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DISSERTATION

Untersuchungen zum Wechselspiel von multi-resistenten *Pseudomonas aeruginosa*, kommensaler Darmmikrobiota und Immunantworten bei muriner Darmentzündung

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1 Zusammenfassung

1.1 Abstrakt

Die weltweite Zunahme von nosokomialen Infektionen mit multiresistenten (multidrug resistant; MDR) Gram-negativen Bakterien wie *Pseudomonas* (*P.*) *aeruginosa* ist eine Bedrohung vor allem für immunsupprimierte, hospitalisierte und chronisch pulmonal erkrankte Patienten. Obwohl der Gastrointestinaltrakt (GIT) eine mögliche Infektionsquelle darstellt, ist wenig darüber bekannt unter welchen Bedingungen es zu einer Kolonisierung des Darms mit MDR *P. aeruginosa* kommt, wie sich diese auf die intestinale, extra-intestinale und systemische Immunantwort des Wirtes auswirkt und welche Rolle eine Darmentzündung dabei spielt. In der vorliegenden Arbeit führten wir Kolonisationsversuche an konventionell kolonisierten sowie sekundär abiotischen Mäusen durch, die zeigten, dass durch die Eliminierung der konventionellen Darmmikrobiota mittels oraler Breitspektrumantibiose, die natürliche Kolonisationsresistenz gegenüber *P. aeruginosa* aufgehoben wird. Die Rekonstitution sekundär abiotischer Mäuse mit humaner oder muriner Darmmikrobiota mittels fäkaler Mikrobiota-Transplantation (FMT) führte nur zu einem partiellen Schutz vor Kolonisierung. Die orale Gabe von *P. aeruginosa* führte sowohl in sekundär abiotischen als auch in mit humaner oder muriner Darmmikrobiota rekonstituierten Mäusen zu intestinalen Immunantworten, die sich in einem Einstrom von Immunzellen wie Makrophagen und Monozyten in die Darmmukosa sowie erhöhten Konzentrationen pro-inflammatorischer Zytokine, wie dem Tumornekrosefaktor (TNF) äußerten. Die gesteigerte Zytokinausschüttung war jedoch nicht nur begrenzt auf den GIT, sondern konnte auch auf systemischer Ebene in der Milz beobachtet werden. Weiterhin konnten wir in Mäusen mit akuter *Toxoplasma* (*T.*) *gondii* induzierter Ileitis sowie IL-10 Knockout (IL-10$^{-/-}$) Mäusen mit chronischer Kolitis zeigen, dass eine Darmentzündung Mäuse empfänglich für eine *P. aeruginosa* Kolonisierung macht. Während wir im akuten Inflammationsmodell keine Verschlimmerung der Darmentzündung nach *T. gondii* Infektion nachweisen konnten, führte die mehrwöchige Darmkolonisierung mit *P. aeruginosa* im chronischen Inflammationsmodell zu einer Exazerbation der chronischen Kolitis, die sich durch einen gesteigerten Einstrom intestinaler Immunzellen und erhöhte Konzentrationen pro-inflammatorischer Zytokine wie TNF und Interferon-γ (IFN-γ) in den mesenterischen Lymphknoten (MLN) und im Serum äußerte. Zusammenfassend konnten wir zeigen, dass die Darmmikrobiota maßgeblich zur Kolonisationsresistenz

gegenüber *P. aeruginosa* beiträgt und dass deren Störung zu einer intestinalen Kolonisierung mit dem opportunistischen Pathogen führen kann. Weiterhin wurde deutlich, dass eine reine intestinale Kolonisierung mit *P. aeruginosa* zu einer pro-inflammatorischen Immunantwort im Wirtsorganismus führt. Darüber hinaus fördert eine Darmentzündung die Kolonisierung mit *P. aeruginosa*, welche wiederum zu einer Verschlechterung des zugrundeliegenden Krankheitsbildes führt.

Abstract

The worldwide growing incidence of nosocomial infections with multidrug resistant (MDR) Gram-negative bacteria such as *Pseudomonas* (*P.*) *aeruginosa* is a threat especially to immunocompromised or hospitalized patients and patients suffering from chronic pulmonary diseases. Although the gastrointestinal tract (GIT) is a potential source for infections, only little is known about conditions leading to intestinal colonization with MDR *P. aeruginosa*, how the latter affects the intestinal, extra-intestinal and systemic immune response of the host and the impact intestinal inflammation has hereon. In the present work colonization experiments in conventionally colonized as well as secondary abiotic mice revealed that elimination of the conventional microbiota through oral treatment with broad-spectrum antibiotics abrogates the natural colonization resistance directed against *P. aeruginosa*. Reconstitution of secondary abiotic mice with either human or murine microbiota through fecal microbiota-transplantation (FMT) only partially protected from colonization. Intestinal carriage of *P. aeruginosa* led to intestinal immune responses not only in secondary abiotic mice but also in mice reconstituted with human or murine microbiota, as indicated by increased numbers of intestinal immune cells such as macrophages and monocytes as well as elevated concentrations of pro-inflammatory cytokines such as tumor necrosis factor (TNF). Pro-inflammatory cytokine responses were not limited to the GIT, but could also be observed systemically (i. e. in the spleen). Furthermore, experiments in mice with acute *Toxoplasma* (*T.*) *gondii* induced ileitis and IL-10 Knockout (IL-$10^{-/-}$) mice with chronic colitis revealed that intestinal inflammation renders mice more susceptible for *P. aeruginosa* carriage. No aggravation of the pronounced immune response could be observed in mice with acute *T. gondii* induced ileitis. Conversely, the intestinal colonization with *P. aeruginosa* in the chronic inflammation model resulted in a distinct exacerbation of chronic colitis, as indicated by an increased influx of intestinal immune cells and elevated concentrations of pro-inflammatory

cytokines such as TNF and interferon-γ (IFN-γ) in the mesenteric lymph nodes (MLN) and serum. Taken together, our studies underline the important impact of the intestinal microbiota on the colonization resistance against *P. aeruginosa* and that the perturbation of the complex intestinal ecosystem may lead to intestinal colonization with the opportunistic pathogen. Furthermore, it became clear that mere intestinal carriage of *P. aeruginosa* results in pro-inflammatory immune responses of the host. Moreover, intestinal inflammation renders mice more susceptible to intestinal *P. aeruginosa* colonization which deteriorates chronic colitis.

1.2 Einführung

Pseudomonas (*P.*) *aeruginosa* ist ein Gram-negatives, obligat aerobes und opportunistisches Pathogen, welches vor allem bei hospitalisierten und immunsupprimierten Patienten zu einer Vielzahl von Infektionen führen kann, welche mit einer hohen Mortalität einhergehen (1). So ist *P. aeruginosa* eine häufige Ursache von nosokomialen Infektionen, wie beatmungsassoziierten Pneumonien, Wundinfektionen, Harnwegsinfektionen oder Septikämien (2, 3). Weiterhin kommt es bei Patienten mit pulmonalen Grunderkrankungen, wie der zystischen Fibrose, häufig zu einer chronischen *P. aeruginosa* Infektion, die über die Jahre erheblich zum Verlust der Lungenfunktion beiträgt (1, 4). Die globale Bedrohung durch *P. aeruginosa* wächst stetig infolge der Zunahme an Stämmen, die aufgrund von Extended-Spektrum-β-Laktamasen, Carbapenemasen und 16S rRNA-Methylasen Resistenzen gegenüber vielen Antibiotikaklassen aufweisen (2, 5, 6).

Bereits sehr frühe Studien beschäftigten sich mit dem Vorkommen des opportunistischen Pathogens im Gastrointestinaltrakt (GIT) sowohl in der Normalbevölkerung als auch bei hospitalisierten Patienten (7-14). So konnten Shooter et al. *P. aeruginosa* aus dem Stuhl von 24% chirurgischer Patienten bereits bei ihrer stationären Aufnahme isolieren, wobei der Anteil von intestinalen *P. aeruginosa*-Trägern im Verlauf des stationären Aufenthaltes weiter anstieg (8). Auch bei gesunden Probanden konnte *P. aeruginosa* nach Inokulation für bis zu sechs Tage im Stuhl nachgewiesen werden (10). Dabei ist der GIT eine wichtige *P. aeruginosa*-Infektionsquelle für den Respirationstrakt (13-16). Weiterhin gibt es Studien, die einen Zusammenhang zwischen der Pathogenese chronisch entzündlicher Darmerkrankungen, wie Morbus Crohn und Pseudomonasspezies, wie *P. fluoreszenz* oder *P. maltophilia* aufzeigen (17-20). So besteht eine Assoziation zu Antikörpern

gegen spezifische bakterielle Antigene, wie dem von *P. fluoreszenz* gebildeten „Crohn's disease-related bacterial sequence (I2)" (18, 21, 22). In Dünndarmgewebeproben von an Morbus Crohn erkrankten Kindern konnten mittels molekulargenetischer Analysen im Vergleich zu Gesunden vermehrt Pseudomonasspezies nachgewiesen werden. *P. aeruginosa* fand sich jedoch nur im Dünndarm gesunder Kinder (23). In Patienten mit chronischem Reizdarmsyndrom hingegen fanden sich signifikant erhöhte *P. aeruginosa*-Lasten in Biopsien aus Duodenalschleimhaut und Stuhlproben (24). Weiterhin wurde in einem Fallbericht ein hoher Anteil pro-inflammatorischer Bakterienspezies in Kolonbiopsien eines pädiatrischen Colitis Ulzerosa Patienten beschrieben. Unter den hierbei analysierten *γ-Proteobacteria* konnte auch ein wesentlicher Anteil an 16S rRNA von *P. aeruginosa* nachgewiesen werden (25).
Die Weltgesundheitsorganisation (WHO) erklärte erst kürzlich, dass *P. aeruginosa* zusammen mit anderen multiresistenten (multidrug resistant; MDR) Gram-negativen Bakterien eine erhebliche Bedrohung der menschlichen Gesundheit darstellt und unterstrich die Wichtigkeit neuer Therapiemöglichkeiten (26). Allerdings ist bis heute wenig über das Wechselspiel von MDR *P. aeruginosa*, kommensaler Darmmikrobiota und Immunantworten bei muriner Darmentzündung bekannt.

1.3 Zielsetzung

Das Ziel der vorliegenden Arbeit war es zu untersuchen, unter welchen Bedingungen es zu einer stabilen Kolonisierung des GIT mit MDR *P. aeruginosa* kommt, welche Rolle dabei die komplexe murine und humane Darmmikrobiota sowohl im gesunden als auch im entzündeten Mäusedarm spielt und wie sich eine antibiotische Therapie auf das Kolonisationsverhalten des opportunistischen Pathogens auswirkt. Darüber hinaus beschäftigten wir uns mit der Frage, welchen Einfluss die Kolonisierung des Darms mit *P. aeruginosa* auf intestinale, extra-intestinale und systemische Immunantworten hat. Dafür verwendeten wir neben gesunden, konventionell mit muriner Darmmikrobiota kolonisierten Mäusen auch sekundär abiotische Mäuse, deren Mikrobiota durch eine orale Breitspektrumantibiose eliminiert wurde. Weiterhin führten wir Untersuchungen an Mäusen durch, die nach fäkaler Mikrobiota-Transplantation (FMT) mit humaner Darmmikrobiota besiedelt waren und arbeiteten mit Mausmodellen für akute und chronische Darmentzündungen.

1.4 Methodik

1.4.1 Ethische Erklärung

Sämtliche Tierversuche wurden entsprechend des europäischen Tierschutzgesetzes (2010/63/EU) mit Zustimmung der Tierversuchskommission (Landesamt für Gesundheit und Soziales, LaGeSo, Berlin; Registrierungsnummer G0097/12 und G0039/15), durchgeführt. Das Wohlergehen der Tiere wurde täglich mittels Erfassung des klinisches Status und der Gewichtsentwicklung überwacht. Bei einem Gewichtsverlust von >20% erfolgte die Tötung mittels Isofluran-Inhalation (Abott, Deutschland).

1.4.2 Versuchstiere und Tierhaltung

Für die vorliegende Arbeit wurden C57BL/6j Wildtyp (WT) und IL-10 Knockout (IL-$10^{-/-}$) Mäuse verwendet, welche den Räumlichkeiten der Forschungseinrichtung für Experimentelle Medizin der Charité Universitätsmedizin entstammten und unter spezifisch-pathogen freien (SPF) Bedingungen gezüchtet wurden. Sekundär abiotische Mäuse mit nahezu kompletter Eliminierung der Darmmikrobiota wurden erzeugt (27): So erhielten acht Wochen alte, in sterilen Käfigen gehaltene Mäuse für 8 bis 10 Wochen eine dem Trinkwasser zugeführte Breitspektrumantibiose (*ad libidum*), bestehend aus Ampicillin/ Sulbactam (1 g/l; Ratiopharm, Deutschland), Ciprofloxacin (200 mg/l; Bayer Vital, Deutschland), Vancomycin (500 mg/l; Cell Pharm, Deutschland), Metronidazol (1 g/l; Fresenius, Deutschland) und Imipenem/ Cilastatin (250 mg/l; MSD, Deutschland). Wenn in Thioglycolat-Boullion (Oxoid, Deutschland) bebrüteten Fäzesproben in drei aufeinanderfolgenden Wochen keine kultivierbaren Bakterien mehr nachweisbar waren, galt die Maus als sekundär abiotisch.

1.4.3 Generierung von mit humaner und muriner Mikrobiota assoziierten Mäusen mittels fäkaler Mikrobiota-Transplantation

Für die Generierung von mit humaner Mikrobiota assoziierten (hma) Mäusen wurden frische humane Stuhlproben von fünf gesunden Probanden in steriler phosphat-gepufferter Salzlösung (phosphate buffered saline, PBS, Gibco, Life Technologies, GB) aufgelöst, aliquotiert und bei -80℃ aufbewahrt (28). Unmittelbar vor FMT wurden die Proben aufgetaut und zusammengeführt. Anschließend wurden 0.3 ml der Stuhlsuspension sekundär abiotischen Mäusen an zwei aufeinanderfolgenden Tagen per oraler Gavage zugeführt. Drei Tage vor FMT war die Breitspektrumantibiose durch steriles Leitungswasser ersetzt worden. Die wichtigsten Bakteriengruppen der

Darmmikrobiota wurden mittels Kultur und 16S rRNA basierter quantitativer real-time Polymerase-Kettenreaktion (qRT-PCR) in der Stuhlsuspension quantifiziert (28, 29). Die Gesamtbakterienlast in den einzelnen Experimenten betrug ca. 10^{10} Kolonie-bildende Einheiten (KBE) und variierte um weniger als 0.5 logarithmische Einheiten. Nach Etablierung der transplantierten Mikrobiota drei Wochen nach FMT quantifizierten wir die wichtigsten Bakteriengruppen der Darmmikrobiota mittels Kultur und 16S rRNA basierter qRT-PCR in individuellen Fäzesproben der Mäuse (30). Die Generierung von mit muriner Mikrobiota assoziierten (mma) Mäusen erfolgte entsprechend. So wurde eine Suspension aus Fäzesproben von 10 gesunden SPF-Kontrollmäusen in 10 ml steriler PBS Lösung hergestellt und direkt für die orale FMT verwendet (31, 32).

1.4.4 Induktion der akuten Ileitis

Zur Induktion einer akuten Ileitis wurden die Mäuse peroral mit 50 *Toxoplasma* (*T.*) *gondii* (ME49 Stamm) Zysten infiziert (27, 30, 33-35).

1.4.5 Infektion mit *Pseudomonas aeruginosa* und *Escherichia coli*

Die Mäuse wurden mit 10^9 KBE des MDR *P. aeruginosa* oder *Escherichia* (*E.*) *coli* in 0.3 ml PBS per oraler Gavage an zwei konsekutiven Tagen infiziert (30, 31, 36, 37). Das *P. aeruginosa*-Isolat wurde ursprünglich aus Respirationstraktsekret eines Patienten mit nosokomialer Pneumonie isoliert und uns freundlicherweise von Prof. Dr. Bastian Opitz (Charité-Universitätsmedizin Berlin) zur Verfügung gestellt. Es zeigte lediglich antimikrobielle Sensibilität gegenüber Fosfomycin und Colistin (30, 31, 36, 37). Das *E. coli*-Isolat entstammt dem Fäzes einer konventionellen C57BL/6j Maus und enthielt keine der folgenden Virulenzfaktor-kodierenden Gene wie z.B. stx 1 und 2, catA, hlyA oder cspA (27, 38).

1.4.6 Kulturelle Analyse der Darmmikrobiota und bakteriellen Translokation

Sowohl die Zusammensetzung der Darmmikrobiota als auch die in extra-intestinale Organsysteme translozierenden Bakterien wurden mittels kultureller Analyse ermittelt. Zu definierten Zeitpunkten entnommene Fäzesproben, luminale Darminhalte und *ex vivo* Gewebeproben von mesenterischen Lymphknoten (MLN), Milz, Leber, Niere und Lunge wurden in sterilem PBS homogenisiert und in Verdünnungsreihen auf verschiedenen Nährmedien ausgestrichen und für mindestens 48 Stunden bei 37 ℃ in aerober und anaerober Atmosphäre inkubiert (27, 30, 31, 33, 36, 37, 39). Für die Bestimmung der *P. aeruginosa*-Lasten wurde zusätzlich Cetrimid Agar

(Oxoid, Deutschland) verwendet. Zudem wurde steril entnommenes Herzblut für eine Woche in Thioglycolat-Bouillon bei 37℃ inkubiert und anschließend auf solide Nährmedien ausgestrichen, welche mindestens 48 weitere Stunden unter aerober und anaerober Atmosphäre inkubiert wurden (30, 31, 36, 37).

1.4.7 Erfassen des klinischen Zustandes

Sowohl Körpergewicht als auch das makroskopische oder mikroskopische Auftreten von fäkalem Blut wurden mittels der Guajac Methode (Haemoccult, Beckman Coulter/ PCD, Deutschland) täglich erfasst (30, 31, 36, 37).

1.4.8 Probengewinnung

Die Mäuse wurden zu definierten Zeitpunkten mittels Isofluran-Inhalation getötet. Herzblut, Gewebeproben von MLN, Milz, Leber, Niere und Lunge sowie luminale Inhalte von Magen, Duodenum, Ileum und Kolon wurden unter sterilen Bedingungen entnommen. Gewebeproben von Ileum und Kolon wurden für immunologische, immunhistochemische und histopathologische Analysen verwendet (30, 31, 36, 37).

1.4.9 Dünndarmlänge und histopathologische Bewertung

Zu Erfassung der Dünndarmlänge wurde der Abstand zwischen Magenausgang und ileo-caecalem Übergang gemessen (30, 31, 36, 37). *Ex vivo* Gewebeproben von terminalem Ileum und Kolon wurden unverzüglich in 5%-igem Formalin fixiert und in Paraffin eingebettet. Mittels Hämatoxylin und Eosin (H&E) wurden 5 µm dicke Schnitte gefärbt (30, 31, 36, 37). Die histopathologische Bewertung der Schnitte erfolgte nach einem standardisierten, histopathologischen Score (27, 33).

1.4.10 Molekulare Analyse der Mikrobiota von Ileum und Kolon

DNA wurde aus Fäzesproben und luminalen Darminhalten zu definierten Zeitpunkten extrahiert und mittels qRT-PCR analysiert (27, 28, 40-42).

1.4.11 Zytokinmessungen in Ileum, Kolon, MLN, Milz, Leber, Niere und Serum

Zytokinkonzentrationen in Serumproben und kulturellen Überständen von Ileum, Kolon, MLN, Milz, Leber und Niere wurden quantifiziert (30, 31, 36, 37): *Ex vivo* Gewebeproben mit einer Größe von ca. 1 cm^2 (Ileum und Kolon longitudinal aufgeschnitten) wurden nach Spülung mit PBS für 18 Stunden bei 37℃ in 24-well-Platten (Falcon, Deutschland) mit jeweils 500 µl serumfreien RPMI-1640 Medium (Gibco, life technologies) mit Penicillin (100 U/ml, Biochrom, Deutschland) und

Streptomycin (100 µg/ml, Biochrom, Deutschland) kultiviert. Mit dem „Mouse Inflammation Cytometric Bead Assay" (CBA, BD Bioscience) und dem FACS Canto II Durchflusszytometer wurden die Konzentrationen von Tumornekrosefaktor (TNF), Interferon-γ (IFN-γ), Monozyten chemotaktischen Protein (MCP), Interleukin (IL)-6, IL-10 und IL-12p70 in den kulturellen Überständen und Serumproben bestimmt. Stickstoffmonooxid (NO) wurde mittels Grieß-Test analysiert (27, 30).

1.4.12 Immunhistochemie

In situ immunhistochemische Analysen von Ileum- und Kolongewebeproben wurden von 5 µm dünnen Paraffin Schnitten durchgeführt (38, 39, 43, 44): Hierfür wurden spezifische Antikörper gegen CD3 für T-Lymphozyten (#IR50361-2, Dako, USA; 1:5), FOXP3 für regulatorische T-Zellen (Tregs) (clone FJK-165, #14-5773, eBioscience, Deutschland; 1:100), B220 für B-Lymphozyten (#14-0452-81, eBioscience, Deutschland; 1:200), Caspase-3 für apoptotische Zellen (Asp175, #9661, Cell Signaling, Niederlande; 1:200), Ki67 für proliferative Zellen (clone 16A8, #652401, BioLegend/Bizol, Deutschland; 1:200) und F4/80 für Monozyten/ Makrophagen (clone BM8, #MF48015, Life Technologies, Deutschland; 1:00) verwendet. Ein geblindeter Untersucher bestimmte die durchschnittliche Anzahl positiv gefärbter Zellen in mindestens sechs hochauflösenden Feldern (High Power Fields, HPF; 0.287 mm^2; 400-fache Vergrößerung) (30, 31, 36, 37).

1.4.13 Statistische Analysen

Es wurden Mittelwerte, Mediane und Standartabweichungen von erhobenen Daten ermittelt sowie Unterschiede zwischen Stichproben auf ihre Signifikanz hin getestet (doppelter t-Test und Mann-Whitney-U-Test bzw. Varianzanalysen mittels one-way ANOVA und Kruskall-Wallis-Test). Wahrscheinlichkeits- (*p*-) Werte ≤0.05 wurden als statistisch signifikant gewertet. Zur Berechnung und graphischen Darstellung wurde das Programm GraphPad Prism Version 6 und 7 (GraphPad Software, USA) verwendet.

1.5 Ergebnisse

Die wichtigsten Ergebnisse werden aus vier Publikationen (Publikation 1, 2, 3 und 4) zum Wechselspiel zwischen multi-resistenten *P. aeruginosa*, kommensaler Darmmikrobiota und Immunantworten bei muriner Darmentzündung anhand verschiedener Mausmodelle zusammengefasst. Die bibliographischen Angaben zu den Publikationen sind dem Kapitel 3 „Druckexemplare der ausgewählten Publikationen" zu entnehmen. Hinsichtlich der graphischen Darstellung der Ergebnisse wird auf die Abbildungsnummer (Abb.) in der jeweiligen Originalpublikation verwiesen.

1.5.1 Einfluss der komplexen Darmmikrobiota auf die intestinale Kolonisationskapazität von MDR *Pseudomonas aeruginosa*

Um die Kolonisationskapazität von MDR *P. aeruginosa* im murinen GIT zu untersuchen, begannen wir mit Kolonisierungsversuchen an C57BL/6j WT Mäusen mit konventioneller Darmmikrobiota. An zwei konsekutiven Tagen wurden die Mäuse mittels peroraler Gavage mit MDR *P. aeruginosa* infiziert. Kulturelle Analysen der fäkalen Proben zeigten, dass *P. aeruginosa* bereits an Tag 4 (d4) nach Infektion nur noch in 15.8% der Fälle und zu späteren Zeitpunkten (bis d28) nur noch in vereinzelten Mäusen mit konventioneller muriner Mikrobiota nachweisbar war (**Publikation 1, Abb. 1 A**). Konventionell besiedelte Mäuse weisen somit eine natürliche Kolonisationsresistenz gegenüber MDR *P. aeruginosa* auf. Anschließende Kolonisierungsversuche an sekundär abiotischen Mäusen, d. h. an Mäusen, deren Darmmikrobiota mittels 8 bis 10-wöchiger oraler Breitspektrumantibiose eliminiert wurde, zeigten, dass von d2 bis d28 nach Infektion im Fäzes sämtlicher sekundär abiotischer Mäuse stabile *P. aeruginosa* Lasten von ca. 10^8 KBE pro Gramm Fäzes nachweisbar waren (**Publikation 1, Abb. 1 B**). Als Gram-negative Kontrollen dienende, mit einem kommensalen *E. coli*-Stamm kolonisierte Mäuse wiesen im gleichen Zeitraum vergleichbare Bakterienlasten im Fäzes auf (**Publikation 1, Abb. 2**). Daraus lässt sich schlussfolgern, dass die Eliminierung der konventionellen murinen Darmmikrobiota mittels oraler Breitspektrumantibiose eine stabile Kolonisierung von MDR *P. aeruginosa* im GIT von WT Mäusen ermöglicht.

