteria per 1000 human cells).[40] Both gut and lung compartments, nonetheless, are largely colonized by Bacteroidetes and Firmicutes[41-43] and it has been suggested that they are in constant communication with each other and that fluctuations in the abundance of bacterial diversity occurs in an identical manner in both compartments.[44] It is therefore plausible that the changes we now describe in gut flora composition (Figure 7) are identically present in the lung flora. Additionally, studies have reported correlations between lung microbiota and disease severity in murine LPS-induced lung injury[45] and in ARDS.[46] Unfortunately, we were not able to obtain sufficient amounts of DNA for murine lung microbiota characterization (supplemental Figure 5), therefore not allowing us to investigate if the lung microbiota may also contribute to the onset of murine TRALI.

Of interest, IL-8, the human equivalent of murine MIP-2, is a known risk factor for human TRALI[3-5]; pulmonary PMN accumulation has also been reported in autopsy reports of TRALI patients.[47] As we have previously shown, using the same established murine model of TRALI, PMN are key effector cells in inducing TRALI.[12] Our current data suggest that the gut microbiota influences the ability of PMN to migrate toward the lungs, at least via regulation of plasma MIP-2 levels, upon induction of antibody-mediated TRALI. Other studies have also shown the ability of gut microbiota to drive PMN migration toward sites of injury.[48,49] Interestingly, resident microbiota were shown to also modify the interaction of dendritic cells with T-regulatory

cells (both found to be major protective cells in TRALI[12]), which increased the susceptibility to inflammatory disorders such as colitis.[29,50] How this is exactly occurring in TRALI will need to be further dissected in subsequent studies. Additionally, treatment with antibiotics may perhaps be an efficient treatment strategy for human TRALI. Regarding risk assessment of TRALI development, patients treated with antibiotics before transfusion may perhaps be protected against TRALI. This may, for instance, apply to orthopedic surgery patients that are prophylactically treated with antibiotics because of high infection risk. For example, a previously described cohort of antibiotic-treated orthopedic surgery patients did not develop TRALI.[7] These results will, however, need to be further validated. On the other hand, it cannot be excluded that critically ill patients receiving antibiotics may still suffer from TRALI. Caution is advised, because antibiotics treatment may also skew the gut microbial balance in a manner that may still enable TRALI. It will therefore be important to further investigate this as well as to see if TRALI patients display alterations in their gut microbiota composition.

In summary, we have identified a previously unestablished link between the GI flora and the onset of antibody-mediated murine TRALI. Only BF mice were susceptible to TRALI because of their GI bacterial composition compared with SPF mice or antibiotic-treated BF mice. The depleted GI flora in these mice protected them from antibody-mediated TRALI induction and TRALI could be restored in SPF mice by pretreating the mice with a low dose of LPS. Sequencing of the gut microbiome confirmed a varying GI bacterial composition in BF vs SPF mice, and BF fecal transfer into SPF restored TRALI susceptibility. Additional studies are warranted to further dissect the communication link between the gut and the lungs during TRALI.

Acknowledgments

This work was supported by grants from Lund University, Crafoordska Stiftelsen (#20170829), Vetenskapsrådet (Swedish Research Council, VR, #2017-01779), Avtal om Läkarutbildning och Forskning, and the Health Canada and Canadian Blood Services (#340668) (J.W.S.). R.K. was the recipient of a young investigator award by the Royal Physiographic Society of Lund and was previously the recipient of a postdoctoral fellowship from Canadian Blood Services.

Authorship

Contribution: R.K. designed all research, performed experiments, collected data, analyzed and interpreted data, performed statistical analyses, made the figures, and wrote and edited the paper; M.K., J.R., A.T.-F., N.K., S.S., S.M., M.J.M., and E.R.S. performed experiments and collected data; B.H. and J.T.B. performed 16S rRNA microbial profiling; and J.W.S. provided financial resources and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: R.K., 0000-0002-1608-876X; J.R., 0000-0003-1673-0684; J.W.S., 0000-0002-1510-0077.

Correspondence: John W. Semple, Lund University, BMC C14, Klinikgatan 26, 221 84 Lund, Sweden; e-mail: john_w.semple@med.lu.se.

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