Supplementary file

THE GUT MICROBIOTA PLAYS A PROTECTIVE ROLE IN THE HOST DEFENSE AGAINST PNEUMOCOCCAL PNEUMONIA

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MATERIAL AND METHODS

Experimental infection and determination of bacterial growth

Pneumonia was induced by intranasal inoculation with 1 x 10^6 colony forming units (CFU) *S. pneumoniae* D39 serotype in 50 µl isotonic saline using previously described methods[1-3]. Mice were euthanized 6, 24 or 48 h after induction of pneumonia (n=8 mice per group at each time point in each experiment) or observed for 12 days (n=20 per group). Blood was obtained from the inferior vena cava. Lung, spleen and liver were harvested and homogenized in five volumes of sterile isotonic saline. The left lung was fixed in 10% buffered formalin and embedded in paraffin. For bacterial quantification blood and organ homogenates were serially diluted by 10-fold in sterile isotonic saline and plated onto sheep-blood agar plates. Following 16 h of incubation at 37°C CFUs were counted. For cytokine measurements, homogenates were diluted 1:1 with lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% (v/v) Triton X-100, pH 7.4) with protease inhibitor mix (Complete protease inhibitor cocktail tablets, Roche) and incubated for 30 min on ice, followed by centrifugation at 680 g for 10 min. Supernatants were stored at -20°C until analysis.

Assays

TNF-α, IL-1β, IL-6, IL-10 and CXCL-1 were measured by ELISA or cytometric bead array (CBA) multiplex assay (BD Biosciences) in accordance with the manufacturers' recommendations. Lactate dehydrogenase (LDH), aspartate aminotranspherase (ASAT), alanine aminotranspherase (ALAT), urea and creatinine were determined with commercial available kits (Sigma-Aldrich), using a Hitachi analyzer (Boehringer) according to the

manufacturers' instructions. Protein content of BALF from naïve (control or Abx treated) mice was measured using a BCA protein assay (Merck Millipore) according to the manufacturer's instructions.

Pathology and immunohistochemistry

Hematoxylin and eosin (H&E) stained paraffin sections from lung, spleen and liver tissue, collected 6, 24 and 48 h after *S. pneumoniae* infection were scored as described[4,5]. The parameters for bronchitis, edema, interstitial inflammation, intraalveolar inflammation, pleuritis, and endothelialitis were by a blinded specialized pathologist graded on a scale of 0 to 4, with 0 as "absent" and 4 as "severe." The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 24. Liver and spleen sections were scored on inflammation, necrosis/abscess formation, and thrombus formation using the scale given above with a maximum score of 12. Granulocyte staining was performed using fluorescein isothiocyanate–labeled rat anti-mouse Ly-6G mAb (Pharmingen) as described[4]. Immunohistochemistry for intestinal tissue was performed on paraffin-embedded sections as described[6]. Primary antibodies, anti-Ki67 (1:1000, Abcam), mucin2 H300 (1:500, Santa Cruz), anti-CHGA (1:100, Santa Cruz) and anti-beta-catenin (1:500, Abcam) were diluted in PBS containing 1% BSA and incubated on slides overnight at 4°C. The day after, slides were washed three times in PBS and incubated with the appropriate HRP-conjugated secondary antibodies (1:2, powervision) for 45 min at room temperature; staining was developed using DAB+ Chromogen (Dako).

Microbiota analysis

Data analyses were performed using the microbiome R-script package (R-package "microbiome"; URL:https://github.com/microbiome/microbiome/wiki/Installation#install-microbiome-package). Multivariate statistics were performed as implemented in CANOCO 5.0 (Microcomputer Power).

RNA preparation and microarray profiling

RNA was isolated from alveolar macrophage homogenates using the RNeasy mini kit (Qiagen). RNA integrity (RIN>7) was assessed by bioanalyzer (Agilent). Biotinylated cRNA was hybridized onto the Illumina MouseRef-8v2 Expression BeadChip. Samples were scanned using Illumina iScan array scanner. Preparation of cRNA, chip hybridization, washing, staining and scanning were carried out at ServiceXS. The raw scan data were read using the *beadarray* package (version 1.12.1)[7], available through Bioconductor[8] in the R statistical environment (version 2.13.2; R Foundation for Statistical Computing). All non-normalized and *neqc* normalized[9] data are available at the gene expression omnibus of NCBI (GEO) with accession number GSE53174. Differential gene expression analysis was performed by means of the *limma* package (version 3.8.3), which implements linear models for microarray data[10]. p-values were obtained from moderated t statistics, which were then adjusted for multiple comparisons with Benjamini and Hochberg's method to control the false discovery rate. Bioinformatics analysis was performed on differentially expressed genes as defined by multiple-test corrected $P < 0.05$ by means of the Ingenuity Pathway Analysis software (IPA, www.ingenuity.com). These analyses were performed using the IPA gene-only knowledgebase as reference set for p-value calculations and specifying "mouse" as species. All other parameters were maintained as default.