Um herauszufinden, welche Rolle die komplexe humane und murine Darmmikrobiota bei der Kolonisierung des GIT mit MDR *P aeruginosa* spielt, untersuchten wir im nächsten Schritt die Kolonisationskapazität von MDR *P. aeruginosa* in, bezogen auf ihre Darmmikrobiota, „humanisierten" Mäusen. Zu diesem Zweck wurden diese hma und die

als Kontrollen dienenden mma Mäuse aus sekundär abiotischen Mäusen mittels oraler FMT generiert. Nach stabiler Etablierung der Mikrobiota im GIT drei Wochen nach FMT und unmittelbar vor peroraler *P. aeruginosa* Infektion, zeigten quantitative molekulare Analysen der fäkalen Mikrobiota beider Gruppen signifikante Unterschiede in deren Zusammensetzung. So war zu dem Zeitpunkt in hma Mäusen die Anzahl der Genkopien von der „*Bacteroides/ Prevotella"*-Gruppe und der „*Clostridium coccoides"*-Untergruppe signifikant höher ($p<0.001$ vs. M; **Publikation 2, Abb. 1 B**), die Anzahl Lactobacillen und „*Mouse Intestinal Bacteroides"* hingegen deutlich geringer als in mma Mäusen ($p<0.001$ vs. M; **Publikation 2, Abb. 1 B**). Bereits an d7 nach peroraler *P. aeruginosa* Infektion glichen sich die anfänglich unterschiedlichen fäkalen *P. aeruginosa* Lasten mit zwischen 10^2 und 10^6 KBE pro Gramm Fäzes an (n.s. vs. M; **Publikation 2, Abb. 2**). An d28 nach Infektion waren 64.7% der hma und 77.8% der mma Mäuse intestinale *P. aeruginosa* Träger (**Publikation 2, Abb. 2**). Somit führte eine Rekonstitution sekundär abiotischer Mäuse mit humaner oder muriner Darmmikrobiota mittels FMT nur zu einem partiellen Schutz vor intestinaler Kolonisierung mit MDR *P. aeruginosa*.

1.5.2 Auszüge der Untersuchungsergebnisse zu intestinalen und systemischen Immunantworten nach Kolonisierung des Darms von sekundär abiotischen Mäusen mit MDR *Pseudomonas aeruginosa*

Im nächsten Schritt untersuchten wir, wie sich die intestinale Kolonisierung mit MDR *P. aeruginosa* auf intestinale, extra-intestinale und systemische Immunantworten in sekundär abiotischen Mäusen, d.h. ohne Vorhandensein einer komplexen Darmmikrobiota, auswirkt. Im Vergleich zu nicht-kolonisierten, sekundär abiotischen Mäusen kam es sowohl in der mit *P. aeruginosa* kolonisierten Gruppe als auch in der mit *E. coli* kolonisierten Kontrollgruppe, bei ausbleibender klinischer Beeinträchtigung, an d28 nach Infektion im Kolonepithel ($p<0.001$ vs. None; **Publikation 1, Abb. 3 A**) und nur bei der *P. aeruginosa* kolonisierten Gruppe im Ileumepithel ($p<0.05$ vs. None; **Publikation 1, Abb. 3 B**) zu einem Anstieg proliferierender Zellen, während die Anzahl apoptotischer Zellen im *P. aeruginosa* kolonisierten Ileum und Kolon nicht anstieg (n. s. vs. None; **Publikation 1, Abb. 3 AB**). Makrophagen und Monozyten waren an d28 nach Infektion im Ileum- und Kolonepithel ($p<0.001$ vs. None; **Publikation 1, Abb. 4 AB**) sowie T-Lymphozyten im Kolonepithel ($p<0.01$ vs. None; **Publikation 1, Abb. 4 A**) ausschließlich in der mit *P. aeruginosa* kolonisierten Gruppe erhöht. Unabhängig von

der Kolonisierung mit *E. coli* oder *P. aeruginosa* war die Anzahl an Tregs und B-Lymphozyten im Kolonepithel ($p<0.05 – 0.001$ vs. None; **Publikation 1, Abb. 4 A**) und die Anzahl an Tregs im Ileumepithel ($p<0.001$ vs. None; **Publikation 1, Abb. 4 B**) im Vergleich zu nicht-kolonisierten sekundär abiotischen Mäusen erhöht. Weiterhin war eine gesteigerte TNF Sekretion ausschließlich im Kolon von *P. aeruginosa* kolonisierten sekundär abiotischen Mäusen zu verzeichnen ($p<0.05$ vs. None; **Publikation 1, Abb. 5 A**), wohingegen sich erhöhte IFN-γ Konzentrationen in den MLN beider Gruppen fanden ($p<0.05$ und 0.001 vs. None; **Publikation 1, Abb. 5 B**). Auf systemischer Ebene kam es in der Milz ausschließlich in *P. aeruginosa* kolonisierten Mäusen zu einer gesteigerten Sekretion von TNF ($p<0.01$ vs. None; **Publikation 1, Abb. 5 C**) und IFN-γ ($p<0.001$ vs. None; **Publikation 1, Abb. 5 C**) sowie zu einer verminderten Sekretion des anti-inflammatorischen Zytokins IL-10 ($p<0.05$ vs. None; **Publikation 1, Abb. 5 C**). Folglich führt die Kolonisierung des Darms sekundärer abiotischer Mäuse mit MDR *P. aeruginosa* bei fehlenden makroskopischen (d.h. klinischen) und mikroskopischen (d.h. apoptotischen) Folgen zu ausgeprägten intestinalen und systemischen Immunantworten.

1.5.3 Auszüge der Untersuchungsergebnisse zu intestinalen und systemischen Immunantworten nach Kolonisierung des Darms von mit humaner und muriner Mikrobiota assoziierten Mäusen mit MDR *Pseudomonas aeruginosa*

Im Folgenden untersuchten wir intestinale und systemische Immunantworten nach Kurz- (d7) und Langzeit (d28) Kolonisierung mit MDR *P. aeruginosa* im mit humaner oder muriner Mikrobiota kolonisierten GIT von zuvor sekundär abiotischen Mäusen. Es zeigte sich, bei fehlender klinischer Beeinträchtigung und unabhängig von der zugrunde liegenden humanen oder murinen Mikrobiota, an d28 nach peroraler *P. aeruginosa* Infektion ein signifikanter Anstieg apoptotischer Zellen in der Kolon- ($p<0.001$ und 0.05 d28 vs. d0; **Publikation 2, Abb. 5 AB**) und Ileummukosa ($p<0.001$ d28 vs. d0; **Publikation 2, Abb. S2 AB**). Dies war begleitet von einem kurzfristigen Anstieg proliferierender Zellen im Kolonepithel von hma Mäusen ($p<0.01$ d7 vs. d0; **Publikation 2, Abb. 5 B**) und einem kurzfristigen Anstieg von Makrophagen und Monozyten im Kolonepithel von hma und mma Mäusen ($p<0.001$ d7 vs. d0; **Publikation 2, Abb. 5 AB**). Nur in mma Mäusen kam es zu einem längerfristigen Anstieg von B-Lymphozyten in der Kolonmukosa ($p<0.05$ d28 vs. d0; **Publikation 2, Abb. 5 A**). Weiterhin kam es im Vergleich zu nicht (*P. aeruginosa*) kolonisierten

Kontrollen zu einem vorrübergehenden intestinalen Konzentrationsanstieg pro-inflammatorischer Zytokine wie TNF im Kolon von mma Mäusen ($p<0.05$ d7 vs. d0; **Publikation 2, Abb. 6 A**) und IFN-γ in den MLN von hma und mma Mäusen ($p<0.001$ und $p<0.01$; d7 vs. d0; **Publikation 2, Abb. 7 AB**). Unabhängig von der zugrunde liegenden Darmmikrobiota bewirkte die intestinale *P. aeruginosa* Kolonisierung keine vermehrte Sekretion des anti-inflammatorischen Zytokins IL-10 (**Publikation 2, Abb. 6 AB und Abb. 7 AB**). Auf systemischer Ebene stiegen sowohl bei hma als auch bei mma Mäusen die IFN-γ Konzentrationen und bei mma noch zusätzlich die TNF Konzentrationen in der Milz nach *P. aeruginosa* Infektion an (**Publikation 2, Abb. 8 AB**). Zusammenfassend lässt sich sagen, dass die intestinale Kolonisierung mit *P. aeruginosa* sowohl in hma als auch in mma Mäusen zu differentiellen sowohl kurz- als auch langfristigen Immunantworten auf intestinaler und systemischer Ebene führt.

1.5.4 Auszüge der Untersuchungsergebnisse zur Kolonisierung von mit humaner Mikrobiota assoziierten Mäusen mit akuter Ileitis mit MDR *Pseudomonas aeruginosa*

Anschließend untersuchten wir den Einfluss von akuter Darmentzündung auf die intestinale MDR *P. aeruginosa* Kolonisationskapazität und deren Auswirkung auf das Immunsystem im „humanisierten“ Mausmodell. Hierfür generierten wir hma Mäuse und induzierten mittels peroraler Infektion mit 50 *T. gondii* Zysten eine akute Ileitis. An d3 nach Ileitis Induktion führten wir die orale Infektion mit *P. aeruginosa* durch. Zu diesem Zeitpunkt können die ersten histopathologischen Veränderungen im akuten *T. gondii*-Inflammationsmodel beobachtet werden (27).

Bereits an d3 nach *P. aeruginosa* Infektion wiesen hma Mäuse mit akuter Ileitis im Vergleich zu gesunden hma Mäusen signifikant höhere *P. aeruginosa*-Lasten auf ($p<0.01$ vs. N; **Publikation 3, Abb. 2**) und waren zum Zeitpunkt der Sektion an d4 noch zu 95% intestinale *P. aeruginosa* Träger (**Publikation 3, Abb. 2**). Daraus lässt sich schließen, dass die akute Ileitis die intestinale Kolonisierung von hma Mäusen mit MDR *P. aeruginosa* begünstigt. Bereits in einer früheren Arbeit zeigten wir, dass eine Infektion mit 50 Zysten *T. gondii* im „humanen“ Mausmodell innerhalb von sieben Tagen zu einer akuten, fulminanten Ileitis mit deutlichen Veränderungen in der Zusammensetzung der Darmmikrobiota, wie einem erheblichen Anstieg Gram-negativer und obligat anaerober Bakteriengruppen zu Lasten aerober Gram-positiver Stäbchen, führt (35). Hier konnten wir zeigen, dass eine Ko-Infektion mit *P. aeruginosa* keinen

Einfluss auf die Zusammensetzung der Darmmikrobiota hat (n.s. vs. N; **Publikation 3, Abb. 3**). Nur die Bifidobakterienlasten waren interessanterweise in luminalen Ileum-Inhalten der *P. aeruginosa* ko-infizierten Gruppe erniedrigt ($p<0.05$ vs. N; **Publikation 3, Abb. 3 B**).

Im nächsten Schritt konnten wir zeigen, dass eine *P. aeruginosa* Ko-Infektion von hma Mäusen mit akuter Darmentzündung nicht zu einer weiteren Verschlechterung des klinischen Zustandes oder vermehrten histopathologischen und apoptotischen Veränderungen in deren Ileummukosa führte (**Publikation 3, Abb. 4 A-D**). Ebenfalls waren Zellzahlen des angeborenen (d.h. Makrophagen/ Monozyten) und erworbenen (d.h. Tregs und T-Lymphozyten) Immunsystems sowie Konzentrationen des pro-inflammatorischen Zytokins IFN-γ in Ileum, Colon und MLN vergleichbar in *P. aeruginosa* ko-infizierten und nicht-infizierten hma Mäusen mit akuter Ileitis (**Publikation 3, Abb. 5 A-D und Abb. 6 A-C**). In Leber, Niere, Milz und Serum konnten sowohl in *P. aeruginosa* ko-infizierten als auch nicht-infizierten hma Mäusen 7 Tage nach Ileitis-Induktion vergleichbare Konzentrationen pro-inflammatorischer Mediatoren wie z. B. IFN-γ und NO gemessen werden (**Publikation 3, Abb. 7 und Abb. 8**). Einzig die hepatische TNF Konzentration war in *P. aeruginosa* ko-infizierten Mäusen im Vergleich zur Kontrollgruppe erhöht ($p<0.01$ vs. Naive; **Publikation 3, Abb. 7 A**). Darüber hinaus translozierten häufiger lebende Bakterien vom Darm in systemische Kompartimente, wie Milz und Herzblut von *P. aeruginosa* ko-infizierten Mäusen im Vergleich zu hma Kontrollen mit Ileitis (**Publikation 3, Abb. 9 A**). Eine akute Ileitis fördert demnach die intestinale Kolonisierung von hma Mäusen mit MDR *P. aeruginosa*, welche die bestehende Immunpathologie in intestinalen, extra-intestinalen und systemischen Kompartimenten nicht wesentlich verschlimmert.

1.5.5 Auszüge der Untersuchungsergebnisse zur Kolonisierung von IL-10 Knockout Mäusen mit chronischer Kolitis mit MDR *Pseudomonas aeruginosa*

Im Folgenden untersuchten wir den Einfluss einer chronischen Darmentzündung auf die intestinale MDR *P. aeruginosa* Kolonisierung und deren Auswirkung auf das Immunsystem von IL-$10^{-/-}$ Mäusen mit konventioneller Darmmikrobiota. Bereits an d2 nach *P. aeruginosa* Infektion wiesen die IL-$10^{-/-}$ Mäuse mir chronischer Kolitis im Fäzes signifikant höhere *P. aeruginosa* Lasten auf als ihre gesunden WT Kontrollen, in denen *P. aeruginosa* bereits eine Woche nach Infektion kulturell nicht mehr nachweisbar war ($p<0.001$; **Publikation 4, Abb. 1 A**). In 78.9% der IL-$10^{-/-}$ Mäuse hingegen konnte

P. aeruginosa noch sechs Wochen nach Infektion nachgewiesen werden (**Publikation 4, Abb. 1 B**). Daraus lässt sich schließen, dass die intestinale Kolonisierung durch MDR *P. aeruginosa* durch chronische intestinale Inflammation begünstigt wird.

Bei vergleichbaren histopathologischen Veränderungen der Kolonmukosa sechs Wochen nach *P. aeruginosa* Infektion (n.s. vs. N; **Publikation 4, Suppl. Fig. 3**) zeigten immunhistochemische Färbungen, dass die Anzahl apoptotischer Zellen ($p<0.001$ vs. N; **Publikation 4, Abb. 3 A**) sowie der Zellen des angeborenen (Makrophagen/ Monozyten: $p<0.001$ vs. N; **Publikation 4, Abb. 4 A**) und erworbenen Immunsystems (T-Lymphozyten: $p<0.001$ vs. N; **Publikation 4, Abb. 4 B**, Tregs; $p<0.005$ vs. N; **Publikation 4, Abb. 4 C** und B-Lymphozyten: $p<0.001$ vs. N; **Publikation 4, Abb. 4 D**) im Kolonepithel *P. aeruginosa* kolonisierter IL-$10^{-/-}$ Mäuse im Vergleich zu nicht-kolonisierten IL-$10^{-/-}$ Mäusen gesteigert war. Zusätzlich wiesen *P. aeruginosa* kolonisierter IL-$10^{-/-}$ Mäuse im Vergleich zu nicht-kolonisierten IL-$10^{-/-}$ Mäusen erhöhte Konzentrationen der pro-inflammatorischen Zytokine TNF ($p<0.05$ vs. N; **Publikation 4, Abb. 5 A**) und IFN-γ ($p<0.05$ vs. N; **Publikation 4, Abb. 5 B**) in den MLN auf. In der Milz kam es zu keiner gesteigerten Sekretion pro-inflammatorischer Zytokine in den *P. aeruginosa* kolonisierte IL-$10^{-/-}$ Mäusen, verglichen mit der nicht-kolonisierten Kontrollgruppe (**Publikation 4, Abb. 6**). Dem stehen erhöhte Serumkonzentrationen von TNF und IFN-γ in der mit *P. aeruginosa* kolonisierten Gruppe entgegen ($p<0.05$ vs. N; **Publikation 4, Abb. 7 AB**). Interessanterweise kam es nur in der mit *P. aeruginosa* kolonisierten Gruppe zu einer Translokation von Vertretern der kommensalen Darmmikrobiota in die MLN und extra-intestinalen Kompartimente wie Milz, Leber und Niere (**Publikation 4, Suppl. Fig. 5**). Durch die chronische Kolitis kann somit die natürliche Kolonisationsresistenz gegenüber MDR *P. aeruginosa* in konventionell kolonisierten Mäusen aufgehoben. werden. Eine *P. aeruginosa* Kolonisierung des Darms von IL-$10^{-/-}$ Mäusen mit chronischer Kolitis führt zu einer verstärkten intestinalen sowie systemischen Immunantwort, die mit einer vermehrten Translokation lebender Bakterien aus der kommensalen Darmmikrobiota in extra-intestinale Organe einhergeht.

1.6 Diskussion

MDR *P. aeruginosa* wurden kürzlich von der WHO zu einer erheblichen Bedrohung der menschlichen Gesundheit erklärt (26). Der GIT ist dabei eine wichtige Infektionsquelle (13-16). Ziel der vorliegenden Arbeit war es, das Wechselspiel zwischen MDR *P. aeruginosa*, der kommensalen Darmmikrobiota und dem Immunsystem des Wirts sowie die Auswirkung einer *P. aeruginosa* Kolonisierung auf eine Darmentzündung in der Maus zu untersuchen.

Unsere kulturellen Untersuchungen zeigten, dass WT Mäuse mit konventioneller Darmmikrobiota eine natürliche Kolonisationsresistenz gegenüber *P. aeruginosa* aufweisen, die durch Eliminierung der Mikrobiota mittels oraler Breitspektrumantibiose aufgehoben werden kann. Diese Ergebnisse decken sich mit früheren Untersuchungen am Mausmodell, in denen sowohl durch orale als auch systemische Antibiose die ursprüngliche Kolonisationsresistenz gegenüber *P. aeruginosa* im murinen GIT aufgehoben wurde (45-47). Auch gegenüber anderen Pathogenen, wie *Campylobacter* (*C.*) *jejuni*, *Clostridium difficile*, *Listeria monocytogenes* oder *Salmonella* (*S.*) t*yphimurium*, zeigen Mäuse, nach Veränderungen der komplexen murinen Darmmikrobiota durch z.B. Antibiose, eine aufgehobene physiologische Kolonisationsresistenz (28, 48-51). Doch nicht nur im murinen, sondern auch im humanen GIT kann eine antibiotische Behandlung zur Herabsetzung der Kolonisationsresistenz gegenüber *P. aeruginosa* führen (8, 10). So konnte *P. aeruginosa* in einem Probanden mit zeitgleicher Ampicillin-Behandlung bis zu 14 Tage nach Inokulation im Stuhl nachgewiesen werden (10). Dies kann vor allem für kritisch kranke Patienten auf Intensivstationen eine wichtige Rolle spielen, in welchen die intestinale *P. aeruginosa* Kolonisierung ein Hauptrisikofaktor für systemische Infektionen, wie z.B. Pneumonien, Wundinfektionen oder Harnwegsinfektionen darstellt (15, 16).

Weiterhin konnten wir zeigen, dass eine Rekonstitution sekundär abiotischer Mäuse mittels FMT unabhängig davon, ob mit humaner oder muriner Darmmikrobiota, nur zu einem partiellen Schutz vor Kolonisierung des Darms mit MDR *P. aeruginosa* führt. Auch frühere Studien zeigten, dass sich die Kolonisationsresistenz gegenüber *P. aeruginosa* nach Beendigung der antibiotischen Therapie mit Streptomycin und Neomycin und erneuter Besiedlung des murinen GIT mit überlebenden Anaerobiern sofort erholt (45). Vier Wochen nach Infektion wiesen bei unseren Experimenten 64.7% der hma und 77.8% der mma Mäuse *P. aeruginosa* im Fäzes auf, wobei die

P. aeruginosa-Lasten deutlich geringer ausfielen als in sekundär abiotischen Mäusen. Demnach könnte man argumentieren, dass die Kolonisationsresistenz durch komplexe intestinale Mikrobiota, nach oraler Breitspektrumantibiose und anschließender FMT, noch nicht wieder vollständig hergestellt ist. Frühere Untersuchungen unserer Arbeitsgruppe zeigten jedoch, dass die physiologische Kolonisationsresistenz gegenüber *C. jejuni* nach FMT von muriner Mikrobiota im gleichen Zeitraum wieder vollständig hergestellt werden konnte (28).

Zu beachten ist, dass die Rekonstitution des GIT sekundär abiotischer Mäuse mit komplexer humaner Darmmikrobiota mittels FMT Limitationen aufweisen kann. So ist es möglich, dass obligate Anaerobier und andere anspruchsvolle, mitunter schwer kultivierbare Bakterien aus den Stuhlsuspensionen durch die Verarbeitung verloren gegangen sind (52). Weiterhin gilt es zu bedenken, dass wirtsspezifische intrinsische sowie extrinsische Faktoren die Zusammensetzung der Mikrobiota beeinflussen (53). So konnte gezeigt werden, dass der genetischer Hintergrund und die Unterbringung von Mäusen einen großen Einfluss auf die Zusammensetzung von deren Darmmikrobiota haben (54-56). Einen sehr wesentlichen Einfluss auf die intestinale Mikrobiota nimmt auch die Zusammensetzung der Ernährung sowohl bei Mäusen als auch bei Menschen (57-63). Dennoch wurden auch in vorangegangenen wissenschaftlichen Arbeiten hma Mäuse verwendet, um Kolonisationskapazitäten anderer Enteropathogene wie *C. jejuni*, *S. enterica* oder *Clostridium difficile* zu untersuchen (28, 64, 65). Sie stellen somit ein gutes Modell zur Untersuchung der Interaktionen zwischen Wirt, Darmmikrobiota, Pathogenen und Immunsystem dar (31, 52).

Nach unserem Kenntnisstand ist wenig über Immunantworten nach Kolonisierung des GIT mit MDR *P. aeruginosa* bekannt. Unsere Untersuchungen ergaben, dass weder konventionell kolonisierte, noch sekundär abiotische oder hma und mma Mäuse über die gesamten vier Wochen nach MDR *P. aeruginosa* Infektion klinische Symptome aufwiesen. Dies stimmt mit Untersuchungen an gesunden Mäusen und Menschen überein, bei denen es nach intestinaler Kolonisierung mit *P. aeruginosa* ebenfalls zu keinen makroskopisch sichtbaren Folgen kam (10, 46). Mikroskopisch zeigten sekundär abiotische Mäuse vier Wochen nach *P. aeruginosa* Infektion eine ausgeprägte intestinale Immunantwort, wobei die Anzahl proliferierender Zellen und Zellen des angeborenen und erworbenen Immunsystems in der intestinalen Mukosa anstieg. Dies ging einher mit einer vermehrten Ausschüttung der pro-inflammatorischen Zytokine TNF und IFN-γ nicht nur auf intestinaler, sondern auch auf systemischer Ebene.

Auch hma und mma Mäuse wiesen nach intestinaler *P. aeruginosa* Kolonisierung kurzfristige (d.h. an d7) und langfristige (d.h. an d28) intestinale wie systemische Immunantworten auf. Diese waren geprägt von einer erhöhten Anzahl apoptotischer sowie proliferierender Zellen im Darmepithel. Dazu kam es zu einem relativ frühen gesteigerten Einstrom von Makrophagen und Monozyten in die Darmmukosa, während die Anzahl an B-Lymphozyten als Zellen des erworbenen Immunsystems eher spät anstieg. Weiterhin war die zelluläre Immunantwort von erhöhten Konzentrationen der pro-inflammatorischen Zytokine TNF und IFN-γ sowohl im Darm als auch in der Milz begleitet. Eine anti-inflammatorische Immunantwort blieb sowohl auf intestinaler Ebene als auch auf systemischer Ebene aus.

Die durch *P. aeruginosa* ausgelösten Immunantworten lassen sich auf die zahlreichen Virulenzfaktoren des opportunistischen Pathogens zurückführen. Dazu gehören unter anderem eine Flagelle, mehrere Pili, die Sekretion von Exotoxinen, die Expression von Lipopolysacchariden oder die Biofilmbildung mittels „Quorum Sensing“, die zu einer Aktivierung des angeborenen Immunsystems führen (1, 3, 66-69). Im Gegensatz zum GIT gibt es bereits einige Erkenntnisse über die Immunantworten bei *P. aeruginosa* induzierten akuten und chronischen Atemwegsinfektionen (1, 3, 66, 70). So kommt es über die Aktivierung des Toll-like Rezeptor (TLR) 5 durch das extrazelluläre Flagellin und des TLR4 durch das membranständige Lipid A, einem Bestandteil der Lipopolysaccharide, zu einer NFκB induzierten Expression pro-inflammatorischer Zytokine (1, 71-73). Auch der intrazelluläre NOD-like Rezeptor (NLR) NOD1 führt über die Aktivierung durch Peptidoglykane der bakteriellen Zellwand zu einer Aktivierung von NFκB (66, 68, 74-76). Weiterhin binden nach intrazellulär translozierte Exotoxine ExoY, ExoS, ExoT und ExoU des Typ 3 Sekretionssystems sowie Flagellin an den NLRC4 und führen so zu einer Aktivierung der Caspase-1 in Makrophagen (68, 77-81).

Soweit uns bekannt ist, sind wir die Ersten, die sowohl im sekundär abiotischen Mausmodell als auch an hma und mma Mäusen zeigen konnten, dass eine reine intestinale *P. aeruginosa* Kolonisierung zu pro-inflammatorischen intestinalen sowie systemischen Immunantworten führt. Im gesunden Wirt gilt *P. aeruginosa* nicht als ein übliches Enteropathogen, auch wenn es wenige Fälle von Antibiotika-assoziierten Diarrhoen, verursacht durch *P. aeruginosa*, gibt (82-84). Erstmals wurde bereits vor 90 Jahren das „Shanghai Fieber“ in der asiatischen Bevölkerung beschrieben, ein ambulant erworbenes Krankheitsbild, welches sich durch Fieber, Diarrhö und eine *P. aeruginosa* assoziierte Sepsis auszeichnet, Neugeborene und Kinder ohne jegliche

Vorerkrankungen betrifft und durch einen komplikationsreichen Verlauf mit einer hohen Mortalität einhergeht (84-87).

Wie bereits in der Einleitung aufgeführt, gibt es mehrere sowohl experimentelle als auch klinische Studien, die einen Zusammenhang zwischen der Pathogenese chronisch entzündlicher Darmerkrankungen, wie Morbus Crohn und dem Vorkommen von Pseudomonasspezies, wie etwa *P. aeruginosa* untersuchen (17-25). Um der Frage nachzugehen, inwieweit eine intestinale Entzündung eine Kolonisierung des Darms mit MDR *P. aeruginosa* begünstigt und ob die *P. aeruginosa* Kolonisierung zu einer Verschlimmerung der Entzündung führt, wendeten wir sowohl ein akutes, *T. gondii* induziertes Ileitis-Modell als auch ein chronisches Kolitis-Modell unter Verwendung von IL-10 $^{-/-}$ Mäusen an.