Phagocytosis

Cells were mobilized, washed and plated in duplo in 96-well plates at a density of $1x10^5$ cells/well. A working solution with a multiplicity of infection (MOI) of 1:100 of growth arrested, CFSE-labeled *S. pneumoniae* D39 was added to the wells. Plates were centrifuged at 2000 RPM for 5 min at 4 °C and samples were incubated at 37°C or 4°C (control) for 10 min or 60 min. Cells were washed with cold RPMI medium and resuspended in FACS-buffer for FACS analysis on a FACSCalibur flow cytometer (Beckton Dickinson). The phagocytosis index of each sample was calculated as mean fluorescence intensity (MFI)×percentage (%) positive cells) at 37°C minus (MFI×% positive cells) at 4°C as described[11].

Stimulation

Cells were mobilized, washed and plated in duplo in a 96-well flat-bottom plate at a density of $1x10^5$ cells/well. The cells were incubated overnight at 37°C and 5% humidified CO2 in the incubator to allow the macrophages to adhere. Working solutions were added to the cells containing 100 ng/ml LPS (*Escherichia coli*, Invitrogen), 10 µg/ml LTA (*Staphylococcus aureus*, Invivogen), or *S. pneumoniae* D39 with a MOI of 1:50 for 18 hours at 37°C. Supernatants were analyzed for cytokine release.

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FIGURES

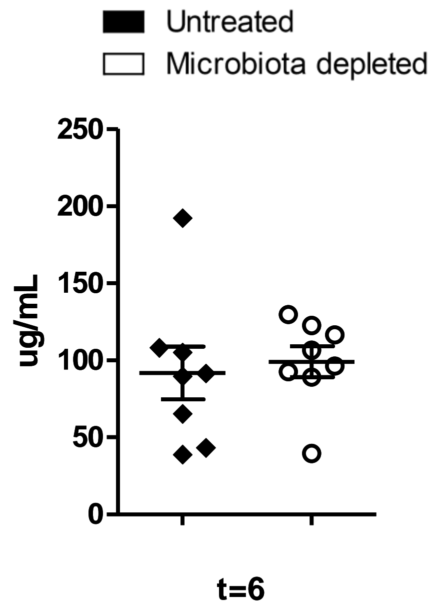

Figure S1. Influence of gut microbiota depletion on lung protein content. Protein content of bronchoalveolar lavage fluid (BALF) from naïve untreated control (black) and microbiota depleted (white) mice using a BCA protein assay. Group size is 8 per group; results are shown as means ± s.e.m;

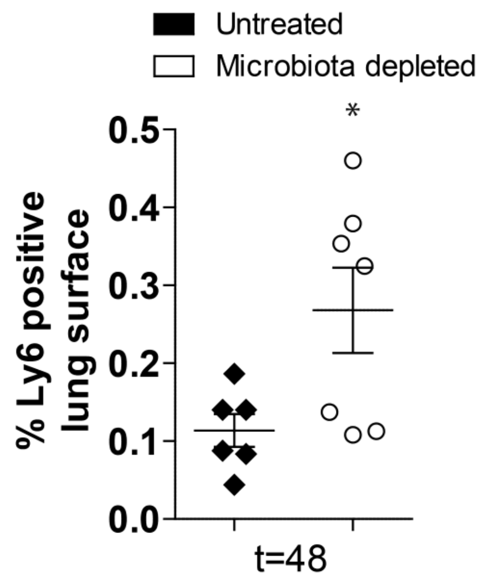

Figure S2. Increased pulmonary neutrophil influx in gut microbiota depleted mice during pneumococcal pneumonia. Ly6G-immunostaining for granulocytes in lung tissue 48 h after intranasal *S. pneumoniae* infection in untreated (black) and microbiota depleted (white) mice. Group size is 8 per group; results are shown as means ± s.e.m; * $P < 0.05$.