Mäuse entwickeln innerhalb einer Woche nach peroraler Infektion mit 50 Zysten des intrazellulären Parasiten *T. gondii* eine sowohl von T Helferzellen 1 (Th1) als auch von TLR4 abhängige Ileitis, die mit einem massiven pro-inflammatorischen „Zytokinsturm“, ausgeprägten intestinalen Nekrosen, einer Überwucherung des Lumens mit Gram-negativen Spezies wie Enterobakterien und *Bacteroides/ Prevotella* spp. sowie einer extra-intestinalen Immunreaktionen einhergeht (27, 33, 88-91). Diese Veränderungen spiegeln wesentliche pathogenetische Mechanismen während eines akuten Schubes beim Morbus Crohn wider (89, 91). In einer kürzlich durchgeführten Arbeit konnten wir zeigen, dass es auch in hma Mäusen nach peroraler *T. gondii* Infektion zu entsprechenden immunopathologischen Veränderungen kam (35). Aus unseren Experimenten ging hervor, dass die akute *T. gondii* induzierte Ileitis hma Mäuse empfänglich für eine intestinale Kolonisierung mit MDR *P. aeruginosa* macht. Vier Tage nach MDR *P. aeruginosa* Infektion ließ sich mit 95% bei hma Mäusen mit akuter Ileitis wesentlich häufiger *P. aeruginosa* im Fäzes nachweisen als bei deren gesunden Kontrollen. Dies geht einher mit früheren Untersuchungen unserer Arbeitsgruppe, bei der die natürliche Kolonisationsresistenz gegenüber dem Enteropathogen *C. jejuni* im Zuge akuter intestinaler Inflammation bei konventionell kolonisierten Mäusen aufgehoben werden konnte (43). Aber auch andere z. B. durch den Parasiten *Plasmodium yoelii*, enteropathogene *S. typhimurium* oder genetische Immundefekte hervorgerufene Auslöser intestinaler Inflammation und Dysbiose führen zu einer gesteigerten Kolonisationskapazität von Enteropathogenen (92, 93). Dies lässt vermuten, dass es im entzündlichen Milieu durch ein z. B. erhöhtes intraluminales Aufkommen an Zelltrümmern und Veränderungen in der chemischen und mikrobiellen

Zusammensetzung zu Wachstumsvorteilen nicht nur für kommensale Enterobakterien, sondern auch für (opportunistische) Pathogene kommen kann (27, 33, 40, 90, 94-96).
Interessanterweise hat eine intestinale Kolonisierung der hma Mäuse mit *P. aeruginosa* bei akuter Ileitis nicht zu einer weiteren Exazerbation der Darmentzündung im Vergleich zu den ebenfalls *T. gondii* infizierten, kranken hma Mäusen ohne zusätzliche *P. aeruginosa* Kolonisierung geführt. Es kam lediglich zu einer gesteigerten bakteriellen Translokation in Milz und Blut im Vergleich zur nicht-infizierten Kontrollgruppe. Dies könnte auf eine ausgeprägtere Störung der epithelialen Barrierefunktion durch *P. aeruginosa* hinweisen. Da die *T. gondii*-induzierte Ileitis durch Aktivierung von TLR4 durch Lipopolysaccharide Gram-negativer Kommensale verstärkt wird, könnte man annehmen, dass eine Ko-infektion mit dem Gram-negativen, opportunistischen Pathogen *P. aeruginosa* zu einer weiteren Exazerbation der *T. gondii* induzierten intestinalen Immunantwort führt (27, 90). Dass dies nicht der Fall ist, könnte an dem durch die Th1-Typ Immunantwort ausgelösten Zytokinsturm liegen, durch den eine zusätzliche *P. aeruginosa* Kolonisierung nicht weiter biologisch relevant ins Gewicht fällt (91).
Um das Wechselspiel zwischen intestinaler Inflammation und MDR *P. aeruginosa* Kolonisierung weiter zu differenzieren, wendeten wir im nächsten Schritt ein chronisches Kolitis Modell an. IL-10$^{-/-}$ Mäuse werden häufig als Modell für chronische GIT Inflammation verwendet, da sie, unter SPF Bedingungen gehalten, eine spontane, chronische Kolitis entwickeln, bei deren Entstehung die Darmmikrobiota eine wesentliche Rolle spielt (97-99). Sechs Wochen nach Infektion konnte trotz konventioneller muriner Besiedlung noch in 79% der IL-10$^{-/-}$ Mäuse MDR *P. aeruginosa* im Fäzes nachgewiesen werden, während bei den gesunden WT Kontrollgruppen bereits eine Woche nach Infektion kein *P. aeruginosa* mehr nachweisbar war. Wie im akuten *T. gondii* induzierten Ileitis Modell konnte auch bei IL-10$^{-/-}$ Mäusen mit chronischer Kolitis bereits gezeigt werden, dass die natürliche Kolonisationsresistenz gegenüber Enteropathogenen wie *S. typhimurium* aber auch einem nicht pathogenen *E. coli* Stamm durch die chronische Entzündung und den damit einhergehenden Veränderungen in der Zusammensetzung der Darmmikrobiota aufgehoben wurde (93, 100). Weiterhin konnten wir zeigen, dass eine Kolonisierung des chronisch entzündeten Kolons mit MDR *P. aeruginosa* zu einer weiter verstärkten Immunantwort führt. Diese äußerte sich in einer gesteigerten Apoptoserate von Darmepithelzellen und vermehrtem Einstrom von Immunzellen in die Kolonmukosa und Lamina propria sowie in einer

gesteigerten Sekretion pro-inflammatorischer Zytokine. Auch auf systemischer Ebene kam es zu erhöhten Serumkonzentrationen von TNF und IFN-γ und einer bakteriellen Translokation ausschließlich in den *P. aeruginosa* kolonisierten IL-$10^{-/-}$ Mäusen.

Zusammenfassung und Forschungsausblick: Zusammenfassend zeigt die vorliegende Arbeit, dass die Darmmikrobiota wesentlich zur Kolonisationsresistenz gegenüber MDR *P. aeruginosa* beiträgt und dass ihre Störung (z.B. durch antibiotische Therapien oder intestinale Inflammation) zu einer intestinalen Kolonisierung mit dem opportunistischen Pathogen führen kann. Eine reine Kolonisierung mit *P. aeruginosa* führt bereits im gesunden Wirtsorganismus zu pro-inflammatorischen Immunantworten und fördert darüber hinaus eine bereits bestehende chronische Inflammation. Um mögliche Alternativen zur antibiotischen Therapie wie z.B. FMT oder probiotische Interventionen entwickeln zu können, sollten in zukünftigen Arbeiten die jeweiligen intraluminalen Moleküle identifiziert werden, die zu einer Kolonisierung von MDR Gram-negativen Bakterien im gesunden und kranken GIT führt.

1.7 Literaturverzeichnis

1. Gellatly SL, Hancock RE. *Pseudomonas aeruginosa*: New insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159-73.
2. Vincent JL. Nosocomial infections in adult intensive-care units. Lancet. 2003;361(9374):2068-77.
3. Driscoll JA, Brody SL, Kollef MH. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs. 2007;67(3):351-68.
4. Davies JC. *Pseudomonas aeruginosa* in cystic fibrosis: Pathogenesis and persistence. Paediatr Respir Rev. 2002;3(2):128-34.
5. Oliver A, Mulet X, Lopez-Causape C, Juan C. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updat. 2015;21-22:41-59.
6. Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. Int J Antimicrob Agents. 2015;45(6):568-85.
7. Darrell JH, Wahba AH. Pyocine-typing of hospital strains of *Pseudomonas pyocyanea*. J Clin Pathol. 1964;17:236-42.
8. Shooter RA, Walker KA, Williams VR, Horgan GM, Parker MT, Asheshov EH, Bullimore JF. Faecal carriage of *Pseudomonas aeruginosa* in hospital patients. Possible spread from patient to patient. Lancet. 1966;2(7477):1331-4.
9. Kessner DM, Lepper MH. Epidemiologic studies on gram-negative bacilli in the hospital and community. Am J Epidemiol. 1967;85(1):45-60.
10. Buck AC, Cooke EM. The fate of ingested *Pseudomonas aeruginosa* in normal persons. J Med Microbiol. 1969;2(4):521-5.
11. Stoodley BJ, Thom BT. Observations on the intestinal carriage of *Pseudomonas aeruginosa*. J Med Microbiol. 1970;3(3):367-75.
12. Bleday R, Braidt J, Ruoff K, Shellito PC, Ackroyd FW. Quantitative cultures of the mucosal-associated bacteria in the mechanically prepared colon and rectum. Dis Colon Rectum. 1993;36(9):844-9.
13. Speert DP, Campbell ME, Davidson AG, Wong LT. *Pseudomonas aeruginosa* colonization of the gastrointestinal tract in patients with cystic fibrosis. J Infect Dis. 1993;167(1):226-9.
14. Bonten MJ, Bergmans DC, Speijer H, Stobberingh EE. Characteristics of polyclonal endemicity of *Pseudomonas aeruginosa* colonization in intensive care units. Implications for infection control. Am J Respir Crit Care Med. 1999;160(4):1212-9.
15. Marshall JC, Christou NV, Meakins JL. The gastrointestinal tract. The "undrained abscess" of multiple organ failure. Ann Surg. 1993;218(2):111-9.
16. Gomez-Zorrilla S, Camoez M, Tubau F, Canizares R, Periche E, Dominguez MA, Ariza J, Pena C. Prospective observational study of prior rectal colonization status as a predictor for subsequent development of *Pseudomonas aeruginosa* clinical infections. Antimicrob Agents Chemother. 2015;59(9):5213-9.
17. Graham DY, Yoshimura HH, Estes MK. DNA hybridization studies of the association of *Pseudomonas maltophilia* with inflammatory bowel diseases. J Lab Clin Med. 1983;101(6):940-54.
18. Wei B, Huang T, Dalwadi H, Sutton CL, Bruckner D, Braun J. *Pseudomonas fluorescens* encodes the Crohn's disease-associated I2 sequence and T-cell superantigen. Infect Immun. 2002;70(12):6567-75.
19. Eckburg PB, Relman DA. The role of microbes in Crohn's disease. Clin Infect Dis. 2007;44(2):256-62.
20. Forbes JD, Van Domselaar G, Bernstein CN. Microbiome survey of the inflamed and noninflamed gut at different compartments within the gastrointestinal tract of inflammatory bowel disease patients. Inflamm Bowel Dis. 2016;22(4):817-25.
21. Mow WS, Vasiliauskas EA, Lin YC, Fleshner PR, Papadakis KA, Taylor KD, Landers CJ, Abreu-Martin MT, Rotter JI, Yang H, Targan SR. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. Gastroenterology. 2004;126(2):414-24.
22. Tsianos EV, Katsanos K. Do we really understand what the immunological disturbances in inflammatory bowel disease mean? World J Gastroenterol. 2009;15(5):521-5.
23. Wagner J, Short K, Catto-Smith AG, Cameron DJ, Bishop RF, Kirkwood CD. Identification and characterisation of *Pseudomonas* 16s ribosomal DNA from ileal biopsies of children with Crohn's disease. PLoS One. 2008;3(10):e3578.

24. Kerckhoffs AP, Ben-Amor K, Samsom M, van der Rest ME, de Vogel J, Knol J, Akkermans LM. Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in irritable bowel syndrome. J Med Microbiol. 2011;60(Pt 2):236-45.
25. Wang M, Molin G, Ahrne S, Adawi D, Jeppsson B. High proportions of proinflammatory bacteria on the colonic mucosa in a young patient with ulcerative colitis as revealed by cloning and sequencing of 16s rrna genes. Dig Dis Sci. 2007;52(3):620-7.
26. Tacconelli E, Magrini N. Global priority list of antibiotic-resistamt bacteria to giude reserachm discovery, and development of new antibiotics. WHO. 2017.
27. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, Jahn HK, Dunay IR, Moter A, Gescher DM, Schumann RR, Gobel UB, Liesenfeld O. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. J Immunol. 2006;177(12):8785-95.
28. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kuhl AA, Dasti JI, Zautner AE, Munoz M, Loddenkemper C, Gross U, Gobel UB, Heimesaat MM. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. PLoS One. 2011;6(6):e20953.
29. Heimesaat MM, Boelke S, Fischer A, Haag LM, Loddenkemper C, Kuhl AA, Gobel UB, Bereswill S. Comprehensive postmortem analyses of intestinal microbiota changes and bacterial translocation in human flora associated mice. PLoS One. 2012;7(7):e40758.
30. von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM. Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. Gut Pathog. 2017;9:4.
31. von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM. Intestinal and systemic immune responses upon multi-drug resistant *Pseudomonas aeruginosa* colonization of mice harboring a human gut microbiota. Front Microbiol. 2017;8:2590.
32. Ekmekciu I, von Klitzing E, Fiebiger U, Escher U, Neumann C, Bacher P, Scheffold A, Kuhl AA, Bereswill S, Heimesaat MM. Immune responses to broad-spectrum antibiotic treatment and fecal microbiota transplantation in mice. Front Immunol. 2017;8:397.
33. Heimesaat MM, Fischer A, Jahn HK, Niebergall J, Freudenberg M, Blaut M, Liesenfeld O, Schumann RR, Gobel UB, Bereswill S. Exacerbation of murine ileitis by Toll-like Receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. Gut. 2007;56(7):941-8.
34. Struck D, Frank I, Enders S, Steinhoff U, Schmidt C, Stallmach A, Liesenfeld O, Heimesaat MM. Treatment with interleukin-18 binding protein ameliorates *Toxoplasma gondii*-induced small intestinal pathology that is induced by bone marrow cell-derived interleukin-18. Eur J Microbiol Immunol (Bp). 2012;2(3):249-57.
35. von Klitzing E, Ekmekciu I, Kuhl AA, Bereswill S, Heimesaat MM. Intestinal, extra-intestinal and systemic sequelae of *Toxoplasma gondii* induced acute ileitis in mice harboring a human gut microbiota. PLoS One. 2017;12(4):e0176144.
36. von Klitzing E, Bereswill S, Heimesaat MM. Multidrug-resistant *Pseudomonas aeruginosa* induce systemic pro-inflammatory immune responses in colonized mice. Eur J Microbiol Immunol (Bp). 2017;7(3):200-9.
37. von Klitzing E, Ekmekciu I, Kuhl AA, Bereswill S, Heimesaat MM. Multidrug-resistant *Pseudomonas aeruginosa* aggravates inflammatory responses in murine chronic colitis. Sci Rep. 2018;8(1):6685.
38. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, Bereswill S, Heimesaat MM. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic il-10-/- mice via Toll-like-Receptor-2 and -4 signaling. PLoS One. 2012;7(7):e40761.
39. Heimesaat MM, Fischer A, Siegmund B, Kupz A, Niebergall J, Fuchs D, Jahn HK, Freudenberg M, Loddenkemper C, Batra A, Lehr HA, Liesenfeld O, Blaut M, Gobel UB, Schumann RR, Bereswill S. Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like Receptors 2 and 4. PLoS One. 2007;2(7):e662.
40. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, Steinhoff U, Tchaptchet S, Thiel E, Freudenberg MA, Gobel UB, Uharek L. Myd88/tlr9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut. 2010;59(8):1079-87.
41. Rausch S, Held J, Fischer A, Heimesaat MM, Kuhl AA, Bereswill S, Hartmann S. Small intestinal nematode infection of mice is associated with increased enterobacterial loads alongside the intestinal tract. PLoS One. 2013;8(9):e74026.

42. Bereswill S, Kuhl AA, Alutis M, Fischer A, Mohle L, Struck D, Liesenfeld O, Gobel UB, Dunay IR, Heimesaat MM. The impact of Toll-like-Receptor-9 on intestinal microbiota composition and extra-intestinal sequelae in experimental *Toxoplasma gondii* induced ileitis. Gut Pathog. 2014;6:19.
43. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, Bereswill S, Heimesaat MM. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. PLoS One. 2012;7(5):e35988.
44. Alutis ME, Grundmann U, Hagen U, Fischer A, Kuhl AA, Gobel UB, Bereswill S, Heimesaat MM. Matrix metalloproteinase-2 mediates intestinal immunopathogenesis in *Campylobacter jejuni*-infected infant mice. Eur J Microbiol Immunol (Bp). 2015;5(3):188-98.
45. van der Waaij D, Berghuis-de Vries JM, Lekkerkerk L-v. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg (Lond). 1971;69(3):405-11.
46. Hentges DJ, Stein AJ, Casey SW, Que JU. Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: Influence of antibiotics on colonization resistance. Infect Immun. 1985;47(1):118-22.
47. Koh AY, Priebe GP, Pier GB. Virulence of *Pseudomonas aeruginosa* in a murine model of gastrointestinal colonization and dissemination in neutropenia. Infect Immun. 2005;73(4):2262-72.
48. Wilson KH, Sheagren JN, Freter R, Weatherbee L, Lyerly D. Gnotobiotic models for study of the microbial ecology of *Clostridium difficile* and *Escherichia coli*. J Infect Dis. 1986;153(3):547-51.
49. Reeves AE, Koenigsknecht MJ, Bergin IL, Young VB. Suppression of *Clostridium difficile* in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the family lachnospiraceae. Infect Immun. 2012;80(11):3786-94.
50. Inagaki H, Suzuki T, Nomoto K, Yoshikai Y. Increased susceptibility to primary infection with *Listeria monocytogenes* in germfree mice may be due to lack of accumulation of l-selectin+ CD44+ T cells in sites of inflammation. Infect Immun. 1996;64(8):3280-7.
51. Stecher B, Macpherson AJ, Hapfelmeier S, Kremer M, Stallmach T, Hardt WD. Comparison of *Salmonella enterica* serovar typhimurium colitis in germfree mice and mice pretreated with streptomycin. Infect Immun. 2005;73(6):3228-41.
52. von Klitzing E, Oz F, Ekmekciu I, Escher U, Bereswill S, Heimesaat MM. Comprehensive survey of intestinal microbiota changes in offspring of human microbiota-associated mice. Eur J Microbiol Immunol (Bp). 2017;7(1):65-75.
53. Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? Dis Model Mech. 2015;8(1):1-16.
54. Kovacs A, Ben-Jacob N, Tayem H, Halperin E, Iraqi FA, Gophna U. Genotype is a stronger determinant than sex of the mouse gut microbiota. Microb Ecol. 2011;61(2):423-8.
55. McCafferty J, Muhlbauer M, Gharaibeh RZ, Arthur JC, Perez-Chanona E, Sha W, Jobin C, Fodor AA. Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. Isme j. 2013;7(11):2116-25.
56. Thoene-Reineke C, Fischer A, Friese C, Briesemeister D, Gobel UB, Kammertoens T, Bereswill S, Heimesaat MM. Composition of intestinal microbiota in immune-deficient mice kept in three different housing conditions. PLoS One. 2014;9(11):e113406.
57. Hirayama K, Mishima M, Kawamura S, Itoh K, Takahashi E, Mitsuoka T. Effects of dietary supplements on the composition of fecal flora of human-flora-associated (hfa) mice. Bifidobacteria and Microflora. 1994;13(1):1-7.
58. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. Nature. 2009;457(7228):480-4.
59. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med. 2009;1(6):6ra14.
60. Bereswill S, Plickert R, Fischer A, Kuhl AA, Loddenkemper C, Batra A, Siegmund B, Gobel UB, Heimesaat MM. What you eat is what you get: Novel *Campylobacter* models in the quadrangle relationship between nutrition, obesity, microbiota and susceptibility to infection. Eur J Microbiol Immunol (Bp). 2011;1(3):237-48.
61. Faith JJ, McNulty NP, Rey FE, Gordon JI. Predicting a human gut microbiota's response to diet in gnotobiotic mice. Science. 2011;333(6038):101-4.
62. Neyrinck AM, Possemiers S, Druart C, Van de Wiele T, De Backer F, Cani PD, Larondelle Y, Delzenne NM. Prebiotic effects of wheat arabinoxylan related to the increase in *Bifidobacteria*, *Roseburia* and *Bacteroides/prevotella* in diet-induced obese mice. PLoS One. 2011;6(6):e20944.

63. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334(6052):105-8.
64. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, Reading NC, Villablanca EJ, Wang S, Mora JR, Umesaki Y, Mathis D, Benoist C, Relman DA, Kasper DL. Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 2012;149(7):1578-93.
65. Collins J, Auchtung JM, Schaefer L, Eaton KA, Britton RA. Humanized microbiota mice as a model of recurrent *Clostridium difficile* disease. Microbiome. 2015;3:35.
66. Alhede M, Bjarnsholt T, Givskov M, Alhede M. *Pseudomonas aeruginosa* biofilms: Mechanisms of immune evasion. Adv Appl Microbiol. 2014;86:1-40.
67. Korneev KV, Arbatsky NP, Molinaro A, Palmigiano A, Shaikhutdinova RZ, Shneider MM, Pier GB, Kondakova AN, Sviriaeva EN, Sturiale L, Garozzo D, Kruglov AA, Nedospasov SA, Drutskaya MS, Knirel YA, Kuprash DV. Structural relationship of the lipid a acyl groups to activation of murine Toll-like Receptor 4 by lipopolysaccharides from pathogenic strains of *Burkholderia mallei*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Front Immunol. 2015;6:595.
68. Alhazmi A. Nod-like Receptor(s) and host immune responses with *Pseudomonas aeruginosa* infection. Inflamm Res. 2018.
69. Yong VFL, Soh MM, Jaggi TK, Mac Aogain M, Chotirmall SH. The microbial endocrinology of *Pseudomonas aeruginosa*: Inflammatory and immune perspectives. Arch Immunol Ther Exp (Warsz). 2018.
70. Kipnis E, Sawa T, Wiener-Kronish J. Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Med Mal Infect. 2006;36(2):78-91.
71. Miao EA, Andersen-Nissen E, Warren SE, Aderem A. Tlr5 and Ipaf: Dual sensors of bacterial flagellin in the innate immune system. Semin Immunopathol. 2007;29(3):275-88.
72. Teghanemt A, Zhang D, Levis EN, Weiss JP, Gioannini TL. Molecular basis of reduced potency of underacylated endotoxins. J Immunol. 2005;175(7):4669-76.
73. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
74. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, Tedin K, Taha MK, Labigne A, Zahringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ, Philpott DJ. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science. 2003;300(5625):1584-7.
75. Inohara N, Nunez G. Nods: Intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol. 2003;3(5):371-82.
76. Diacovich L, Gorvel JP. Bacterial manipulation of innate immunity to promote infection. Nat Rev Microbiol. 2010;8(2):117-28.
77. Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, Aderem A. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via ipaf. Nat Immunol. 2006;7(6):569-75.
78. Miao EA, Ernst RK, Dors M, Mao DP, Aderem A. *Pseudomonas aeruginosa* activates caspase 1 through ipaf. Proc Natl Acad Sci U S A. 2008;105(7):2562-7.
79. Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozoren N, Jagirdar R, Inohara N, Vandenabeele P, Bertin J, Coyle A, Grant EP, Nunez G. Cytosolic flagellin requires ipaf for activation of caspase-1 and interleukin 1beta in *Salmonella*-infected macrophages. Nat Immunol. 2006;7(6):576-82.
80. Franchi L, Stoolman J, Kanneganti TD, Verma A, Ramphal R, Nunez G. Critical role for ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation. Eur J Immunol. 2007;37(11):3030-9.
81. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like Receptors in microbial recognition and host defense. Immunol Rev. 2009;227(1):106-28.
82. Adlard PA, Kirov SM, Sanderson K, Cox GE. *Pseudomonas aeruginosa* as a cause of infectious diarrhoea. Epidemiol Infect. 1998;121(1):237-41.
83. Kim SW, Peck KR, Jung SI, Kim YS, Kim S, Lee NY, Song JH. *Pseudomonas aeruginosa* as a potential cause of antibiotic-associated diarrhea. J Korean Med Sci. 2001;16(6):742-4.
84. Chuang CH, Wang YH, Chang HJ, Chen HL, Huang YC, Lin TY, Ozer EA, Allen JP, Hauser AR, Chiu CH. Shanghai fever: A distinct *Pseudomonas aeruginosa* enteric disease. Gut. 2014;63(5):736-43.
85. Dold H. On pyocyaneus sepsis and intestinal infections in shanghai due to *Bacillus pyocyaneus*. Chin Med J. 1918;32:435.
86. Chusid MJ, Hillmann SM. Community-acquired *Pseudomonas* sepsis in previously healthy infants. Pediatr Infect Dis J. 1987;6(7):681-4.

87. Martin-Ancel A, Borque C, del Castillo F. *Pseudomonas* sepsis in children without previous medical problems. Pediatr Infect Dis J. 1993;12(3):258-60.
88. Liesenfeld O, Kang H, Park D, Nguyen TA, Parkhe CV, Watanabe H, Abo T, Sher A, Remington JS, Suzuki Y. TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with *Toxoplasma gondii*. Parasite Immunol. 1999;21(7):365-76.
89. Liesenfeld O. Oral infection of c57bl/6 mice with *Toxoplasma gondii*: A new model of inflammatory bowel disease? J Infect Dis. 2002;185 Suppl 1:S96-101.
90. Erridge C, Duncan SH, Bereswill S, Heimesaat MM. The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5. PLoS One. 2010;5(2):e9125.
91. Munoz M, Liesenfeld O, Heimesaat MM. Immunology of *Toxoplasma gondii*. Immunol Rev. 2011;240(1):269-85.
92. Mooney JP, Lokken KL, Byndloss MX, George MD, Velazquez EM, Faber F, Butler BP, Walker GT, Ali MM, Potts R, Tiffany C, Ahmer BM, Luckhart S, Tsolis RM. Inflammation-associated alterations to the intestinal microbiota reduce colonization resistance against non-typhoidal *Salmonella* during concurrent malaria parasite infection. Sci Rep. 2015;5:14603.
93. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, Chaffron S, Macpherson AJ, Buer J, Parkhill J, Dougan G, von Mering C, Hardt WD. *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol. 2007;5(10):2177-89.
94. Wohlgemuth S, Keller S, Kertscher R, Stadion M, Haller D, Kisling S, Jahreis G, Blaut M, Loh G. Intestinal steroid profiles and microbiota composition in colitic mice. Gut Microbes. 2011;2(3):159-66.
95. Otto B, Haag LM, Fischer A, Plickert R, Kuhl AA, Gobel UB, Heimesaat MM, Bereswill S. *Campylobacter jejuni* induces extra-intestinal immune responses via Toll-like-Receptor-4 signaling in conventional IL-10 deficient mice with chronic colitis. Eur J Microbiol Immunol (Bp). 2012;2(3):210-9.
96. Heimesaat MM, Grundmann U, Alutis ME, Fischer A, Bereswill S. Microbiota composition and immune responses during *Campylobacter jejuni* infection in conventionally colonized IL-10-/- mice lacking nucleotide oligomerization domain 2. Eur J Microbiol Immunol (Bp). 2017;7(1):1-14.
97. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993;75(2):263-74.
98. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun. 1998;66(11):5224-31.
99. Wohlgemuth S, Haller D, Blaut M, Loh G. Reduced microbial diversity and high numbers of one single *Escherichia coli* strain in the intestine of colitic mice. Environ Microbiol. 2009;11(6):1562-71.
100. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of enterobacteriaceae. Cell Host Microbe. 2007;2(3):204.

2 Eidesstattliche Versicherung

„Ich, Eliane von Klitzing, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Untersuchungen zum Wechselspiel von multi-resistenten *Pseudomonas aeruginosa*, kommensaler Darmmikrobiota und Immunantworten bei muriner Darmentzündung“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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_____

Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Eliane von Klitzing hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:

von Klitzing E, Bereswill S, Heimesaat MM

Multidrug-resistant *Pseudomonas aeruginosa* induce systemic pro-inflammatory immune responses in colonized mice. Eur J Microbiol Immunol (Bp) 2017

Beitrag im Einzelnen: Versuchsplanung- und Vorbereitung, Tierarbeit mit antibiotischer Behandlung, Infektion mit *P. aeruginosa* und *E. coli*, tägliche Tiervisiten, klinische Beurteilung der Tiere, mikrobiologische Auswertung der fäkalen Proben, Sektion, Analyse der entnommenen Proben, Anfertigung und Auswertung immunhistochemischer Schnitte, statistische Ausarbeitung der Daten, Literaturrecherche, Bearbeitung des Manuskripts.

Publikation 2:

Von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM

Intestinal and systemic immune responses upon multi-drug resistant *Pseudomonas aeruginosa* colonization of mice harboring a human gut microbiota. Front Microbiol 2017

Beitrag im Einzelnen: Versuchsplanung- und Vorbereitung, Tierarbeit mit antibiotischer Behandlung, Aufarbeitung humaner und muriner Spenderlösungen, Fäkale-Mikrobiota Transplantation mit humaner und muriner Spenderlösung, Infektion mit *P. aeruginosa*, tägliche Tiervisiten, klinische Beurteilung der Tiere, mikrobiologische Auswertung der fäkalen Proben, Sektion, Analyse der entnommenen Proben, Anfertigung und Auswertung immunhistochemischer Schnitte, statistische Ausarbeitung der Daten, Literaturrecherche, Bearbeitung des Manuskripts.

Publikation 3:

von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM

Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. Gut Pathog 2017

Beitrag im Einzelnen: Versuchsplanung- und Vorbereitung, Tierarbeit mit antibiotischer Behandlung, Aufarbeitung humaner Spenderlösung, Fäkale-Mikrobiota Transplantation mit humaner und muriner Spenderlösung, Assistenz bei *T. gondii* Infektion, Infektion mit *P. aeruginosa*, tägliche Tiervisiten, klinische Beurteilung der Tiere, mikrobiologische Auswertung fäkaler Proben, Sektion, Analyse der entnommenen Proben, Anfertigung und Auswertung immunhistochemischer Schnitte, statistische Ausarbeitung der Daten, Literaturrecherche, Verfassung des Manuskripts.

Publikation 4:

von Klitzing E, Ekmekciu I, Kühl AA, Bereswill S, Heimesaat MM

Multidrug-resistant *Pseudomonas aeruginosa* infection aggravates inflammatory responses in murine chronic colitis. Sci Rep 2018

Beitrag im Einzelnen: Versuchsplanung- und Vorbereitung, Infektion mit *P. aeruginosa*, tägliche Tiervisiten, klinische Beurteilung der Tiere, mikrobiologische Auswertung fäkaler Proben, Sektion, Analyse der entnommenen Proben, Anfertigung und Auswertung immunhistochemischer Schnitte, Literaturrecherche, Bearbeitung des Manuskripts.

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

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Unterschrift der Doktorandin

3 Druckexemplare der ausgewählten Publikationen

Publikation 1:

von Klitzing E, Bereswill S, Heimesaat MM. Multidrug-resistant *Pseudomonas aeruginosa* induce systemic pro-inflammatory immune responses in colonized mice. Eur J Microbiol Immunol (Bp). 2017;7(3):200-9.

Aktuell noch kein Impact Factor, laut Research Gate taxiert auf: 2.03

Publikation 2:

von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM. Intestinal and systemic immune responses upon multi-drug resistant *Pseudomonas aeruginosa* colonization of mice harboring a human gut microbiota. Front Microbiol. 2017;8:2590.

2017 Journal Impact Factor: 4.019

Publikation 3:

von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM. Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. Gut Pathog. 2017;9:4.

2017 Journal Impact Factor: 2.809

Publikation 4:

von Klitzing E, Ekmekciu I, Kuhl AA, Bereswill S, Heimesaat MM. Multidrug-resistant *Pseudomonas aeruginosa* aggravates inflammatory responses in murine chronic colitis. Sci Rep. 2018;8(1):6685.

2017 Journal Impact Factor: 4.122

Original article

European Journal of Microbiology and Immunology 7 (2017) 3, pp. 200–209
DOI: 10.1556/1886.2017.00022

MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* INDUCE SYSTEMIC PRO-INFLAMMATORY IMMUNE RESPONSES IN COLONIZED MICE

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The World Health Organization has rated multidrug-resistant (MDR) *Pseudomonas aeruginosa* as a critical threat to human health. In the present study, we performed a survey of intestinal colonization, and local and systemic immune responses following peroral association of secondary abiotic mice with either a clinical MDR *P. aeruginosa* or a commensal murine *Escherichia coli* isolate. Depletion of the intestinal microbiota following antibiotic treatment facilitated stable intestinal colonization of both *P. aeruginosa* and *E. coli* that were neither associated with relevant clinical nor histopathological sequelae. Either stable bacterial colonization, however, resulted in distinct innate and adaptive immune cell responses in the intestines, whereas a pronounced increase in macrophages and monocytes could be observed in the small as well as large intestines upon *P. aeruginosa* challenge only, which also applied to colonic T lymphocytes. In addition, TNF secretion was exclusively elevated in large intestines of *P. aeruginosa*-colonized mice. Strikingly, association of secondary abiotic mice with MDR *P. aeruginosa,* but not commensal *E. coli,* resulted in pronounced systemic pro-inflammatory responses, whereas anti-inflammatory responses were dampened. Hence, intestinal carriage of MDR *P. aeruginosa* as compared to a mere commensal Gram-negative strain in otherwise healthy individuals results in distinct local and systemic pro-inflammatory sequelae.

Keywords: *Pseudomonas aeruginosa,* multi-drug resistant Gram-negative bacteria, susceptibility to infection, intestinal microbiota, colonization resistance, broad-spectrum antibiotic treatment, secondary abiotic (gnotobiotic) mice, pro-inflammatory immune responses, systemic sequelae of infection, commensal *Escherichia coli*

Introduction

The strictly aerobic, non-fermenting Gram-negative bacterium *Pseudomonas aeruginosa* can be ubiquitously found and particularly thrives in moist environments. Whereas a single flagellum and multiple cell surface pili contribute to bacterial motility and adherence to surfaces, biofilm formation, alginate secretion, quorum-sensing, intrinsic antibiotic resistance, and an elaborated secretion system constitute further virulence factors of *P. aeruginosa* facilitating immune escape and establishment in the host ecosystem [1, 2]. In the hospital setting, water bottles, respiratory equipment, and sinks are typical environmental reservoirs [3]. *P. aeruginosa* is considered an opportunistic pathogen and one of the most frequent causative agents of acute nosocomial infections, particularly affecting immune-compromised (such as neutropenic) individuals or patients admitted to the intensive care unit (ICU) [4, 5]. *P. aeruginosa*-induced infections including ventilator-associated pneumonia, burn wound, and surgical site infections, as well as urinary tract infections are associated with very high (i.e., >30%) mortality rates [4]. In addition, *P. aeruginosa* drives chronic respiratory infections in patients suffering from cystic fibrosis, chronic

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obstructive lung disease, or bronchiectasis [5–7]. In recent years, emerging infections with strains that were resistant to a plethora of antibiotic classes due to β-lactamases, 16S rRNA methylases, and carbapenemases have been associated with significant morbidity and increasing mortality [5, 7, 8]. This fatal scenario has prompted the World Health Organization (WHO) in the beginning of 2017 to rate multidrug-resistant (MDR) Gram-negative species including *P. aeruginosa* as serious threats for human health, further emphasizing the urgent need for novel treatment approaches [9]. In addition to colonized patients, contaminated surfaces, and objects in the hospital setting as potential external sources for *P. aeruginosa* acquisition, the human gastrointestinal tract might be considered as important internal source for *P. aeruginosa* infection [10, 11]. Although *P. aeruginosa* is not considered part of the human commensal gut microbiota, intestinal colonization usually precedes *P. aeruginosa* infection. This might particularly be the case upon antibiotic treatment compromising the intestinal microbiota integrity and, hence, physiological colonization resistance subsequently facilitating establishment of the opportunistic pathogen in the intestinal ecosystem [5, 12]. In fact, a recent study revealed that prior rectal colonization was a predictor of subsequent development of *P. aeruginosa* infection of ICU patients [13].

To date, however, no data are available whether intestinal *P. aeruginosa* carriage by otherwise healthy individuals that are treated with antibiotic compounds is accompanied with host immune responses. This prompted us in the present study to treat mice with broad-spectrum antibiotics and to challenge them with either a clinical MDR *P. aeruginosa* or a murine commensal *Escherichia coli* strain perorally. We determined intestinal colonization capacities of respective strains either under continuous antibiotic treatment or after antibiotic withdrawal as compared to uncompromised gut microbiota conditions and further assessed intestinal as well as systemic pro- and anti-inflammatory responses in otherwise healthy bacterial carriers.

Materials and methods

Mice and broad-spectrum antibiotic treatment

C57BL/6j wildtype mice were reared and maintained under specific pathogen-free (SPF) conditions in the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité – University Medicine Berlin). At the age of 8 to 10 weeks, female mice were subjected to broad-spectrum antibiotic treatment. In brief, mice were transferred to sterile cages and treated with a quintuple antibiotic cocktail consisting of ampicillin plus sulbactam (1 g/l; Ratiopharm, Ulm, Germany), vancomycin (500 mg/l; Cell Pharm, Hannover, Germany), ciprofloxacin (200 mg/l; Bayer Vital, Leverkusen, Germany), imipenem (250 mg/l; MSD, Haar, Germany), and metronidazole (1 g/l; Fresenius, Bad Homburg, Germany) via the drinking water *ad libitum* for 8 weeks [14, 15]. Cultural and culture-independent (i.e., 16S rRNA based molecular) quality control measures revealed virtual absence of bacteria in fecal samples as described earlier [15, 16]. In one group of thus generated secondary abiotic (i.e., gnotobiotic) mice, the antibiotic cocktail was replaced by sterile water three days before *P. aeruginosa* infection, whereas in another group, antibiotic treatment was continued until the end of the experiment. Sex- and age-matched conventionally colonized mice served as antibiotics-untreated control group.

Bacterial strains

The MDR *P. aeruginosa* isolate was initially cultured from respiratory material of a patient with nosocomial pneumonia and kindly provided by Prof. Dr. Bastian Opitz (Charité – University Medicine Berlin, Berlin, Germany). Notably, the bacterial strain displayed antimicrobial sensitivity to fosfomycin and colistin only [17].

The commensal *E. coli* strain had been isolated from a conventional wildtype mouse recently [14]. Of note, the genome of the applied *E. coli* strain did not contain known virulence genes of pathogenic *E. coli* such as *stx 1* and *2, hlyA, cspA, catA, astA,* and *katP* as confirmed by PCR analysis in a reference laboratory [18].

Prior infection, the *P. aeruginosa* and *E. coli* strains were grown on cetrimid agar (Oxoid) for 48 h and MacConkey agar (Oxoid) for 24 h (both from Oxoid), respectively, in an aerobic atmosphere at 37 °C.

Bacterial challenge

On two consecutive days (i.e., days 0 and 1), mice were perorally challenged either with 10^9 colony forming units (CFU) of the MDR *P. aeruginosa* or the commensal *E. coli* strain by gavage in a total volume of 0.3 ml PBS as reported earlier [14, 17, 18].

Cultural analysis of P. aeruginosa *and* E. coli

Fecal samples were homogenized in sterile PBS. Serial dilutions were then streaked onto Columbia agar supplemented with 5% sheep blood (Oxoid, Germany) and cetrimid or MacConkey agar and incubated in an aerobic atmosphere at 37 °C for 48 and 24 h in order to assess intestinal *P. aeruginosa* and *E. coli* loads, respectively, as described previously [14, 17, 18].

Clinical conditions

Macroscopic and/or microscopic abundance of fecal blood was assessed in individual mice on a daily basis by the Guajac method using Haemoccult (Beckman Coulter/PCD, Germany) as described earlier [18–20].

Sampling procedures

Mice were sacrificed 4 weeks (28 days) postinfection (p.i.) by isoflurane treatment (Abott, Germany). Cardiac blood (for serum) and tissue samples from spleen, mesenteric lymph nodes (MLN), ileum, and colon were removed under sterile conditions. Intestinal samples were collected from each mouse in parallel for microbiological, immunological, and immunohistochemical analyses.

Immunohistochemistry

Five-micrometer thin paraffin sections of colonic and ileal *ex vivo* biopsies were used for *in situ* immunohistochemical analysis as reported previously [19–21]. In brief, primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Glostrup, Denmark, 1:100), F4/80 (no. 14-4801, clone BM8, eBioscience, 1:50), CD3 (no. N1580, Dako, 1:10), FOXP3 (FJK-16s, eBioscience, San Diego, CA, USA, 1:100), and B220 (eBioscience, 1:200) were used to assess apoptotic cells, proliferating cells, macrophages/monocytes, T lymphocytes, regulatory T cells (Treg), and B lymphocytes, respectively. The average numbers of positively stained cells within at least six high power fields (HPF, 0.287 mm^2; 400× magnification) were determined by an independent blinded investigator.

Cytokine detection

Ex vivo biopsies (approximately 1 cm^2) derived from colon (cut longitudinally and washed in PBS), MLN, and spleen were placed in 24-well flat-bottom culture plates (Falcon, Germany) containing 500 ml serum-free RPMI 1640 medium (Gibco, life technologies) supplemented with penicillin (100 U/ml, Biochrom, Germany) and streptomycin (100 µg/ml; Biochrom). After 18 h at 37 °C, culture supernatants and serum samples were tested for TNF, IFN-γ, and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Bioscience) on a BD FACSCanto II flow cytometer (BD Bioscience).

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney *U* test. Two-sided probability (*p*) values of ≤0.05 were considered significant. Experiments were reproduced twice.

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration numbers G0039/15). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice.

Results

Broad-spectrum antibiotic treatment facilitates intestinal colonization of mice with MDR P. aeruginosa

We first addressed how efficient an MDR *P. aeruginosa* strain was able to colonize the intestinal tract of clinically uncompromised wildtype mice with a conventional gut microbiota. The first 3 days following peroral challenge with 10^9 CFU *P. aeruginosa,* fecal loads between 10^3 and 10^5 per gram could be assessed on two consecutive days ($p < 0.001$ versus uninfected mice; *Fig. 1A*) but were progressively declining thereafter. In fact, only 15.8% of conventional mice harbored *P. aeruginosa* in their intestines between days 4 and 6 postinfection (p.i.) (with median loads of 0 CFU per gram; n.s. versus uninfected mice; *Fig. 1A*), whereas *P. aeruginosa* could be isolated from fecal samples in single cases only later on.

Given that the commensal intestinal microbiota is known to provide the host with a physiological colonization resistance thereby preventing from (opportunistic) pathogenic infection [15, 22], we next generated secondary abiotic mice following broad-spectrum antibiotic treatment. Three days following an antibiotic withdrawal, mice with a virtually depleted gut microbiota were perorally challenged with 10^9 CFU *P. aeruginosa* on days 0 and 1. Within 24 h p.i., *P. aeruginosa* could establish within the intestinal tract of secondary abiotic mice at high median loads of 10^8 CFU per gram feces that remained stable until day 28 p.i. ($p < 0.01$–0.001; *Fig. 1B*).

We further perorally associated secondary abiotic mice under continuous antibiotic treatment with 10^9 CFU *P. aeruginosa,* but this time without antibiotic withdrawal and washout. Whereas within 24 h p.i. viable bacteria could be isolated from almost all fecal samples with median loads of 10^4 CFU per gram ($p < 0.001$ versus day 0; *Fig. 1C*), *P. aeruginosa* detection rates progressively declined until day 6 p.i. After this nadir, however, *P. aeruginosa* could again be isolated from fecal samples in 90% of mice at day 7 p.i., and median bacterial loads further increased up to 10^8 CFU per gram until day 28 p.i. *(Fig. 1C)* which had been observed in secondary abiotic mice *(Fig. 1B)*. Notably, under continuous antibiotic treatment, also small colony variants of *P. aeruginosa* could be detected, particularly during the nadir between days 4 and 6 p.i., but also thereafter.

Hence, depletion of the intestinal microbiota following antibiotic treatment facilitated stable MDR *P. aeruginosa* colonization of the intestinal tract.

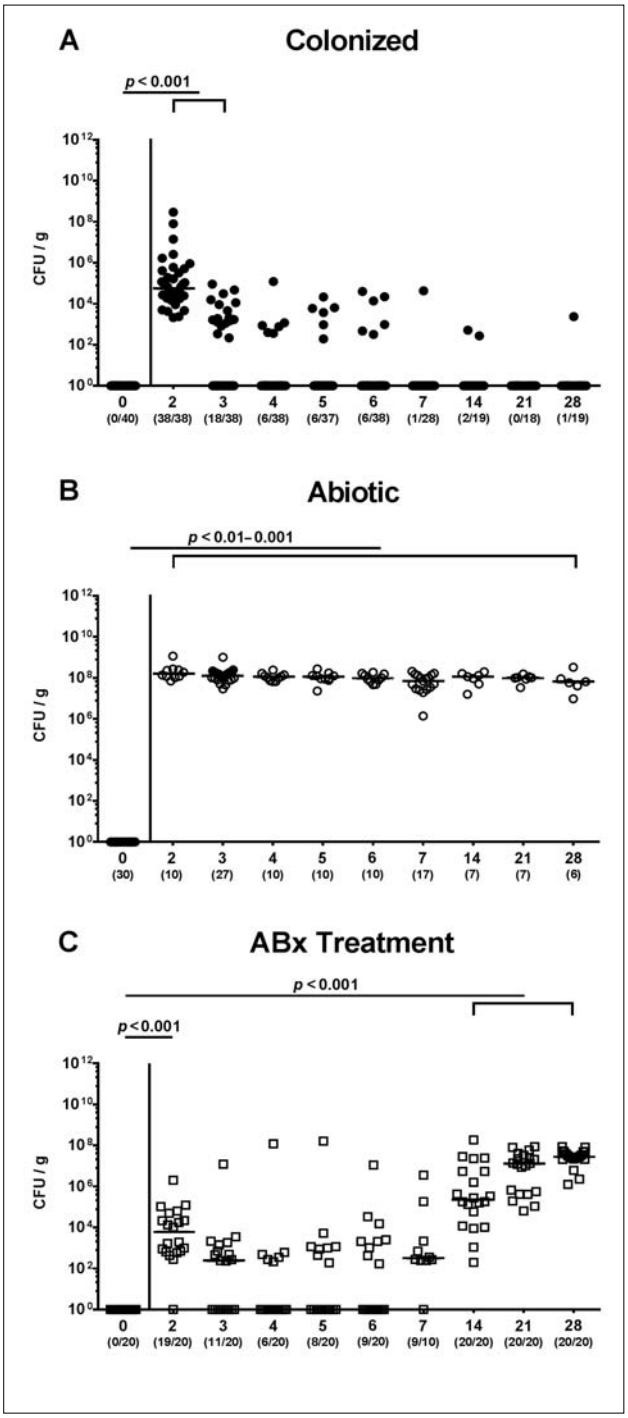

Fig. 1. Fecal *P. aeruginosa* loads over time following peroral challenge of mice. (A) Conventionally colonized wildtype mice (black circles), (B) secondary abiotic mice generated by broad-spectrum antibiotic (ABx) treatment as described in methods (ABx treatment withdrawn 3 days prior infection; white circles), and (C) secondary abiotic mice under continuous ABx treatment (white squares) were perorally associated with a multidrug resistant *P. aeruginosa* strain on day (d) 0. Intestinal colonization densities were determined in fecal samples until day 28 postinfection by culture and expressed as colony forming units per gram (CFU/g). Numbers of mice harboring *P. aeruginosa* out of the total number of analyzed mice (in parentheses), medians (black bars), and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown were pooled from at least three independent experiments

Comparative colonization properties of MDR P. aeruginosa *and commensal* E. coli *in secondary abiotic mice*

We next addressed whether the intestinal colonization properties of the applied MDR *P. aeruginosa* strain differed from those of a commensal murine Gram-negative strain such as *E. coli* that had been isolated from feces of a conventionally colonized wildtype mouse previously. Therefore, after a 3-day antibiotic washout period, secondary abiotic mice were either perorally associated with 10^9 CFU MDR *P. aeruginosa* or commensal *E. coli* by gavage and the bacterial loads were determined in fecal samples thereafter. Whereas until day 7 p.i. intestinal *E. coli* loads were approximately one order of magnitude higher as compared to *P. aeruginosa* ($p < 0.01$–0.001; *Fig. 2*), respective fecal bacterial burdens were comparable thereafter until the end of the experiment (i.e., 10^8 CFU per gram at day 28 p.i.; n.s., *Fig. 2*).

Hence, MDR *P. aeruginosa* displayed virtually comparable intestinal colonization properties like a commensal Gram-negative strain such as *E. coli*.

Macroscopic and microscopic inflammatory sequelae following MDR P. aeruginosa *colonization of secondary abiotic mice*

We further assessed whether potential inflammatory sequelae of MDR *P. aeruginosa* colonization of secondary abiotic mice could be observed on macroscopic (i.e., clinical) or microscopic (i.e., histological) level. Notably, neither following MDR *P. aeruginosa* nor upon commensal

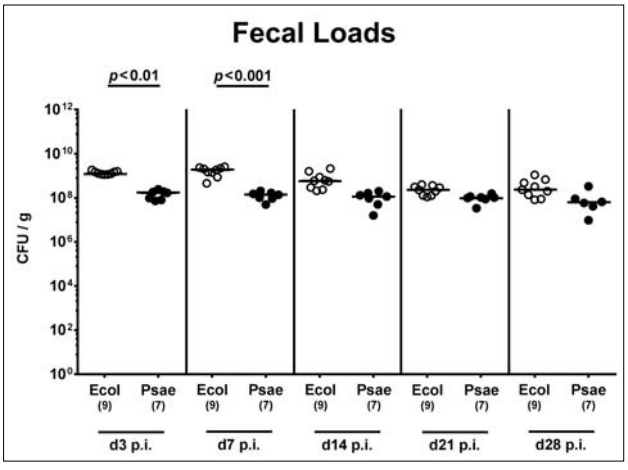

Fig. 2. Fecal bacterial loads over time following peroral MDR *P. aeruginosa* association of secondary abiotic mice. Secondary abiotic mice (antibiotic treatment withdrawn 3 days prior infection) were perorally associated with a murine commensal *E. coli* (Ecol, white circles) or a multidrug resistant *P. aeruginosa* strain (Psae, black circles) on day (d) 0. Intestinal colonization densities were determined in fecal samples over time until day 28 postinfection (p.i.) by culture and expressed as colony forming units per gram (CFU/g). Numbers of mice, medians (black bars), and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown were pooled from two independent experiments

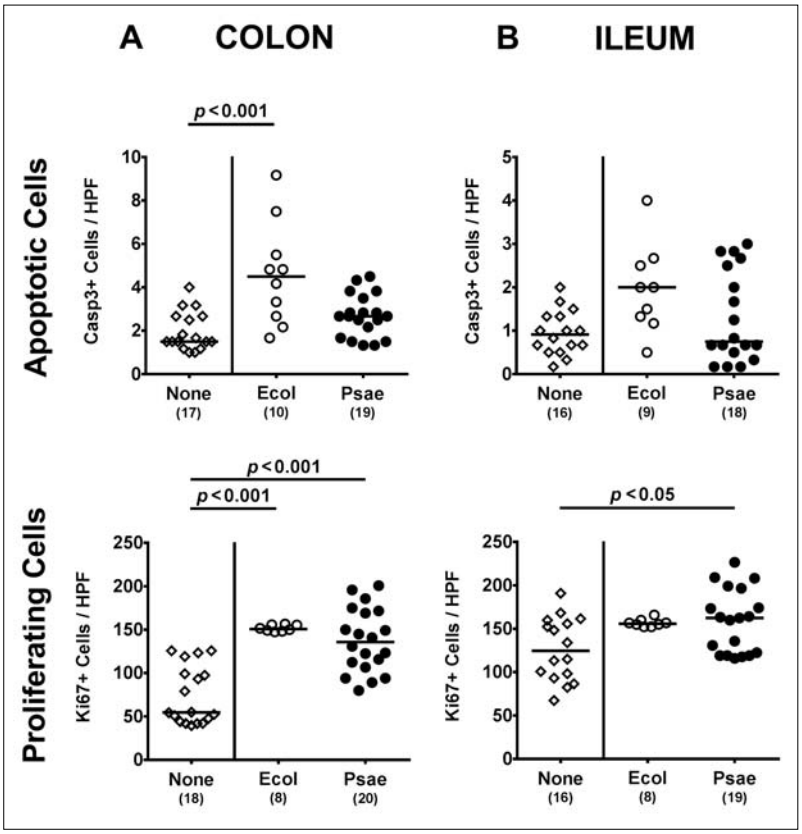

Fig. 3. Apoptotic and proliferating intestinal epithelial cells following peroral MDR *P. aeruginosa* association of secondary abiotic mice. Secondary abiotic mice were perorally associated with a murine commensal *E. coli* (Ecol, white circles) or a multidrug resistant *P. aeruginosa* strain (Psae, black circles) on day (d) 0. Four weeks thereafter (i.e., on day 28 postinfection), the average numbers of epithelial apoptotic (positive for caspase 3, Casp3; upper panel) and proliferating cells (positive for Ki67; lower panel) were determined in *ex vivo* biopsies derived from the (A) colon (left panel) and (B) ileum (right panel) in six high power fields (HPF, 400× magnification) per animal in immunohistochemically stained intestinal paraffin sections. Uninfected mice (none; open diamonds) served as negative controls. Numbers of mice, medians (black bars), and significance levels (p values) determined by the Mann–Whitney U test are indicated. Data shown were pooled from three independent experiments

E. coli challenge, secondary abiotic mice were clinically compromised and exhibited symptoms such as weight loss, wasting, diarrhea, or macroscopic/microscopic abundance of fecal blood (data not shown). We next quantitatively assessed apoptotic cell numbers in intestinal epithelia applying *in situ* immunohistochemistry. Neither in the colon nor in the ileum of MDR *P. aeruginosa*-associated secondary abiotic mice increased apoptotic epithelial cell numbers could be observed (n.s., *Fig. 3*), whereas caspase3+ cells were even higher in the large intestinal epithelia of mice harboring commensal *E. coli* as compared to naive controls ($p < 0.001$; *Fig. 3A*).