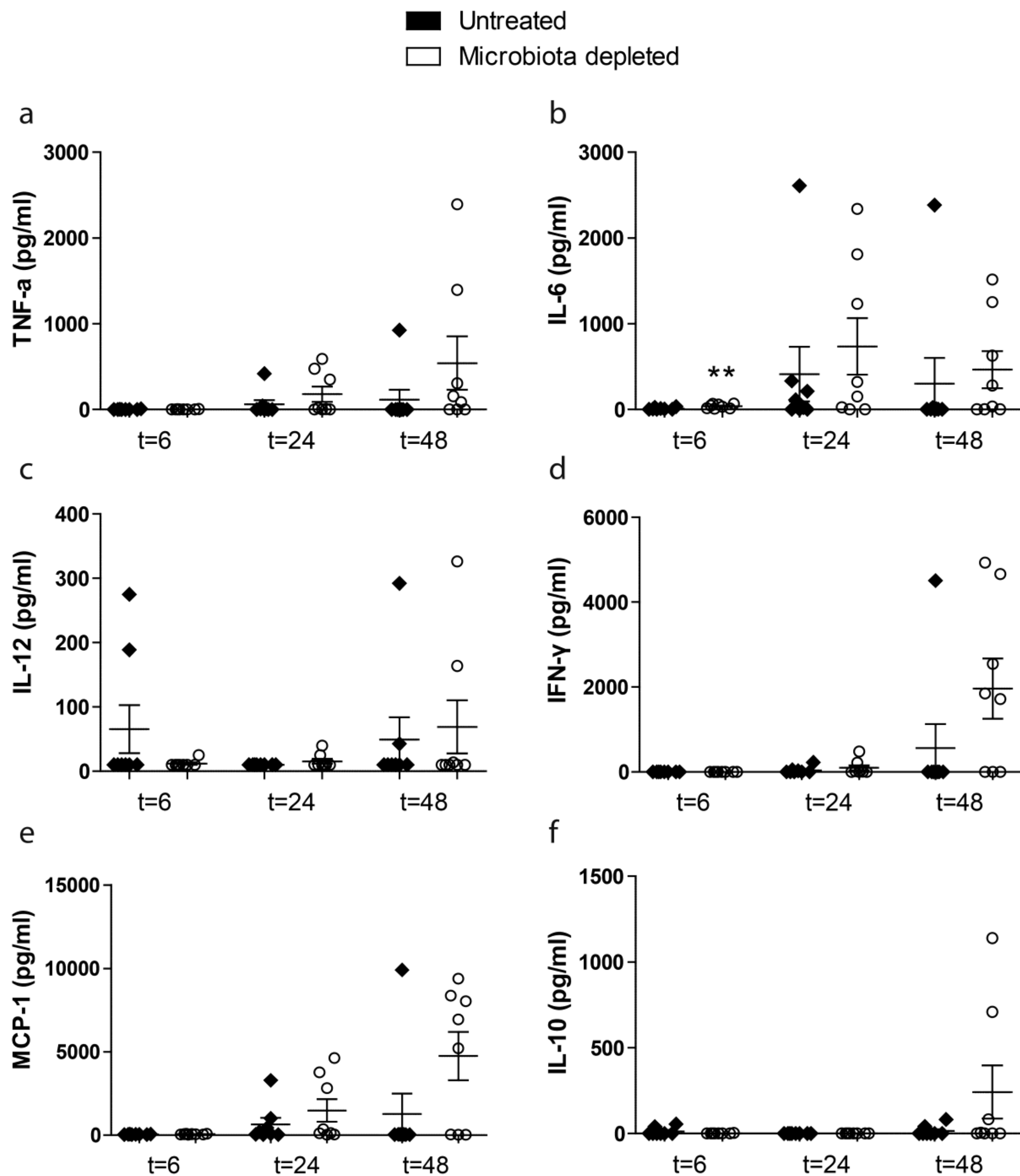

Figure S3. Influence of gut microbiota on plasma cytokine levels during pneumococcal pneumonia. *(A)* TNF-α, *(B)* IL-6, *(C)* IL-12, *(D)* IFN-γ, *(E)* MCP-1 and *(F)* IL-10 levels in plasma 6, 24 and 48 h after intranasal *S. pneumoniae* infection in untreated (black) and microbiota depleted (white) mice. Group size is 8 per group; results are shown as means ± s.e.m.