We further determined numbers of Ki67+ cells in intestinal epithelia indicative for cell proliferation and regeneration counteracting potential bacteria-induced intestinal inflammatory responses. Remarkably, Ki67+ counts were multifold increased in large intestinal epithelia at day 28 following association of secondary abiotic mice with either strain ($p < 0.001$; *Fig. 3A*), whereas in the ileal epithelia numbers of proliferating cell were elevated upon MDR *P. aeruginosa* challenge only ($p < 0.05$; *Fig. 3B*).

Hence, MDR *P. aeruginosa* did neither induce macroscopic (i.e., clinical) nor microscopic (i.e., apoptotic) sequelae upon stable intestinal colonization of secondary abiotic mice.

Intestinal immune cell responses following MDR P. aeruginosa *colonization of secondary abiotic mice*

We next determined whether association of secondary abiotic mice with MDR *P. aeruginosa* was accompanied by an increased influx of pro-inflammatory immune cell populations into the intestinal mucosa and lamina propria. To address this, we applied quantitative *in situ* immunohistochemistry of small and large intestinal *ex vivo* biopsies that had been stained with antibodies against distinct innate and adaptive immune cell subsets. Following association with MDR *P. aeruginosa,* but not commensal *E. coli,* numbers of innate immune cells such as macrophages and monocytes increased more than two-fold in both colon and ileum of secondary abiotic mice at day 28 p.i. ($p < 0.001$; *Fig. 4*). Whereas increased T lymphocyte numbers could be determined in the colon of *P. aeruginosa* associated mice only ($p < 0.001$; *Fig. 4A*), this held true for increased ileal CD3+ counts at day 28 upon *E. coli*

challenge ($p < 0.001$; *Fig. 4B*). Following association with either bacterial strain, colonic numbers of Treg and B lymphocytes were higher as compared to naive secondary abiotic mice ($p < 0.01$–0.001; *Fig. 4A*), whereas this held true for Treg counts in the ileal mucosa and lamina propria at day 28 p.i. ($p < 0.001$; *Fig. 4B*).

Hence, both MDR *P. aeruginosa* and commensal *E. coli* association resulted in distinct innate and adap-

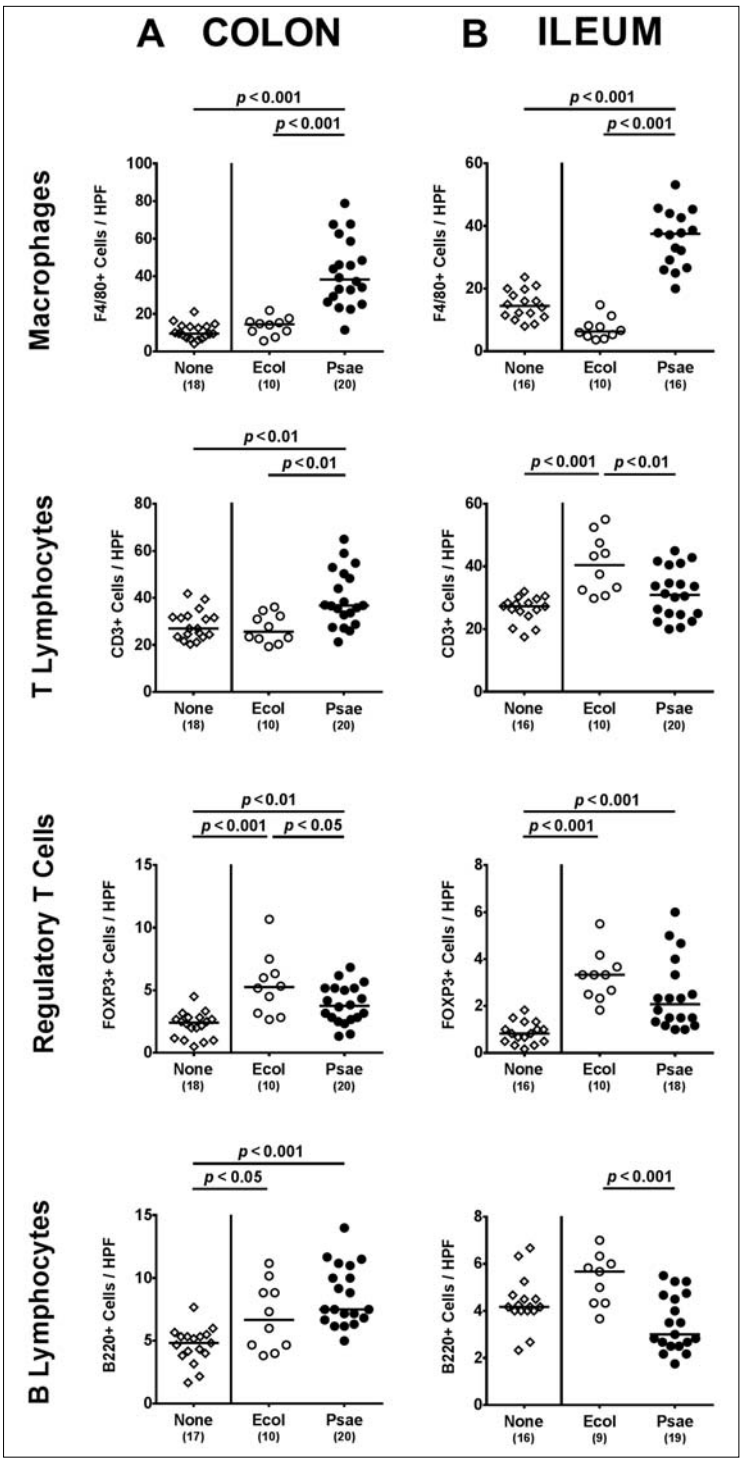

Fig. 4. Intestinal immune cell responses following peroral MDR *P. aeruginosa* association of secondary abiotic mice. Secondary abiotic mice were perorally associated with a murine commensal *E. coli* (Ecol, white circles) or a multidrug resistant *P. aeruginosa* strain (Psae, black circles) on day (d) 0. Four weeks thereafter (i.e., on day 28 postinfection), the average numbers of macrophages and monocytes (positive for F4/80), colonic T lymphocytes (positive for CD3), regulatory T cells (positive for FOXP3), and B lymphocytes (positive for B220) were determined in *ex vivo* biopsies derived from the (A) colon (left panel) and (B) ileum (right panel) in six high power fields (HPF, 400× magnification) per animal in immunohistochemically stained intestinal paraffin sections. Uninfected mice (none; open diamonds) served as negative controls. Numbers of mice, medians (black bars), and significance levels (p values) determined by the Mann–Whitney U test are indicated. Data shown were pooled from three independent experiments

tive immune cell responses in the intestines of secondary abiotic mice, whereas a pronounced increase in macrophages and monocytes could be observed in the small as well as large intestines upon MDR *P. aeruginosa* challenge only, which also applied to colonic T lymphocytes.

Intestinal cytokine secretion following peroral MDR P. aeruginosa *association of secondary abiotic mice*

Next, we addressed whether the observed intestinal immune cell responses upon bacterial challenge were accompanied by pronounced local secretion of pro- and anti-inflammatory cytokines. At day 28 following MDR *P. aeruginosa,* but not commensal *E. coli* association of secondary abiotic mice, colonic TNF concentrations were more than two-fold higher as compared to uninfected controls ($p < 0.05$; *Fig. 5A*), whereas higher TNF secretion could be determined in MLN of *E. coli* associated versus non-associated mice ($p < 0.001$, *Fig. 5B*). At day 28 following challenge with either *E. coli* or *P. aeruginosa,* increased IFN-γ secretion could be measured in MLN as compared to naive controls ($p < 0.05$ and $p < 0.001$, respectively; *Fig. 5B*) with even higher concentrations in the former as compared to the latter associated mice ($p < 0.05$; *Fig. 5B*). Notably, secretion of the anti-inflammatory cytokine IL-10 was virtually unchanged in the colon and MLN at day 28 following either bacterial association.

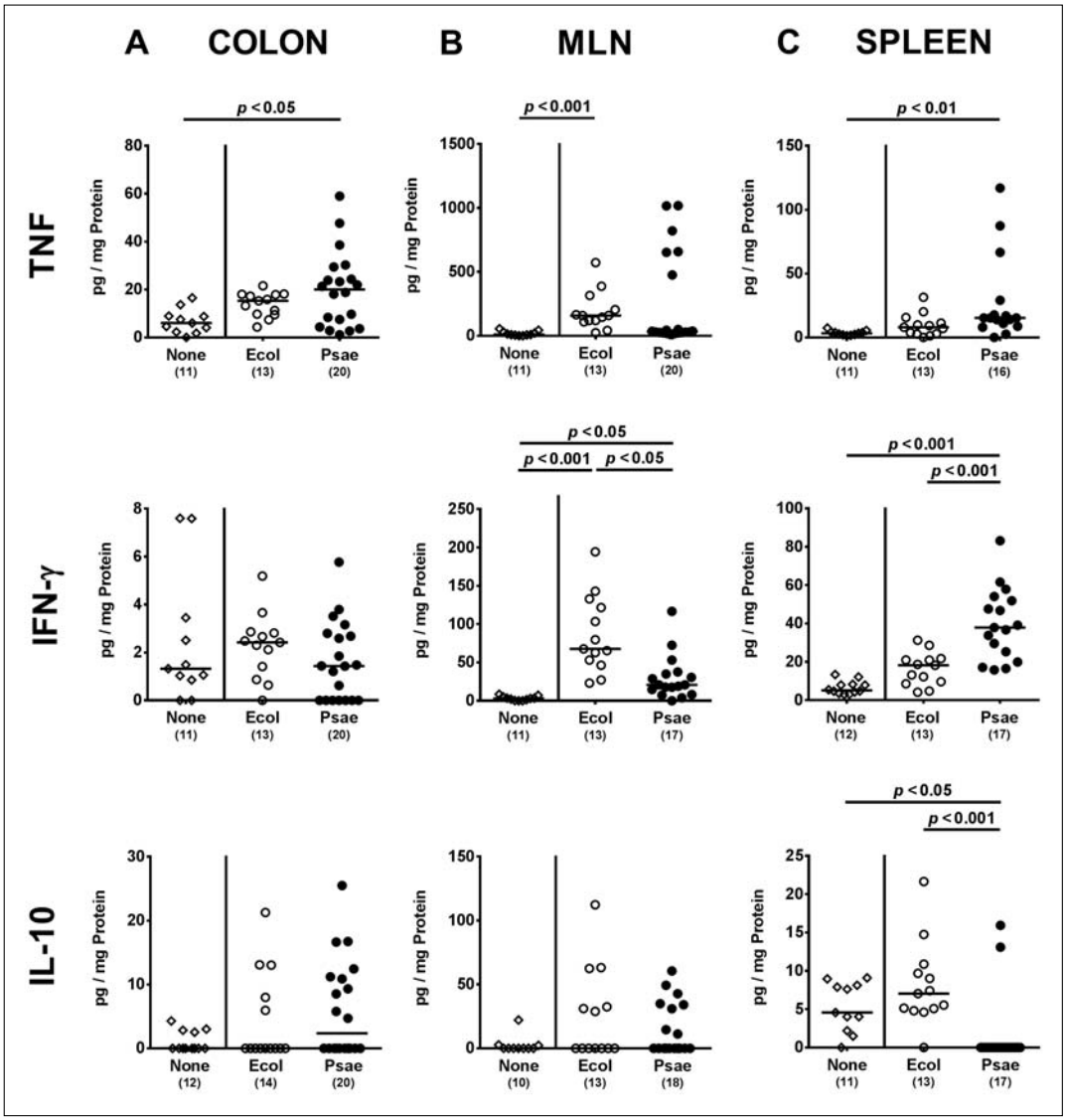

Fig. 5. Intestinal cytokine responses following peroral MDR *P. aeruginosa* association of secondary abiotic mice. Secondary abiotic mice were perorally associated with a murine commensal *E. coli* (Ecol, white circles) or a multidrug resistant *P. aeruginosa* strain (Psae, black circles) on day (d) 0. Four weeks thereafter (i.e., on day 28 postinfection), proinflammatory cytokines including TNF (upper panel) and IFN-γ (middle panel) as well as the anti-inflammatory mediator IL-10 (lower panel) were measured in supernatants of *ex vivo* biopsies derived from the (A) colon (left panel), (B) mesenteric lymph nodes (MLN; middle panel), and (C) spleen (right panel). Uninfected mice (none; open diamonds) served as negative controls. Numbers of mice, medians (black bars), and significance levels (*p* values) determined by the Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments

Hence, TNF secretion was exclusively increased in large intestines of MDR *P. aeruginosa* associated secondary abiotic mice.

Systemic cytokine secretion following peroral MDR P. aeruginosa *association of secondary abiotic mice*

We finally addressed systemic pro- and anti-inflammatory sequelae upon bacterial association of secondary abiotic mice. Remarkably, *P. aeruginosa* but not *E. coli* challenge induced a multifold increased secretion of pro-inflammatory cytokines such as TNF and IFN-γ in the spleen at day 28 ($p < 0.01$ and $p < 0.001$ versus naive, respectively; *Fig. 5C*), whereas anti-inflammatory IL-10 concentrations were downregulated in spleens of *P. aeruginosa* associated mice far below baseline levels ($p < 0.001$ versus naive; *Fig. 5C*).

Hence, association of secondary abiotic mice with MDR *P. aeruginosa* but not commensal *E. coli* resulted in pronounced systemic pro-inflammatory sequelae, whereas anti-inflammatory responses were dampened.

Discussion

Whereas infections with MDR Gram-negative bacteria including *P. aeruginosa* have become a serious threat, particularly in hospitalized patients under antibiotic therapy [2, 11], it is to date unclear, however, whether mere carriage of an MDR *P. aeruginosa* strain in the gastrointestinal tract of otherwise healthy individuals provokes pro-inflammatory immune responses. In the present study, we therefore treated mice with broad-spectrum antibiotic compounds and challenged them perorally with a clinical MDR *P. aeruginosa* strain – either during continuous antibiotic treatment or after antibiotic withdrawal to assure antibiotic wash-out. Upon cessation of antibiotics, mice could be stably colonized by the opportunistic pathogenic strain as early as 24 h p.i. and displayed continuously high bacterial burdens in their feces until the end of the observation period (i.e., day 28 p.i.). These results are indicative for successful disruption of the colonization resistance (that is physiologically provided by the complex and diverse commensal intestinal microbiota) which in turn facilitates establishment and invasion of opportunistic or obligate pathogens in mice and men [15, 22–24]. The vast majority of mice with an uncompromised intestinal microbiota, however, had expelled the clinical isolate within 4 days p.i.; after 7 days p.i., *P. aeruginosa* could be cultured from fecal samples in single cases and at very low loads only. Our results are well in line with an earlier study where mice had been treated with five different antibiotics and exhibited distinct intestinal *P. aeruginosa* loads at day 7 following peroral infection depending on the degree of the compromised colonization resistance due to respective antimicrobial spectra of the applied compound [23]. Furthermore, ingested *P. aeruginosa* could be cultured from fecal samples of healthy volunteers with an intact gut microbiota up to 6 days following peroral bacterial challenge [25]. In subjects who had a 4-day course of oral ampicillin treatment, however, *P. aeruginosa* persisted for 14 days.

Interestingly, under continuous broad-spectrum antibiosis, *P. aeruginosa* could stably establish within the intestinal tract rather late (i.e., after 2 to 3 weeks) p.i., but finally reached comparably high fecal loads as compared to mice in which the antibiotic cocktail had been withdrawn before bacterial challenge. Even though tested resistant against each antibiotic compound in our *in vitro* antimicrobial susceptibility assays, the nadir in fecal *P. aeruginosa* loads between day 3 and day 14 p.i. in addition to the abundance of small colony variants point toward synergistic antimicrobial properties of the components within the quintuple antibiotic cocktail. Remarkably, neither antimicrobial treatment nor respective bacterial colonization was associated with any clinical symptoms in challenged mice. This is in line with an earlier study by Hentges et al. who did not report any clinical symptoms in mice upon treatment with five different antibiotic compounds and subsequent *P. aeruginosa* infection [23], which also held true for healthy volunteers subjected to *P. aeruginosa* with or without preceding ampicillin treatment [25]. Furthermore, following peroral application of the clinical isolate or the commensal *E. coli* strain in our study, secondary abiotic mice (with withdrawn antibiosis) harbored comparable fecal bacterial loads indicating that the clinical *P. aeruginosa* isolate exhibited intestinal colonization like a murine commensal Gram-negative strain.

In line with lacking macroscopic (i.e., clinical) sequelae, *P. aeruginosa* colonization was neither associated with histopathological nor apoptotic responses affecting the intestinal mucosa and epithelial cells in particular. Interestingly, either bacterial association, however, resulted in increased proliferating/regenerating epithelial cell numbers in both small and large intestines thereby counteracting potential cell damage. Remarkably, MDR *P. aeruginosa* colonization induced pronounced innate as well as adaptive immune cell responses as indicated by elevated numbers of macrophages/monocytes as well as T lymphocytes, Treg and B cells, respectively, in intestinal mucosa and lamina propria at day 28 p.i., that were accompanied by multifold increased pro-inflammatory cytokine concentrations such as TNF and IFN-γ in colon and MLN, respectively. Strikingly, *P. aeruginosa* carriage induced not only immune responses in the intestinal tract but also systemically as indicated by increased splenic TNF and IFN-γ secretion at day 28 p.i., whereas anti-inflammatory IL-10 concentration was virtually undetectable by then.

Preceding antibiotic treatment facilitates intestinal *P. aeruginosa* colonization, which in turn bears a 15-fold increased risk for a nosocomial infection on ICU [13]. Whereas the pathogenic potential of *P. aeruginosa* is well known, its potential in initiation and perpetuation of immunopathologies, particularly within the intestinal tract, is only incompletely understood. In a very recent study, we were able to show that acute ileitis facilitated infection

with MDR *P. aeruginosa* in mice harboring a human gut microbiota, but the inflammation model was too acute to decipher additional inflammatory sequelae induced by the MDR bacterial strain [17].

We conclude that upon preceding broad-spectrum antibiotic treatment a clinical MDR *P. aeruginosa* isolate is capable of colonizing the intestinal tract like a commensal Gram-negative strain such as murine *E. coli*. Already mere MDR *P. aeruginosa* carriage, however, is associated with both intestinal and systemic immune responses that might be potentiated in concert with other comorbidities. Further studies need to unravel the orchestrated interplay of MDR Gram-negative strains including *P. aeruginosa,* host microbiota and immunity, particularly under inflammatory conditions.

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Conflict of interest

Stefan Bereswill and Markus M. Heimesaat are Editorial Board members.

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References

1. Driscoll JA, Brody SL, Kollef MH: The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs 67, 351–368 (2007)
2. Gellatly SL, Hancock RE: *Pseudomonas aeruginosa:* new insights into pathogenesis and host defenses. Pathog Dis 67, 159–173 (2013)
3. Stratton CW: *Pseudomonas aeruginosa* revisited. Infect Control Hosp Epidemiol 11, 101–104 (1990)
4. Vincent JL: Nosocomial infections in adult intensive-care units. Lancet 361, 2068–2077 (2003)
5. Oliver A, Mulet X, Lopez-Causape C, Juan C: The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updates 21–22, 41–59 (2015)
6. Lyczak JB, Cannon CL, Pier GB: Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect 2, 1051–1060 (2000)
7. Potron A, Poirel L, Nordmann P: Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii:* mechanisms and epidemiology. Int J Antimicrob Agents 45, 568–585 (2015)
8. Livermore DM: Has the era of untreatable infections arrived? J Antimicrob Chemother 64 Suppl 1, 29–36 (2009)
9. Tacconelli E, Magrini N: Global priority list of antibiotic-resistant bacteria to guide research discovery and development of new antibiotics. World Health Organization (2017)
10. Shooter RA, Walker KA, Williams VR, Horgan GM, Parker MT, Asheshov EH, Bullimore JF: Faecal carriage of *Pseudomonas aeruginosa* in hospital patients. Possible spread from patient to patient. Lancet 2, 1331–1334 (1966)
11. Cohen R, Babushkin F, Cohen S, Afraimov M, Shapiro M, Uda M, Khabra E, Adler A, Ben Ami R, Paikin S: A prospective survey of *Pseudomonas aeruginosa* colonization and infection in the intensive care unit. Antimicrob Resist Infect Control 6, 7 (2017)
12. Ohara T, Itoh K: Significance of *Pseudomonas aeruginosa* colonization of the gastrointestinal tract. Intern Med 42, 1072–1076 (2003)
13. Gomez-Zorrilla S, Camoez M, Tubau F, Canizares R, Periche E, Dominguez MA, Ariza J, Pena C: Prospective observational study of prior rectal colonization status as a predictor for subsequent development of *Pseudomonas aeruginosa* clinical infections. Antimicrob Agents Chemother 59, 5213–5219 (2015)
14. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, Jahn HK, Dunay IR, Moter A, Gescher DM, Schumann RR, Göbel UB, Liesenfeld O: Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. J Immunol 177, 8785–8795 (2006)
15. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kühl AA, Dasti JI, Zautner AE, Muñoz M, Loddenkemper C, Loddenkemper C, Gross U, Göbel UB, Heimesaat MM: Novel murine infection models provide deep insights into the "ménage à trois" of *Campylobacter jejuni,* microbiota and host innate immunity. PLoS One 6, e20953 (2011)
16. Ekmekciu I, von Klitzing E, Fiebiger U, Escher U, Neumann C, Bacher P, Scheffold A, Kuhl AA, Bereswill S, Heimesaat MM: Immune responses to broad-spectrum antibiotic treatment and fecal microbiota transplantation in mice. Front Immunol 8, 397 (2017)
17. von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM: Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. Gut Pathog 9, 4 (2017)
18. Haag LM, Fischer A, Otto B, Plickert R, Kühl AA, Göbel UB, Bereswill S, Heimesaat MM: *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10$^{-/-}$ mice via Toll-like-receptor-2 and -4 signaling. PLoS One 7, e40761 (2012)
19. Alutis ME, Grundmann U, Fischer A, Hagen U, Kühl AA, Göbel UB, Bereswill S, Heimesaat MM: The role of gelatinases in *Campylobacter jejuni* infection of gnotobiotic mice. Eur J Microbiol Immunol (Bp) 5, 256–267 (2015)
20. Alutis ME, Grundmann U, Hagen U, Fischer A, Kühl AA, Göbel UB, Bereswill S, Heimesaat MM: Matrix metallo-

proteinase-2 mediates intestinal immunopathogenesis in *Campylobacter jejuni*-infected infant mice. Eur J Microbiol Immunol (Bp) 5, 188–198 (2015)
21. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, Steinhoff U, Tchaptchet S, Thiel E, Freudenberg MA, Göbel UB, Uharek L: MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut 59, 1079–1087 (2010)
22. Fiebiger U, Bereswill S, Heimesaat MM: Dissecting the interplay between intestinal microbiota and host immunity in health and disease: lessons learned from germfree and gnotobiotic animal models. Eur J Microbiol Immunol (Bp) 6, 253–271 (2016)
23. Hentges DJ, Stein AJ, Casey SW, Que JU: Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: influence of antibiotics on colonization resistance. Infect Immun 47, 118–122 (1985)
24. Marshall JC, Christou NV, Meakins JL: The gastrointestinal tract. The "undrained abscess" of multiple organ failure. Ann Surg 218, 111–119 (1993)
25. Buck AC, Cooke EM: The fate of ingested *Pseudomonas aeruginosa* in normal persons. J Med Microbiol 2, 521–525 (1969)

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Intestinal and Systemic Immune Responses upon Multi-drug Resistant *Pseudomonas aeruginosa* Colonization of Mice Harboring a Human Gut Microbiota

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The World Health Organization has rated multi-drug resistant (MDR) *Pseudomonas aeruginosa* as serious threat for human health. It is, however, unclear, whether intestinal MDR *P. aeruginosa* carriage is associated with inflammatory responses in intestinal or even systemic compartments. In the present study, we generated with respect to their microbiota "humanized" mice by human fecal microbiota transplantation of secondary abiotic mice. Following peroral challenge with a clinical *P. aeruginosa* isolate on two consecutive days, mice harboring a human or murine microbiota were only partially protected from stable intestinal *P. aeruginosa* colonization given that up to 78% of mice were *P. aeruginosa*-positive at day 28 post-infection (p.i.). Irrespective of the host-specificity of the microbiota, *P. aeruginosa* colonized mice were clinically uncompromised. However, *P. aeruginosa* colonization resulted in increased intestinal epithelial apoptosis that was accompanied by pronounced proliferative/regenerative cell responses. Furthermore, at day 7 p.i. increased innate immune cell populations such as macrophages and monocytes could be observed in the colon of mice harboring either a human or murine microbiota, whereas this held true at day 28 p.i. for adaptive immune cells such as B lymphocytes in both the small and large intestines of mice with murine microbiota. At day 7 p.i., pro-inflammatory cytokine secretion was enhanced in the colon and mesenteric lymph nodes, whereas the anti-inflammatory cytokine IL-10 was down-regulated in the former at day 28 p.i. Strikingly, cytokine responses upon intestinal *P. aeruginosa* colonization were not restricted to the intestinal tract, but could also be observed systemically, given that TNF and IFN-γ concentrations were elevated in spleens as early as 7 days p.i., whereas splenic IL-10 levels were dampened at day 28 p.i. of mice with human microbiota. In conclusion, mere intestinal carriage of MDR *P. aeruginosa* by clinically unaffected mice results in pro-inflammatory sequelae not only in intestinal, but also systemic compartments.

Keywords: multidrug-resistant Gram-negative bacteria, intestinal *Pseudomonas aeruginosa* colonization, fecal microbiota transplantation, human microbiota associated mice, colonization resistance, host-pathogen-interaction, intestinal and systemic sequelae of colonization, broad-spectrum antibiotic treatment

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INTRODUCTION

Pseudomonas aeruginosa constitute strictly aerobic Gram-negative bacteria that normally inhabit the soil and surfaces in aqueous environments. Their adaptive capabilities and intrinsic antibiotic resistance support survival in nature as well as in a plethora of settings including healthcare facilities (Gellatly and Hancock, 2013). Whereas a single flagellum and many cell surface pili facilitate bacterial motility and adherence to surfaces (Driscoll et al., 2007), alginate secretion, biofilm formation, quorum-sensing and an elaborate secretion system contribute to *P. aeruginosa* virulence (Driscoll et al., 2007; Gellatly and Hancock, 2013). In the hospital, typical environmental reservoirs of *P. aeruginosa* include sinks, water bottles and respiratory equipment, for instance (Morrison and Wenzel, 1984; Stratton, 1990). *P. aeruginosa* is a leading cause of nosocomial infections in ICUs such as ventilator-associated pneumonia, bloodstream infections, urinary tract infections or superinfections of burn wounds associated with mortality rates of more than 30% (Vincent, 2003). The emergence of MDR *P. aeruginosa* strains particularly due to extended-spectrum β-lactamases, carbapenemases and 16S rRNA methylases, has posed immuno-compromised individuals, patients suffering from cystic fibrosis and other chronic pulmonary morbidities or ICU patients at increased risk for prolonged hospital stay and mortality (Vincent, 2003; Oliver et al., 2015; Potron et al., 2015). In consequence, the WHO has rated MDR Gram-negative bacteria including *P. aeruginosa* as serious threat for human health and emphasized the urgent need for new treatment options (Tacconelli and Magrini, 2017). *P. aeruginosa* infection typically follows colonization that may happen either endogenously (hence arising from the patients' inherent microbiota) or exogenously (if acquired from other patients or from the hospital environment by cross-contamination) (Cohen et al., 2017). Intestinal *P. aeruginosa* colonization before ICU admission has been shown to be associated with an almost 15 times increased risk of subsequent infection as compared to non-colonized individuals (Gomez-Zorrilla et al., 2015). Disruption of the complex and diverse commensal intestinal microbiota physiologically exerting colonization resistance to the host by antimicrobial compounds further facilitates establishment and invasion of opportunistic or obligate pathogens in mice and men (Hentges et al., 1985; Marshall et al., 1993; Bereswill et al., 2011; Fiebiger et al., 2016). We have recently shown that also the host-specificity of the complex intestinal microbiota is pivotal for effective colonization resistance given that mice harboring a murine, but not human gut microbiota were protected from intestinal colonization with enteropathogenic *C. jejuni* (Bereswill et al., 2011). Conversely, perorally with *C. jejuni* challenged "humanized" mice could be stably infected by the enteropathogen and displayed pro-inflammatory immune responses as seen in human campylobacteriosis (Bereswill et al., 2011; Masanta et al., 2013; Fiebiger et al., 2016).

To date, however, no data are available regarding host immune responses in otherwise healthy *P. aeruginosa* carriers with an intact intestinal microbiota composition. In the present study we therefore investigated, whether mice harboring a human vs. murine microbiota could be stably colonized by a clinical MDR *P. aeruginosa* strain and whether bacterial carriage was associated with intestinal or even systemic inflammatory host responses.

Abbreviations: Bif, bifidobacteria; B/P, *Bacteroides/Prevotella* spp.; CFU, colony forming units; *C. jejuni, Campylobacter jejuni*; Clept, *Clostridium leptum* group; Clocc, *Clostridium coccoides* group; EB, enterobacteria; EC, enterococci; FMT, fecal microbiota transplantation; HPF, high power fields; ICU, intensive care unit; LB, lactic acid bacteria; MDR, multi-drug resistant; MIB, *Mouse Intestinal Bacteroides*; MLN, mesenteric lymph nodes; *P,* two-sided probability values; PBS, phosphate buffered saline; p.i., post-infection; qRT-PCR, quantitative real-time polymerase chain reaction; SPF, specific pathogen-free; TL, total eubacterial load; TLR, Toll-like receptor; WHO, World Health Organization.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration numbers G0039/15). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice.

Generation of Secondary Abiotic Mice

Female C57BL/6j mice were bred and maintained under SPF conditions in the Forschungsinstitute für Experimentelle Medizin (Charité – University Medicine, Berlin, Germany). Secondary abiotic mice with a virtually depleted microbiota were generated as described previously (Heimesaat et al., 2006). In brief, 8 weeks old mice were transferred into sterile cages and subjected to a broad-spectrum antibiotic treatment for 8 weeks by adding ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany), vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany) and metronidazole (1 g/L; Fresenius, Germany) to the drinking water (*ad libitum*). Cultural and culture-independent (i.e., 16S rRNA based molecular) quality control measures revealed virtual absence of bacteria in fecal samples as described earlier (Ekmekciu et al., 2017).

Human and Murine Fecal Microbiota Transplantation

Three days before association of secondary abiotic mice with a complex human or murine intestinal microbiota by FMT, the antibiotic cocktail was replaced by autoclaved tap water (*ad libitum*). Fresh fecal samples free of enteropathogenic bacteria (e.g., enteropathogenic *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, *Clostridium difficile*), viruses (e.g., Noro-, Rota-, Enterovirus) and parasites (e.g., *Giardia lamblia*, *Entamoeba histolytica*, *Blastocystis hominis* and worms) were collected from five individual healthy human

volunteers, dissolved in sterile PBS (Gibco, Life Technologies, United Kingdom), aliquoted and stored at −80°C as described earlier (Bereswill et al., 2011). Immediately before FMT, individual fecal aliquots were thawed and pooled (Bereswill et al., 2011). In addition, fresh murine fecal samples were collected from 10 age and sex matched SPF control mice, pooled, dissolved in 10 mL sterile PBS and the supernatant served as murine donor suspension. To generate human intestinal microbiota associated mice or reintroduce murine microbiota into mice, secondary abiotic animals were subjected to peroral FMT either with 0.3 mL of the human or murine donor suspension by gavage on two consecutive days (Bereswill et al., 2011). Of note, each donor suspension was tested negative for *P. aeruginosa* by both culture and culture-independent (i.e., 16S rRNA based) methods. Bacterial groups varied less than 0.5 logarithmic orders of magnitude between independent experiments. To assure proper establishment of the human or murine microbiota in the murine host, mice were kept for 3 weeks until *P. aeruginosa* infection. Immediately before peroral *P. aeruginosa* challenge individual fecal samples were collected for quantitative molecular analyses of main intestinal bacterial communities as described elsewhere (Bereswill et al., 2011).

Molecular Analysis of the Human and Murine Donor Suspensions and the Fecal Microbiota

DNA was extracted from fecal samples as described previously (Heimesaat et al., 2006). In brief, DNA was quantified by using Quant-iT PicoGreen reagent (Invitrogen, United Kingdom) and adjusted to 1 ng per μL. Then, TLs as well as the main bacterial groups abundant in the murine and human intestinal microbiota including EB, EC, LB, Bif, B/P, Clocc, Clept and MIB were assessed by qRT-PCR with species-, genera- or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as described previously (Heimesaat et al., 2010) and numbers of 16S rRNA gene copies per ng DNA of each sample determined.

MDR *P. aeruginosa* Infection and Quantitative Assessment of Fecal Loads

The MDR *P. aeruginosa* isolate was initially cultured from respiratory material of a patient suffering from nosocomial pneumonia and kindly provided by Prof. Dr. Bastian Opitz (Charité – University Medicine, Berlin, Germany). Notably, the clinical bacterial strain was tested resistant against piperacillin/tazobactam, ceftazidime, cefepim ± cefoxitin, imipenem, ciprofloxacin, gentamycin, tobramycin, amikacin, trimethoprim/sulfamethoxazole, and aztreonam (according to EUCAST interpretation guidelines) and displayed antimicrobial sensitivity to fosfomycin and colistin only (von Klitzing et al., 2017a). Prior infection, the *P. aeruginosa* strain was grown on cetrimide agar (Oxoid) for 48 h in an aerobic atmosphere at 37°C.

On days 0 and 1, mice were perorally challenged with 10^9 CFU of the MDR *P. aeruginosa* strain by gavage in a total volume of 0.3 mL PBS as reported earlier (von Klitzing et al., 2017a).

For quantitative assessment of fecal *P. aeruginosa* loads over time p.i., fecal samples were homogenized in sterile PBS, then serial dilutions streaked onto Columbia agar supplemented with 5% sheep blood (Oxoid, Germany) and cetrimide agar and incubated in an aerobic atmosphere at 37°C for 48 h as described previously (von Klitzing et al., 2017a). Fecal weights were determined by the difference of the sample weights before and after asservation. The detection limit of viable bacteria was 100 CFU per g.

Clinical Conditions

Macroscopic and/or microscopic abundance of fecal blood was assessed in individual mice on a daily basis by the Guajac method using Haemoccult (Beckman Coulter/ PCD, Germany) as described earlier (Haag et al., 2012a).

Sampling Procedures

Mice were sacrificed 28 days p.i. by isoflurane treatment (Abott, Germany). Cardiac blood (for serum) and tissue samples from spleen, MLN, ileum and colon were removed under sterile conditions. Intestinal samples were collected from each mouse in parallel for microbiological, immunological and immunohistochemical analyses.

Immunohistochemistry

Five μm thin paraffin sections of colonic and ileal *ex vivo* biopsies were used for *in situ* immunohistochemical analysis as reported previously (Heimesaat et al., 2010; Alutis et al., 2015a,b). In brief, primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, United States, 1:200), Ki67 (TEC3, Dako, Glostrup, Denmark, 1:100), F4/80 (#14-4801, clone BM8, eBioscience, 1:50) and B220 (eBioscience, 1:200) were used to assess apoptotic cells, proliferating cells, macrophages/monocytes, and B lymphocytes, respectively. The average numbers of positively stained cells within at least six HPFs (0.287 mm^2; 400× magnification) were determined by an independent blinded investigator.

Cytokine Detection

Ex vivo biopsies (approximately 1 cm^2) derived from colon and ileum (both cut longitudinally and washed in PBS), as well as MLN and spleen were placed into 24-flat-bottom well culture plates (Falcon, Germany) containing 500 mL serum-free RPMI 1640 medium (Gibco, Life Technologies) supplemented with penicillin (100 U/ml, Biochrom, Germany) and streptomycin (100 μg/ml; Biochrom). After 18 h at 37°C, culture supernatants and serum samples were tested for TNF, IFN-γ and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Bioscience) on a BD FACSCanto II flow cytometer (BD Bioscience) as described earlier (Haag et al., 2012b).

Statistical Analysis

Medians and levels of significance were determined using appropriate tests (Mann–Whitney *U* test, one-way ANOVA and Kruskal–Wallis test) as indicated. Two-sided probability (p) values ≤ 0.05 were considered significant. Experiments were reproduced at least twice.

RESULTS

Intestinal *P. aeruginosa* Colonization in Mice Harboring a Complex Human vs. Murine Gut Microbiota

In the present study we addressed whether a complex human or murine gut microbiota could sufficiently confer colonization resistance against MDR *P. aeruginosa* to the vertebrate host and if not, whether intestinal *P. aeruginosa* carriage resulted in intestinal or even systemic inflammatory sequelae. To accomplish this, secondary abiotic mice were associated with either a human or murine microbiota by peroral FMT. Culture-independent (i.e., molecular) analyses of the microbiota suspensions derived from human or murine donors revealed substantial host-specific differences in bacterial compositions. TLs and those of specific families/genera such as EB, Bif, Clocc, and Clept were higher in human as compared to murine bacterial suspensions ($p < 0.01$–0.001; **Figure 1A**), whereas LB or MIB were lower or even virtually absent in the former as compared to the latter ($p < 0.001$; **Figure 1A**). Three weeks following FMT and immediately before *P. aeruginosa* challenge (i.e., day 0), we further assessed respective microbiota compositions in fecal samples derived from individual mice. Molecular analyses revealed higher fecal gene numbers of B/P and Clept, but lower LB and MIB burdens as well as slightly lower total eubacterial gene numbers (less than one order of magnitude) in human microbiota transplanted mice as compared to murine counterparts ($p < 0.001$; **Figure 1B**). These results thus indicate stable intestinal establishment of distinct microbiota following respective FMT.

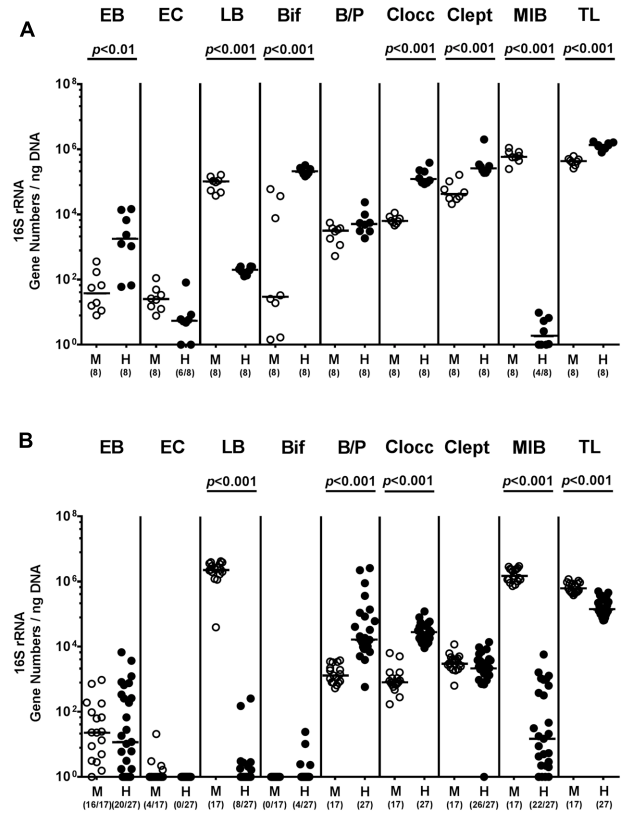

FIGURE 1 | Intestinal bacterial colonization following human and murine FMT of secondary abiotic mice. Secondary abiotic mice were generated by broad-spectrum antibiotic treatment and associated with either a complex murine (M, open circles) or human (H, closed circles) intestinal microbiota by FMT on two consecutive days. **(A)** Respective donor suspensions and **(B)** individual fecal sample taken three weeks following FMT (and hence, immediately before MDR *P. aeruginosa* challenge) were subjected to a comprehensive fecal microbiota survey by quantitative Real-Time PCR amplifying variable regions of the bacterial 16S rRNA gene. The following main intestinal bacterial groups were determined (expressed as 16S rRNA gene numbers per ng DNA): Enterobacteria (EB), enterococci (EC), lactic acid bacteria (LB), bifidobacteria (Bif), *Bacteroides/Prevotella* spp. (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept), *Mouse Intestinal Bacteroides* (MIB), and total eubacterial load (TL). Medians (black bars) and significance levels (*p*-values) determined by Mann–Whitney *U*-test are shown. Numbers of samples harboring the respective bacterial group out of the total number of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

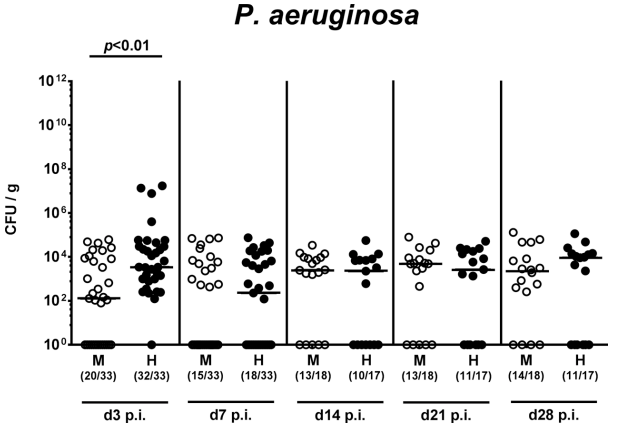

FIGURE 2 | Intestinal colonization densities of MDR *P. aeruginosa* in mice harboring a human vs. murine microbiota. Mice with a murine (M, open circles) or human (H, closed circles) intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. Intestinal colonization densities were assessed in fecal samples at defined time points p.i. by culture. Medians (black bars) and significance levels (*p*-values) determined by Mann–Whitney *U*-test are shown. Numbers of samples harboring the respective bacterial group out of the total number of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

On two consecutive days (i.e., days 0 and 1), we perorally challenged mice with 10^9 viable *P. aeruginosa* by gavage and quantitatively assessed fecal *P. aeruginosa* loads thereafter. Cultural analyses revealed that as early as 48 h following the latest bacterial challenge (i.e., day 3 p.i.), virtually all human microbiota transplanted mice harbored *P. aeruginosa* in their intestines with median loads of approximately 10^4 CFU per g, whereas bacterial counts were approximately two orders of magnitude lower in murine controls ($p < 0.01$; **Figure 2** and **Supplementary Figure S1**). Of note, by day 3 p.i. *P. aeruginosa* could not be detected in 40% of mice with a murine gut microbiota. Until the end of the observation period, however, fecal *P. aeruginosa* loads were comparable in mice with either microbiota, whereas

64.7% of mice following human FMT and 77.8% of murine controls harbored the bacterial strain in their intestinal tract at day 28 p.i. (**Figure 2** and **Supplementary Figure S1**). Hence, the complex intestinal microbiota, irrespective whether of human or murine origin, prevented only a subgroup of mice from long-term intestinal MDR *P. aeruginosa* colonization.

Intestinal Microbiota Changes upon *P. aeruginosa* Colonization of Mice Harboring a Complex Human vs. Murine Gut Microbiota

We next surveyed whether *P. aeruginosa* colonization was accompanied by distinct changes of the intestinal microbiota composition over time (**Figures 3**, **4**). Quantitative culture-independent analysis revealed that four weeks following *P. aeruginosa* association, intestinal gene numbers of MIB had increased in mice that had been subjected to human FMT, but not conventionally colonized mice ($p < 0.05$; **Figure 3**). As early as one week following *P. aeruginosa* challenge, however, the respective gut microbiota did not differ from uninfected control groups, neither in mice harboring a human, nor murine microbiota (**Figures 3**, **4**). Hence, no overt changes in intestinal microbiota composition could be observed relatively early (i.e., 1 week) upon *P. aeruginosa* colonization and were rather subtle thereafter.

Apoptotic and Proliferating Responses in Intestinal Epithelial Cells upon *P. aeruginosa* Colonization of Mice Harboring a Complex Human vs. Murine Gut Microbiota

Given that mice were not clinically compromised following *P. aeruginosa* challenge and did not display symptoms such as weight loss, diarrhea or fecal blood (data not shown), we next assessed potential microscopic sequelae of *P. aeruginosa* colonization. To address this, we quantitatively determined apoptotic epithelial cells in the large intestines applying *in situ* immunohistochemistry. Mice following human FMT displayed more than two-fold increased caspase-3 positive apoptotic cell numbers in their colonic epithelia at both day 7 and day 28 p.i. ($p < 0.001$; **Figure 5B**), whereas this was only the case later in the course of colonization of murine controls ($p < 0.05$; **Figure 5A**). Of note, at day 7 p.i. apoptotic epithelial cells were higher in the large intestines of mice that had been challenged with human gut microbiota as compared to murine controls ($p < 0.001$; **Figures 5A,B**). In the small intestines of mice with either microbiota, however, apoptotic cells were slightly higher at day 7 p.i. as compared to non-challenged controls ($p < 0.05$–0.01; **Supplementary Figures S2A,B**) and further increased until the end of the observation period ($p < 0.05$–0.001 vs. day 7 p.i.; **Supplementary Figures S2A,B**).

We additionally assessed Ki67 positive cell numbers in intestinal epithelia indicative for proliferative/regenerative cell responses counteracting potential *P. aeruginosa*-induced intestinal inflammatory sequelae. Remarkably, only in mice following human FMT, Ki67 positive cells were elevated in large intestines as early as 7 day p.i. ($p < 0.01$ vs. none-challenged controls), but declined thereafter ($p < 0.001$; **Figure 5B**). In the small intestines, proliferative cell responses were highest early in the course of *P. aeruginosa* colonization, whereas Ki67 positive ileal epithelial cell numbers declined thereafter (**Supplementary Figure S2**). Hence, *P. aeruginosa* colonization

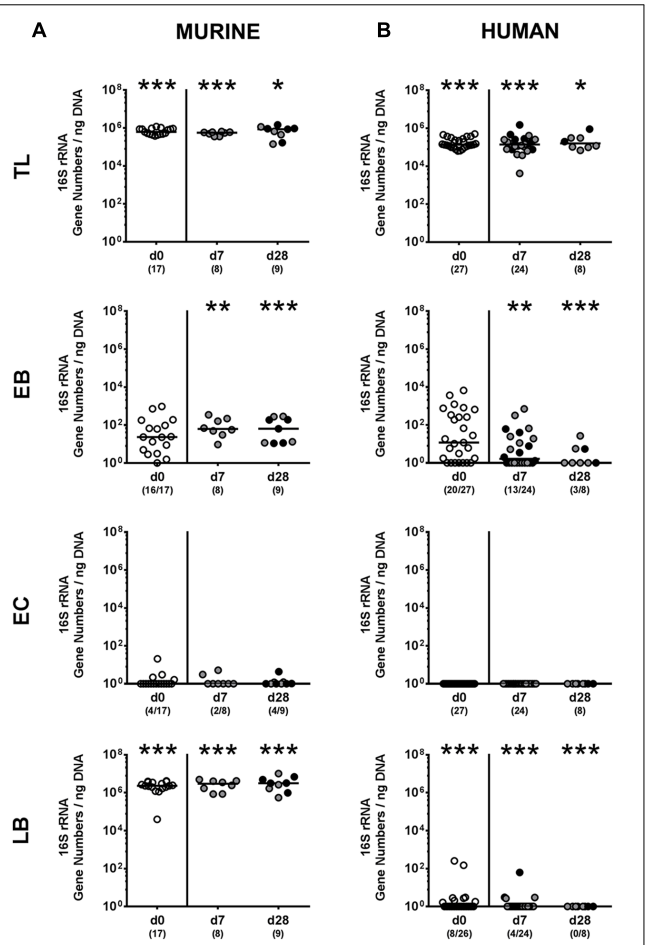

FIGURE 3 | Intestinal total and aerobic bacterial changes following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with *P. aeruginosa* on day 0 and day 1. Immediately before (day 0, open circles) and at day 7 and day 28 post-challenge the intestinal microbiota composition of colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice was surveyed applying quantitative Real-Time PCR amplifying variable regions of the bacterial 16S rRNA gene. The following main intestinal bacterial groups were determined (expressed as 16S rRNA gene numbers per ng DNA): Total eubacterial load (TL), enterobacteria (EB), enterococci (EC), and lactic acid bacteria (LB). Medians (black bars) and significance levels (*p*-values) determined by one-way ANOVA or Kruskal–Wallis test (according to data distribution) are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Numbers of samples harboring the respective bacterial group out of the total number of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

of mice resulted in increased intestinal epithelial apoptosis that was accompanied by proliferative/regenerative epithelial cell responses.