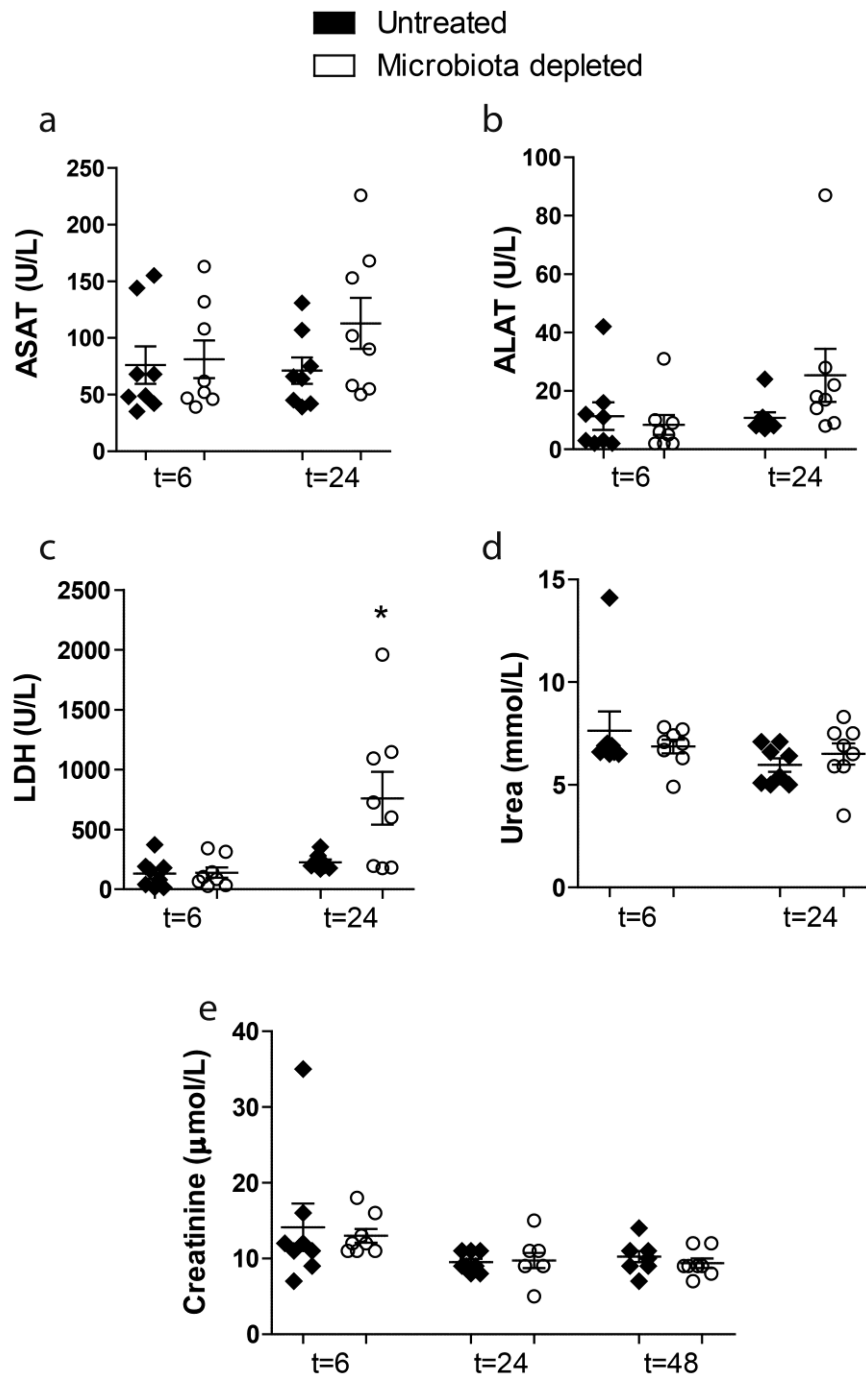

Figure S4. Influence of gut microbiota on markers of organ injury during the early time points of pneumococcal pneumonia. *(A)* Systemic levels of aspartate aminotransferase (AST), *(B)* alanine aminotransferase (ALT), *(C)* lactate dehydrogenase (LDH), *(D)* urea and *(E)* creatinine 6 and 24 h after intranasal *S. pneumoniae* infection in untreated (black) and microbiota depleted (white) mice. Group size is 8 per group; results are shown as means ± s.e.m; * $P < 0.05$.