Intestinal Immune Cell Responses upon *P. aeruginosa* Colonization of Mice Harboring a Complex Human vs. Murine Gut Microbiota

We next addressed whether colonization of human fecal microbiota transplanted mice with MDR *P. aeruginosa* was accompanied by an increased influx of distinct immune cell populations into the intestinal tract, again applying quantitative *in situ* immunohistochemistry. Irrespective of the host-specificity of the intestinal microbiota, numbers of innate immune cells such as F4/80 positive macrophages and monocytes were elevated in the large intestinal mucosa and lamina propria at day 7 p.i., but declined thereafter ($p < 0.001$; **Figure 5**). Only in mice with a murine microbiota, however, *P. aeruginosa*-induced adaptive immune cell responses were more pronounced later in the course of colonization as indicated by increased numbers of colonic B lymphocytes at day 28 p.i. ($p < 0.05$ vs. non-challenged controls; **Figure 5A**). In the small intestinal tract, increases in both numbers of macrophages/monocytes as well as of B lymphocytes could only be observed later in the course of *P. aruginosa* colonization in conventionally colonized, but not human fecal microbiota transplanted mice ($p < 0.01$–0.001; **Supplementary Figure S2A**). Hence, rather early in the course of *P. aeruginosa* colonization increased innate immune cell subsets could be observed in the large intestines of mice harboring either a human or murine gut microbiota, whereas this held true for adaptive immune cells such as B lymphocytes in the small and large intestinal tract of mice with a murine microbiota rather later in the course of *P. aeruginosa* colonization.

Intestinal Cytokine Responses upon *P. aeruginosa* Colonization of Mice Harboring a Complex Human vs. Murine Gut Microbiota

We next addressed whether changes in intestinal innate and adaptive immune cell numbers upon *P. aeruginosa* colonization of human vs. murine fecal microbiota transplanted mice was accompanied by pro- and/or anti-inflammatory cytokine responses within the intestinal tract. At day 7 p.i., increased concentrations of the pro-inflammatory cytokine TNF could be detected in colonic *ex vivo* biopsies derived from mice harboring a murine, but not human microbiota ($p < 0.05$; **Figure 6A**). Large intestinal secretion of the anti-inflammatory cytokine IL-10, however, was dampened at day 7 and day 28 p.i. of mice that had been subjected to human FMT ($p < 0.001$ and $p < 0.05$, respectively; **Figure 6B**) and at the later time point in mice with a murine microbiota ($p < 0.01$; **Figure 6A**). We further analyzed cytokine secretion in MLN. Alike the colon, TNF concentrations were elevated in MLN as early as 7 day p.i. of mice harboring a murine microbiota only ($p < 0.001$), but declined thereafter to basal levels ($p < 0.01$; **Figure 7A**). In addition, at day 7 following *P. aeruginosa* colonization increased IFN-γ secretion could be detected in MLN of both human and murine fecal microbiota transplanted mice ($p < 0.001$ and

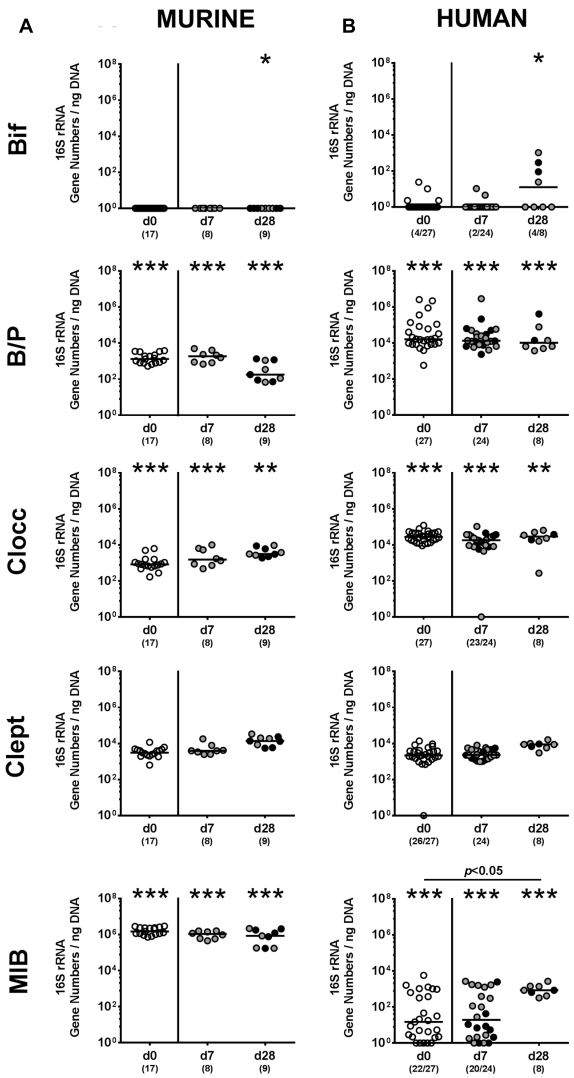

FIGURE 4 | Intestinal anaerobic bacterial changes following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with *P. aeruginosa* on day 0 and day 1. Immediately before (day 0, open circles) and at day 7 and day 28 post-challenge the intestinal anaerobic microbiota composition of colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice was surveyed applying quantitative Real-Time PCR amplifying variable regions of the bacterial 16S rRNA gene. The following main intestinal bacterial groups were determined (expressed as 16S rRNA gene numbers per ng DNA): Bifidobacteria (Bif), *Bacteroides/Prevotella* spp. (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept), and *Mouse Intestinal Bacteroides* (MIB). Medians (black bars) and significance levels (*p*-values) determined by one-way ANOVA or Kruskal–Wallis test (according to data distribution) are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Data were pooled from four independent experiments.

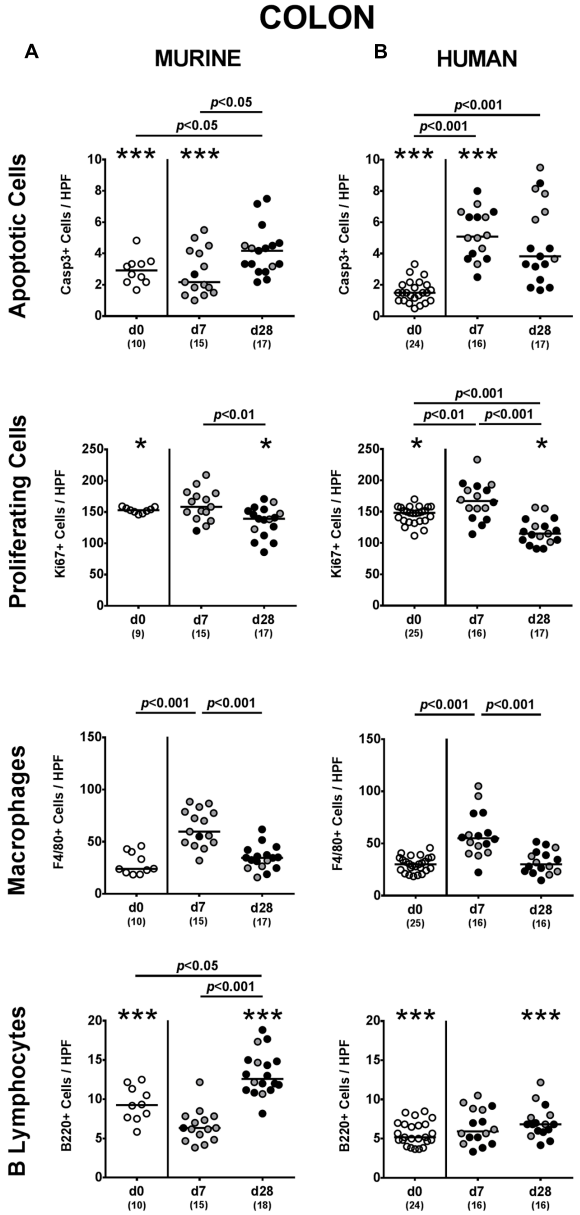

FIGURE 5 | Apoptotic and proliferating epithelial cell as well as immune cell responses in the large intestines following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. At day 7 and day 28 post-challenge, the average number of apoptotic (Casp3+) and proliferating (Ki67+) cells as well as of macrophages/monocytes (F4/80+) and B lymphocytes (B220+) in at least six HPF were quantitatively assessed in colonic paraffin sections derived from colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice applying *in situ* immunohistochemistry. Unchallenged mice (day 0, open circles) harboring a respective murine or human gut microbiota served as negative controls. Medians (black bars) and significance levels (*p*-values) determined by one-way ANOVA or Kruskal–Wallis test (according to data distribution) are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (*$p < 0.05$; ***$p < 0.001$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Numbers of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

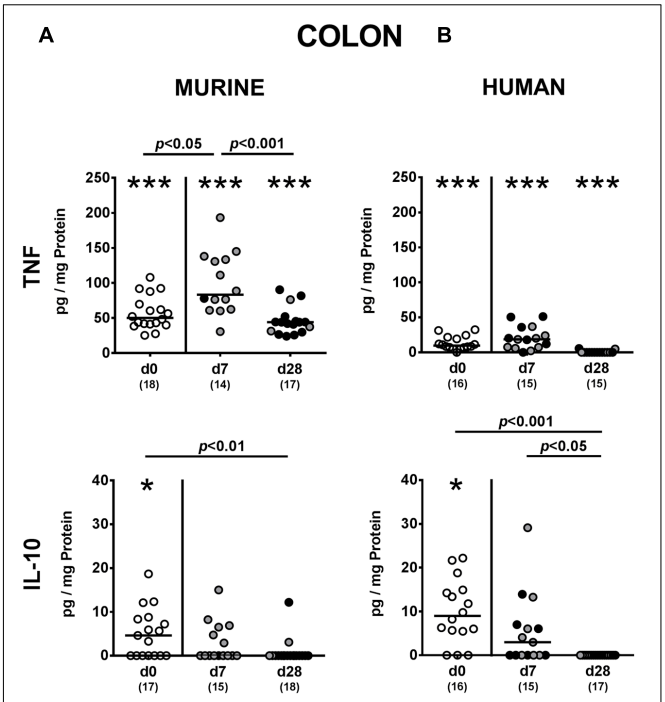

FIGURE 6 | Colonic pro- and anti-inflammatory cytokine responses following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. At day 7 and day 28 post-challenge, TNF and IL-10 concentrations were determined in colonic *ex vivo* biopsies derived from colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice. Unchallenged mice (day 0, open circles) harboring a respective murine or human gut microbiota served as negative controls. Medians (black bars) and significance levels (*p*-values) determined by one-way ANOVA or Kruskal–Wallis test (according to data distribution) are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (*$p < 0.05$; ***$p < 0.001$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Numbers of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

$p < 0.01$, respectively; **Figure 7**), whereas IL-10 levels remained unchanged (**Figure 7**). Hence, rather early in the course of murine *P. aeruginosa* colonization intestinal pro-inflammatory, but not anti-inflammatory cytokine responses were induced.

Systemic Cytokine Responses upon *P. aeruginosa* Colonization of Mice Harboring a Complex Human vs. Murine Gut Microbiota

We finally addressed whether MDR *P. aeruginosa* colonization of human and murine fecal microbiota transplanted mice not only induced local (i.e., intestinal), but also systemic cytokine responses and therefore measured pro- and anti-inflammatory cytokine secretion in splenic *ex vivo* biopsies. Seven days following *P. aeruginosa* challenge, increased TNF concentrations could be detected in spleens derived from mice harboring a murine gut microbiota, but decreased to naive levels until day 28 p.i. ($p < 0.001$; **Figure 8**). In mice that had been

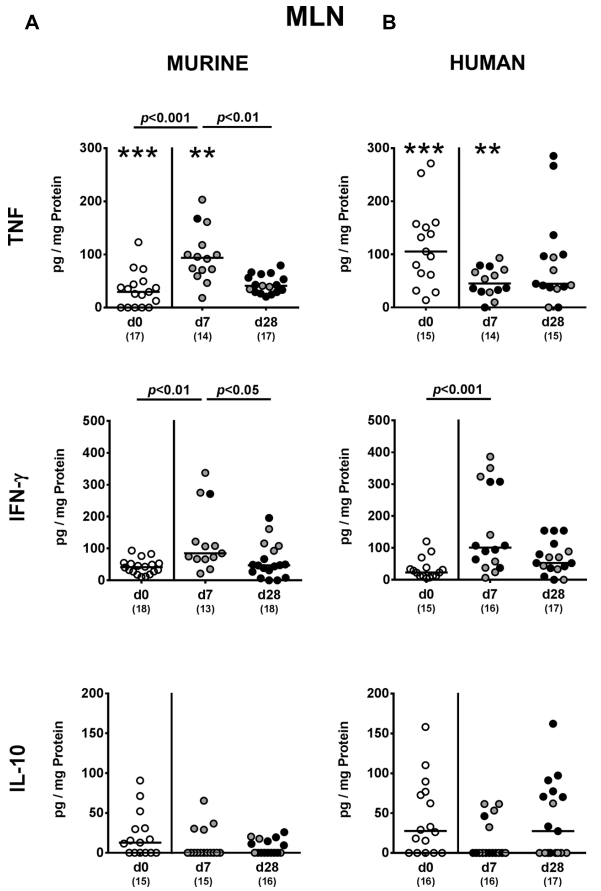

FIGURE 7 | Pro- and anti-inflammatory cytokine responses in MLN following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. At day 7 and day 28 post-challenge, TNF, IFN-γ and IL-10 concentrations were determined in *ex vivo* biopsies derived from MLN of colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice. Unchallenged mice (day 0, open circles) harboring a respective murine or human gut microbiota served as negative controls. Medians (black bars) and significance levels (*p*-values) determined by one-way ANOVA or Kruskal–Wallis test (according to data distribution) are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (**$p < 0.01$; ***$p < 0.001$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Numbers of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

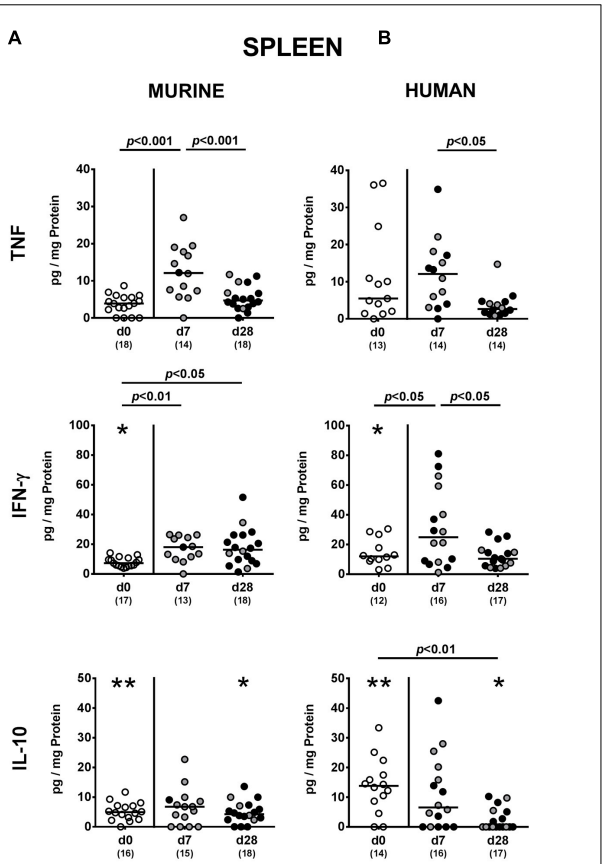

FIGURE 8 | Systemic pro- and anti-inflammatory cytokine responses following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. At day 7 and day 28 post-challenge, TNF, IFN-γ and IL-10 concentrations were determined in splenic *ex vivo* biopsies derived from colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice. Unchallenged mice (day 0, open circles) harboring a respective murine or human gut microbiota served as negative controls. Medians (black bars) and significance levels (*p*-values) determined by one-way ANOVA or Kruskal–Wallis test (according to data distribution) are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (*$p < 0.05$; **$p < 0.01$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Numbers of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

subjected to human FMT, a trend towards elevated splenic TNF concentrations could be observed at day 7 p.i. (n.s. due to high standard deviations; $p < 0.05$ vs. day 28 p.i.; **Figure 8B**). Whereas INF-γ concentrations were higher in spleens of mice with a murine microbiota at both day 7 and day 28 p.i. ($p < 0.01$ and $p < 0.05$, respectively; **Figure 8A**), splenic INF-γ levels increased in human fecal microbiota transplanted mice at day 7 p.i. only and reached basal concentrations thereafter ($p < 0.05$; **Figure 8B**). At day 28 p.i., IL-10 was down-regulated in the spleen of mice with human FMT ($p < 0.01$; **Figure 8B**). Hence, cytokine responses upon intestinal colonization with MDR *P. aeruginosa* were not restricted to the intestinal tract, but could also be observed systemically.

DISCUSSION

In recent years the rising incidences of infections with MDR Gram-negative bacterial including *P. aeruginosa* strains has gained increasing attention not only in the medical field, but also in the general public and global health politics (Livermore, 2009; Oliver et al., 2015). Infection and spread of *P. aeruginosa* have been mainly considered as hospital-associated issues so far, particularly in severely ill patients with antibiotic treatment.

P. aeruginosa has not been considered part of the commensal microbiota and to date, only a few studies are available addressing the prevalence of *P. aeruginosa* carriage in the healthy population ranging from 0 to 24% (Darrell and Wahba, 1964; Shooter et al., 1966; Sutter and Hurst, 1966; Kessner and Lepper, 1967; Stoodley and Thom, 1970; Bleday et al., 1993; Speert et al., 1993; Bonten et al., 1999; Levy, 2000; Kerckhoffs et al., 2011). However, no data are available whether *P. aeruginosa* carriage, particularly of a MDR strain results in distinct host immune responses.

This prompted us to investigate, whether mice harboring a human vs. murine gut microbiota could be stably colonized by a clinical MDR *P. aeruginosa* strain and whether opportunistic pathogenic carriage was associated with intestinal or even systemic inflammatory host responses. Our present study revealed that the complex intestinal microbiota, irrespective whether of human or murine origin, only partially prevented mice from intestinal *P. aeruginosa* colonization. In fact, approximately 50% of mice harboring a human or murine microbiota were *P. aeruginosa*-negative one week upon supra-physiological challenge with 10^9 viable bacteria, whereas *P. aeruginosa* could be cultured at relatively low median loads of less than 10^4 CFU per g feces in up to 78% of mice at day 28 p.i. One might argue that the colonization resistance exerted by the complex intestinal microbiota composition was not fully restored upon broad-spectrum antibiotic pre-treatment and a 3-weeks period following complex bacterial reconstitution by FMT. Our previous infection studies performed at identical timelines, however, revealed that secondary abiotic mice reconstituted with a murine gut microbiota were protected from stable *C. jejuni* colonization and hence, from pathogen-induced pro-inflammatory sequelae (Bereswill et al., 2011). In an earlier study ingested *P. aeruginosa* could be cultured from feces derived from three healthy volunteers up to 6 days following peroral challenge without any clinical sequelae. In line with our results, fecal *P. aeruginosa* counts were lower as compared to those that had been ingested and subsequently declined over time post ingestion (Buck and Cooke, 1969).

One needs to take into consideration, however, that the applied mice harboring a human vs. murine gut microbiota following antibiotic microbiota depletion and subsequent FMT has its limitations – just like any experimental model – and does, hence, only to a certain degree mimic human (patho)physiological conditions. For instance, some members of the human microbiota, particularly obligate anaerobic species might be reduced or even lost upon freezing, thawing and further processing of the fecal donor samples (von Klitzing et al., 2017b) and/or might furthermore not have stably established within the intraluminal ecosystem in some cases as indicated by the observed differences in microbiota compositions of respective donor suspensions and fecal samples derived following FMT. Furthermore, behavioral and environmental factors including housing conditions, diet, genetic background, the distinct anatomic compartment with its inherent intraluminal milieu and respective immunological reportoire all together impact the composition of the complex host-specific gut microbiota (Turnbaugh et al., 2009; Neyrinck et al., 2011; Nguyen et al., 2015; von Klitzing et al., 2017b). Nevertheless, when taking both the strengths and the limitations of the applied experimental model into consideration, with respect to their gut microbiota "humanized" mice might constitute valuable tools in dissecting the interactions between gut commensals, (opportunistic) pathogens and host immunity in health and disease (von Klitzing et al., 2017b). For instance, mice with a human microbiota have been applied to unravel intestinal colonization properties of enteropathogens such as *C. jejuni*, *Salmonella enterica,* and *C. difficile* (Bereswill et al., 2011; Chung et al., 2012; Collins et al., 2015).

In the present study mice were also clinically uncompromised during the entire observation period following peroral *P. aeruginosa* challenge. On microscopic level, however, *P. aeruginosa* induced inflammatory sequelae could be assessed as indicated by increased apoptotic epithelial cell numbers in both small and large intestines as early as 7 days p.i., irrespective of the intestinal microbiota composition of mice. Of note, apoptosis was paralleled by increased proliferative/regenerative epithelial cell responses thereby counteracting potential cell damage. Furthermore, *P. aeruginosa* colonization was accompanied by a rather early innate immune response as indicated by increased numbers of macrophages and monocytes in the colonic mucosa and lamina propria of human fecal microbiota transplanted mice, whereas small and large intestinal numbers of adaptive immune cells such as B lymphocytes were elevated at the end of the observation period. Pro-inflammatory sequelae of *P. aeruginosa* colonization are further characterized by increased TNF secretion in both colon and MLN of mice at day 7 p.i., whereas anti-inflammatory IL-10 levels, however, remained unaffected in the intestinal tract. The *P. aeruginosa* induced immune responses are not surprising given the plethora of virulence factors of the opportunistic pathogen. For instance, *Pseudomonas* lipid A, a core component of bacterial lipopeptide, is capable of activating NFκB signaling via TLR-4 leading to pro-inflammatory cytokine secretion (Korneev et al., 2015). Subsequently, innate immune cells are recruited to the site of infection contributing to inflammatory host responses directed against *P. aeruginosa* (Gellatly and Hancock, 2013).

Strikingly, immune responses upon *P. aeruginosa* carriage were not restricted to the intestinal tract, but could also be observed systemically, given that TNF as well as IFN-γ concentrations were elevated in spleens as early as 7 days p.i., whereas splenic IL-10 levels were dampened in human fecal microbiota transplanted mice later-on. To the best of our knowledge, we here for the first time show that mere intestinal carriage of MDR *P. aeruginosa* by a clinically unaffected host results in pronounced intestinal as well as systemic sequelae. This is even more surprising, given that not all mice under investigation were stable *P. aeruginosa* carriers further underlining the pathogenic potential of MDR *P. aeruginosa*.

Despite several reports of diarrhea caused by *P. aeruginosa* in patients with prior antibiotic treatment, *P. aeruginosa* is not regarded as a common intestinal pathogen in the healthy host (Adlard et al., 1998; Kim et al., 2001). As early as 1918, however, "Shanghai fever", a community acquired syndrome characterized by diarrhea, fever and *P. aeruginosa* sepsis with high mortality has been reported affecting neonates and children without

pre-existing comorbidities primarily in Asian populations (Dold, 1918; Chusid and Hillmann, 1987; Martin-Ancel et al., 1993; Viola et al., 2006; Chuang et al., 2014).

Given the potential cross-talk between (opportunistic) pathogens and commensal intestinal bacteria, we addressed whether changes in respective gut microbiota compositions might occur during *P. aeruginosa* colonization. Interestingly, no overt gut microbiota shifts could be observed early (i.e., 1 week) upon *P. aeruginosa* colonization and were only minor in human fecal microbiota transplanted mice thereafter. Previous studies revealed that both acute and chronic small as well as large intestinal inflammation in mice were associated with increased luminal loads of commensal EB further perpetuating the inflammatory scenario via TLR-4 dependent signaling of lipopolysaccharide (LPS) as Gram-negative bacterial cell wall constituent (Heimesaat et al., 2006, 2007a,b, 2010; Erridge et al., 2010; Haag et al., 2012a,b; Otto et al., 2012). However, despite observed pro-inflammatory immune responses with pronounced intestinal epithelial apoptosis, sequelae of intestinal MDR *P. aeruginosa* carriage was not sufficient enough to drive gut microbiota shifts towards overgrowth with potentially pro-inflammatory commensals.

CONCLUSION

Our study demonstrates that in with a complex microbiota reconstituted secondary abiotic mice the established intestinal microbiota (irrespective whether of human or murine origin) does not sufficiently prevent the host from intestinal colonization with a MDR *P. aeruginosa* strain. Furthermore, mere intestinal carriage of MDR *P. aeruginosa* pose otherwise healthy hosts at risk of developing not only intestinal but even systemic pro-inflammatory sequelae. Future studies need to further unravel the molecular mechanisms underlying the interactions between *P. aeruginosa*, the commensal microbiota and host immunity in health and disease in order to develop strategies preventing from colonization with MDR Gram-negative strains including *P. aeruginosa*.

AUTHOR CONTRIBUTIONS

EvK: designed and performed experiments, analyzed data, co-edited paper. IE: performed experiments, analyzed data, co-edited paper. SB: provided advice in design and performance of experiments, co-edited paper. MH: designed and performed experiments, analyzed data, wrote paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02590/full#supplementary-material

FIGURE S1 | Fecal MDR *P. aeruginosa* loads in mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. Intestinal colonization densities were assessed in fecal samples at defined time points p.i. by culture. Medians (black bars) and significance levels (*p*-values) determined by Kruskal–Wallis test are shown. Numbers of samples harboring the respective bacterial group out of the total number of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

FIGURE S2 | Apoptotic and proliferating epithelial cell as well as immune cell responses in the small intestines following MDR *P. aeruginosa* colonization of mice harboring a human vs. murine microbiota. Mice with a **(A)** murine or **(B)** human intestinal microbiota were perorally challenged with MDR *P. aeruginosa* on day 0 and day 1. At day 7 and day 28 post-challenge, the average number of apoptotic (Casp3+) and proliferating (Ki67+) cells as well as of macrophages/monocytes (F4/80+) and B lymphocytes (B220+) in at least six HPF were quantitatively assessed in ileal paraffin sections derived from colonic *P. aeruginosa* carrying (black circles) and non-carrying (gray circles) mice applying *in situ* immunohistochemistry. Unchallenged mice (day 0, open circles) harboring a respective murine or human gut microbiota served as negative controls. Medians (black bars) and significance levels (*p*-values) determined by Mann–Whitney *U*-test are shown. Significant differences between mice with a murine vs. human microbiota at defined time points are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), whereas numbered *p*-values indicate differences between mice harboring the same microbiota. Numbers of analyzed samples are given in parentheses. Data were pooled from four independent experiments.

REFERENCES

Adlard, P. A., Kirov, S. M., Sanderson, K., and Cox, G. E. (1998). *Pseudomonas aeruginosa* as a cause of infectious diarrhoea. *Epidemiol. Infect.* 121, 237–241. doi: 10.1017/S095026889800106X

Alutis, M. E., Grundmann, U., Fischer, A., Hagen, U., Kuhl, A. A., Gobel, U. B., et al. (2015a). The role of gelatinases in *Campylobacter jejuni* infection of gnotobiotic mice. *Eur. J. Microbiol. Immunol.* 5, 256–267. doi: 10.1556/1886.2015.00033

Alutis, M. E., Grundmann, U., Hagen, U., Fischer, A., Kuhl, A. A., Gobel, U. B., et al. (2015b). Matrix metalloproteinase-2 mediates intestinal immunopathogenesis in *Campylobacter jejuni*-infected infant mice. *Eur. J. Microbiol. Immunol.* 5, 188–198. doi: 10.1556/1886.2015.00020

Bereswill, S., Fischer, A., Plickert, R., Haag, L. M., Otto, B., Kuhl, A. A., et al. (2011). Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. *PLOS ONE* 6:e20953. doi: 10.1371/journal.pone.0020953

Bleday, R., Braidt, J., Ruoff, K., Shellito, P. C., and Ackroyd, F. W. (1993). Quantitative cultures of the mucosal-associated bacteria in the mechanically prepared colon and rectum. *Dis. Colon Rectum* 36, 844–849. doi: 10.1007/BF02047381

Bonten, M. J., Bergmans, D. C., Speijer, H., and Stobberingh, E. E. (1999). Characteristics of polyclonal endemicity of *Pseudomonas aeruginosa* colonization in intensive care units. Implications for infection control. *Am. J. Respir. Crit. Care Med.* 160, 1212–1219. doi: 10.1164/ajrccm.160.4.9809031

Buck, A. C., and Cooke, E. M. (1969). The fate of ingested *Pseudomonas aeruginosa* in normal persons. *J. Med. Microbiol.* 2, 521–525. doi: 10.1099/00222615-2-4-521

Chuang, C. H., Wang, Y. H., Chang, H. J., Chen, H. L., Huang, Y. C., Lin, T. Y., et al. (2014). Shanghai fever: a distinct *Pseudomonas aeruginosa* enteric disease. *Gut* 63, 736–743. doi: 10.1136/gutjnl-2013-304786

Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., et al. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. *Cell* 149, 1578–1593. doi: 10.1016/j.cell.2012.04.037

Chusid, M. J., and Hillmann, S. M. (1987). Community-acquired *Pseudomonas* sepsis in previously healthy infants. *Pediatr. Infect. Dis. J.* 6, 681–684. doi: 10.1097/00006454-198707000-00013

Cohen, R., Babushkin, F., Cohen, S., Afraimov, M., Shapiro, M., Uda, M., et al. (2017). A prospective survey of *Pseudomonas aeruginosa* colonization and infection in the intensive care unit. *Antimicrob. Resist. Infect. Control* 6, 7. doi: 10.1186/s13756-016-0167-7

Collins, J., Auchtung, J. M., Schaefer, L., Eaton, K. A., and Britton, R. A. (2015). Humanized microbiota mice as a model for recurrent *Clostridium difficile* disease. *Microbiome* 3:35. doi: 10.1186/s40168-015-0097-2

Darrell, J. H., and Wahba, A. H. (1964). Pyocine-typing of hospital strains of *Pseudomonas pyocyanea*. *J. Clin. Pathol.* 17, 236–242. doi: 10.1136/jcp.17.3.236

Dold, H. (1918). On pyocyaneus sepsis and intestinal infections in Shanghai due to *Bacillus pyocyaneus*. *Chin. Med. J.* 32:435.

Driscoll, J. A., Brody, S. L., and Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* 67, 351–368. doi: 10.2165/00003495-200767030-00003

Ekmekciu, I., von Klitzing, E., Fiebiger, U., Escher, U., Neumann, C., Bacher, P., et al. (2017). Immune responses to broad-spectrum antibiotic treatment and fecal microbiota transplantation in mice. *Front. Immunol.* 8:397. doi: 10.3389/fimmu.2017.00397

Erridge, C., Duncan, S. H., Bereswill, S., and Heimesaat, M. M. (2010). The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5. *PLOS ONE* 5:e9125. doi: 10.1371/journal.pone.0009125

Fiebiger, U., Bereswill, S., and Heimesaat, M. M. (2016). Dissecting the interplay between intestinal microbiota and host immunity in health and disease: lessons learned from germfree and gnotobiotic animal models. *Eur. J. Microbiol. Immunol.* 6, 253–271. doi: 10.1556/1886.2016.00036

Gellatly, S. L., and Hancock, R. E. (2013). *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathog. Dis.* 67, 159–173. doi: 10.1111/2049-632x.12033

Gomez-Zorrilla, S., Camoez, M., Tubau, F., Canizares, R., Periche, E., Dominguez, M. A., et al. (2015). Prospective observational study of prior rectal colonization status as a predictor for subsequent development of *Pseudomonas aeruginosa* clinical infections. *Antimicrob. Agents Chemother.* 59, 5213–5219. doi: 10.1128/aac.04636-14

Haag, L. M., Fischer, A., Otto, B., Plickert, R., Kuhl, A. A., Gobel, U. B., et al. (2012a). *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10-/- mice via Toll-like-receptor-2 and -4 signaling. *PLOS ONE* 7:e40761. doi: 10.1371/journal.pone.0040761

Haag, L. M., Fischer, A., Otto, B., Plickert, R., Kuhl, A. A., Gobel, U. B., et al. (2012b). Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. *PLOS ONE* 7:e35988. doi: 10.1371/journal.pone.0035988

Heimesaat, M. M., Bereswill, S., Fischer, A., Fuchs, D., Struck, D., Niebergall, J., et al. (2006). Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J. Immunol.* 177, 8785–8795. doi: 10.4049/jimmunol.177.12.8785

Heimesaat, M. M., Fischer, A., Jahn, H. K., Niebergall, J., Freudenberg, M., Blaut, M., et al. (2007a). Exacerbation of murine ileitis by toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. *Gut* 56, 941–948. doi: 10.1136/gut.2006.104497

Heimesaat, M. M., Fischer, A., Siegmund, B., Kupz, A., Niebergall, J., Fuchs, D., et al. (2007b). Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like receptors 2 and 4. *PLOS ONE* 2:e662. doi: 10.1371/journal.pone.0000662

Heimesaat, M. M., Nogai, A., Bereswill, S., Plickert, R., Fischer, A., Loddenkemper, C., et al. (2010). MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut* 59, 1079–1087. doi: 10.1136/gut.2009.197434

Hentges, D. J., Stein, A. J., Casey, S. W., and Que, J. U. (1985). Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: influence of antibiotics on colonization resistance. *Infect. Immun.* 47, 118–122.

Kerckhoffs, A. P., Ben-Amor, K., Samsom, M., van der Rest, M. E., de Vogel, J., Knol, J., et al. (2011). Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in irritable bowel syndrome. *J. Med. Microbiol.* 60(Pt 2), 236–245. doi: 10.1099/jmm.0.022848-0

Kessner, D. M., and Lepper, M. H. (1967). Epidemiologic studies on gram-negative bacilli in the hospital and community. *Am. J. Epidemiol.* 85, 45–60. doi: 10.1093/oxfordjournals.aje.a120674

Kim, S. W., Peck, K. R., Jung, S. I., Kim, Y. S., Kim, S., Lee, N. Y., et al. (2001). *Pseudomonas aeruginosa* as a potential cause of antibiotic-associated diarrhea. *J. Korean Med. Sci.* 16, 742–744. doi: 10.3346/jkms.2001.16.6.742

Korneev, K. V., Arbatsky, N. P., Molinaro, A., Palmigiano, A., Shaikhutdinova, R. Z., Shneider, M. M., et al. (2015). Structural relationship of the lipid a acyl groups to activation of murine toll-like receptor 4 by lipopolysaccharides from pathogenic strains of *Burkholderia mallei*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. *Front. Immunol.* 6:595. doi: 10.3389/fimmu.2015.00595

Levy, J. (2000). The effects of antibiotic use on gastrointestinal function. *Am. J. Gastroenterol.* 95 1(Suppl.), S8–S10. doi: 10.1016/S0002-9270(99)00808-4

Livermore, D. M. (2009). Has the era of untreatable infections arrived? *J. Antimicrob. Chemother.* 64(Suppl. 1), i29–i36. doi: 10.1093/jac/dkp255

Marshall, J. C., Christou, N. V., and Meakins, J. L. (1993). The gastrointestinal tract. The "undrained abscess" of multiple organ failure. *Ann. Surg.* 218, 111–119. doi: 10.1097/00000658-199308000-00001

Martin-Ancel, A., Borque, C., and del Castillo, F. (1993). *Pseudomonas* sepsis in children without previous medical problems. *Pediatr. Infect. Dis. J.* 12, 258–260. doi: 10.1097/00006454-199303000-00022

Masanta, W. O., Heimesaat, M. M., Bereswill, S., Tareen, A. M., Lugert, R., Gross, U., et al. (2013). Modification of intestinal microbiota and its consequences for innate immune response in the pathogenesis of campylobacteriosis. *Clin. Dev. Immunol.* 2013:526860. doi: 10.1155/2013/526860

Morrison, A. J. Jr., and Wenzel, R. P. (1984). Epidemiology of infections due to *Pseudomonas aeruginosa*. *Rev. Infect. Dis.* 6(Suppl. 3), S627–S642. doi: 10.1093/clinids/6.Supplement_3.S627

Neyrinck, A. M., Possemiers, S., Druart, C., Van de Wiele, T., De Backer, F., Cani, P. D., et al. (2011). Prebiotic effects of wheat arabinoxylan related to the increase in bifidobacteria, *Roseburia* and *Bacteroides/Prevotella* in diet-induced obese mice. *PLOS ONE* 6:e20944. doi: 10.1371/journal.pone.0020944

Nguyen, T. L., Vieira-Silva, S., Liston, A., and Raes, J. (2015). How informative ist the mouse for human gut microbiota research? *Dis. Model. Mech.* 8, 1–16. doi: 10.1242/dmm.017400

Oliver, A., Mulet, X., Lopez-Causape, C., and Juan, C. (2015). The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist. Updat.* 22, 41–59. doi: 10.1016/j.drup.2015.08.002

Otto, B., Haag, L. M., Fischer, A., Plickert, R., Kuhl, A. A., Gobel, U. B., et al. (2012). *Campylobacter jejuni* induces extra-intestinal immune responses via toll-like-receptor-4 signaling in conventional IL-10 deficient mice with chronic colitis. *Eur. J. Microbiol. Immunol.* 2, 210–219. doi: 10.1556/EuJMI.2.2012.3.7

Potron, A., Poirel, L., and Nordmann, P. (2015). Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int. J. Antimicrob. Agents* 45, 568–585. doi: 10.1016/j.ijantimicag.2015.03.001

Shooter, R. A., Walker, K. A., Williams, V. R., Horgan, G. M., Parker, M. T., Asheshov, E. H., et al. (1966). Faecal carriage of *Pseudomonas aeruginosa* in hospital patients. possible spread from patient to patient. *Lancet* 2, 1331–1334. doi: 10.1016/S0140-6736(66)92082-4

Speert, D. P., Campbell, M. E., Davidson, A. G., and Wong, L. T. (1993). *Pseudomonas aeruginosa* colonization of the gastrointestinal tract in patients with cystic fibrosis. *J. Infect. Dis.* 167, 226–229. doi: 10.1093/infdis/167.1.226

Stoodley, B. J., and Thom, B. T. (1970). Observations on the intestinal carriage of *Pseudomonas aeruginosa*. *J. Med. Microbiol.* 3, 367–375. doi: 10.1099/00222615-3-3-367

Stratton, C. W. (1990). *Pseudomonas aeruginosa* revisited. *Infect. Control Hosp. Epidemiol.* 11, 101–104. doi: 10.2307/30144269

Sutter, V. L., and Hurst, V. (1966). Sources of *Pseudomonas aeruginosa* infection in burns: study of wound and rectal cultures with phage typing. *Ann. Surg.* 163, 597–602. doi: 10.1097/00000658-196604000-00013

Tacconelli, E., and Magrini, N. (2017). *Global Priority List of Antibiotic-Resistant Bacteria to Giude research Discovery, and Development of New Antibiotics*. Geneva: WHO.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540

Vincent, J. L. (2003). Nosocomial infections in adult intensive-care units. *Lancet* 361, 2068–2077. doi: 10.1016/s0140-6736(03)13644-6

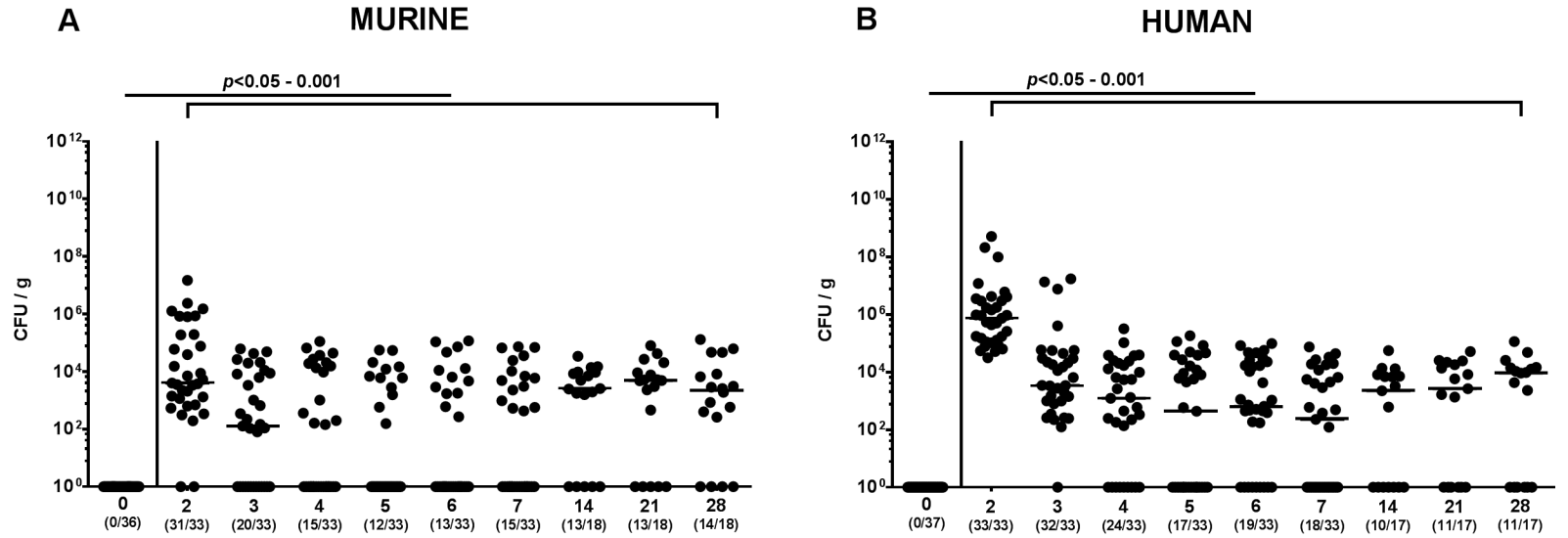
Viola, L., Langer, A., Pulitano, S., Chiaretti, A., Piastra, M., and Polidori, G. (2006). Serious *Pseudomonas aeruginosa* infection in healthy children: case report and review of the literature. *Pediatr. Int.* 48, 330–333. doi: 10.1111/j.1442-200X.2006.02214.x

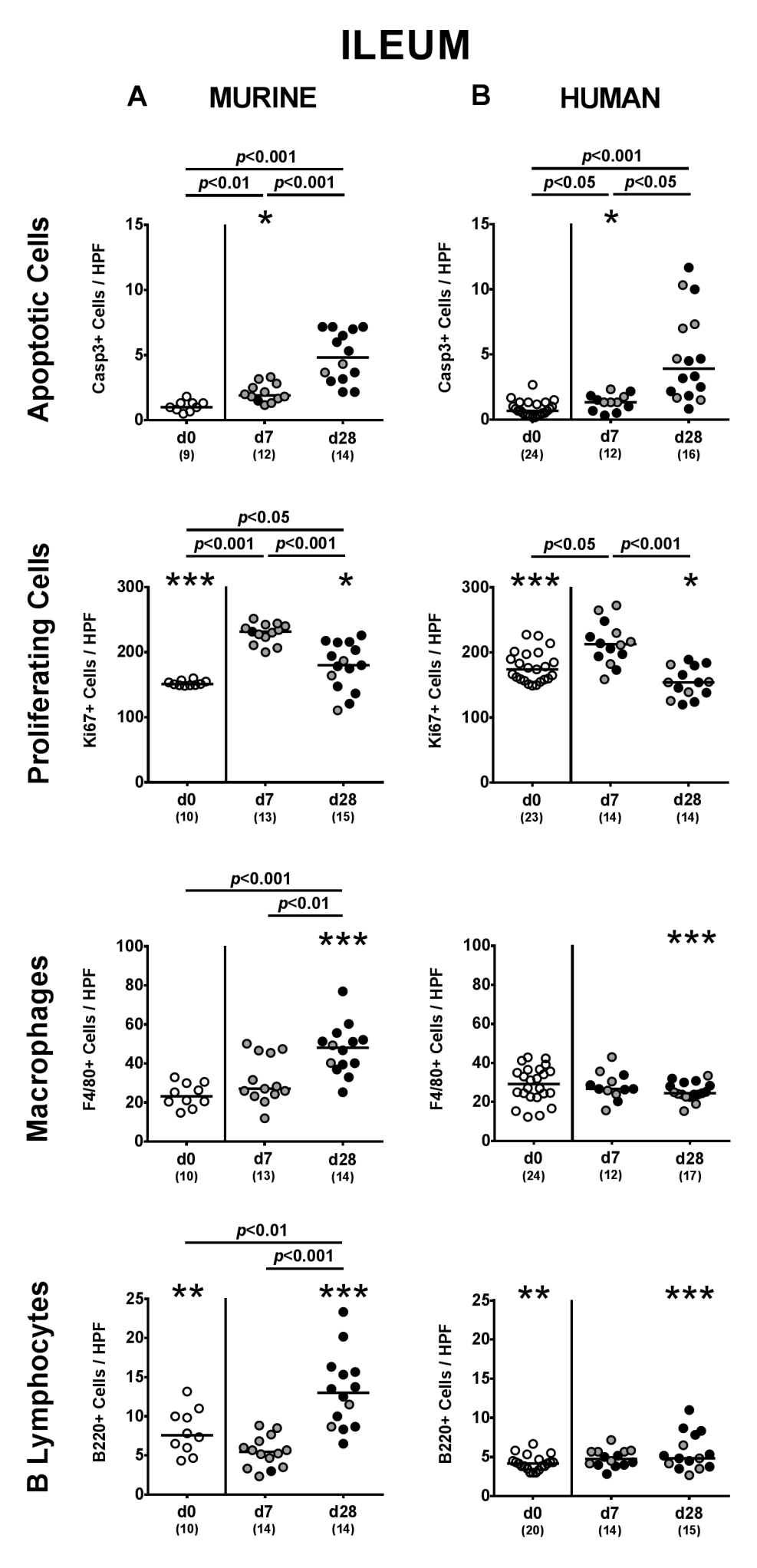
von Klitzing, E., Ekmekciu, I., Bereswill, S., and Heimesaat, M. M. (2017a). Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. *Gut Pathog.* 9:4. doi: 10.1186/s13099-017-0154-4

von Klitzing, E., Oz, F., Ekmekciu, I., Escher, U., Bereswill, S., and Heimesaat, M. M. (2017b). Comprehensive survey of intestinal microbiota changes in offspring of human microbiota-associated mice. *Eur. J. Microbiol. Immunol.* 7, 65–75. doi: 10.1556/1886.2017.00002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Fecal *P. aeruginosa* Loads

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Macrophages
B Lymphocytes
Casp3+ Cells / HPF
Ki67+ Cells / HPF
F4/80+ Cells / HPF
B220+ Cells / HPF
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Gut Pathogens

RESEARCH

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Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice

Eliane von Klitzing, Ira Ekmekciu, Stefan Bereswill and Markus M. Heimesaat*

Abstract

Background: The rising incidence of multidrug resistant (MDR) Gram-negative bacteria including *Pseudomonas aeruginosa* has become a serious issue in prevention of its spread particularly among hospitalized patients. It is, however, unclear whether distinct conditions such as acute intestinal inflammation facilitate *P. aeruginosa* infection of vertebrate hosts.

Methods and results: To address this, we analysed *P. aeruginosa* infection in human microbiota-associated (hma) mice with acute ileitis induced by peroral *Toxoplasma gondii* challenge. When perorally infected with *P. aeruginosa* at day 3 post ileitis induction, hma mice displayed higher intestinal *P. aeruginosa* loads as compared to hma mice without ileitis. However, the overall intestinal microbiota composition was not disturbed by *P. aeruginosa* (except for lowered bifidobacterial populations), and the infection did not further enhance ileal immune cell responses. Pro-inflammatory cytokines including IFN-γ and IL-12p70 were similarly increased in ileum and mesenteric lymph nodes of *P. aeruginosa* infected and uninfected hma mice with ileitis. The anti-inflammatory cytokine IL-10 increased multifold upon ileitis induction, but interestingly more distinctly in *P. aeruginosa* infected as compared to uninfected controls. Immune responses were not restricted to the intestines as indicated by elevated pro-inflammatory cytokine levels in liver and kidney upon ileitis induction. However, except for hepatic TNF-α levels, *P. aeruginosa* infection did not result in more distinct pro-inflammatory cytokine secretion in liver and kidney of hma mice with ileitis. Whereas viable intestinal bacteria were more frequently detected in systemic compartments such as spleen and cardiac blood *of P. aeruginosa* infected than uninfected mice at day 7 following ileitis induction, *P. aeruginosa* infection did not exacerbate systemic pro-inflammatory sequelae, but resulted in lower IL-10 serum levels.

Conclusion: Acute intestinal inflammation facilitates infection of the vertebrate host with MDR bacteria including *P. aeruginosa* and might also pose particularly hospitalized patients at risk for acquisition. Since acute *T. gondii* induced inflammation might mask immunopathology caused by *P. aeruginosa*, a subacute or chronic inflammation model might be better suited to investigate the potential role of *P. aeruginosa* infection in the aggravation of intestinal disease.

Keywords: *Pseudomonas aeruginosa*, Multidrug resistant Gram-negative bacteria, Susceptibility to infection, Intestinal microbiota, Fecal transplantation, Human microbiota-associated mice, *Toxoplasma gondii* induced acute ileitis, Pro-inflammatory immune responses, Extra-intestinal and systemic sequelae of infection, Bacterial translocation

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Background

Pseudomonas aeruginosa, a non-fermenting Gram-negative rod, is one of the most important bacterial pathogens responsible for a multitude of opportunistic infections in humans, especially in hospitalized patients [1]. The strictly aerobic pathogen is the primary cause of ventilator-associated pneumonia or superinfection of burn wounds associated with a mortality of more than 30% [2]. Its worldwide increasing resistance to manifold antibiotic classes particularly due to extended-spectrum β-lactamases, carbapenemases and 16S rRNA methylases makes *P. aeruginosa* to a continuously growing threat for immune-compromised individuals, patients suffering from cystic fibrosis and other pulmonary diseases or patients admitted to intensive care units [2–4]. A single flagellum and multiple cell surface pili allow motility and adherence of *P. aeruginosa* with surfaces [5], whereas biofilm formation, alginate secretion, quorum-sensing and an elaborated secretion system contribute to the virulence of *P. aeruginosa* [1, 5]. Earlier studies revealed that ingested *P. aeruginosa* are detectable in human fecal samples of healthy volunteers up to 6 days following oral administration without occurrence of clinical symptoms. Fecal *P. aeruginosa* loads were lower than those that had been ingested and decreased over time post challenge [6]. However, admission to a surgical ward may increase the risk of peroral *P. aeruginosa* acquisition, since the percentage of patients' stool samples carrying *P. aeruginosa* increased during hospital stay [7]. In addition to objects and materials present on wards, the human gastrointestinal tract may be an important source of *P. aeruginosa* infection [7]. Recent clinical surveys revealed that *P. aeruginosa* detection rates were significantly higher in fecal and mucosal samples derived from patients suffering from irritable bowel syndrome [8] or in the colonic mucosa of a pediatric patient with ulcerative colitis [9] as compared to a healthy individual. Whereas the pathogenic potential of *P. aeruginosa* is well-known, its potential contribution to initiation and perpetuation of intestinal immunopathological conditions are not yet understood. Hence, there is currently a large gap in knowledge regarding the interplay of *P. aeruginosa*, the host microbiota and immune system, particularly under conditions of intestinal inflammation.

Within 1 week following peroral high dose infection with the intracellular parasite *Toxoplasma gondii*, susceptible mice develop acute inflammation of the terminal ileum with massive necrosis (pan-ileitis) as well as extra-intestinal and systemic sequelae and succumb to infection within 7–10 days [10–12]. This fatal hyperinflammatory scenario is due to a typical T helper cell 1 (Th1)-type immunopathology and characterized by an overproduction of pro-inflammatory mediators including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, nitric oxide, interleukin (IL)-6, IL-12 and monocyte chemoattractant protein (MCP)-1 evolving upon parasitic interacting with antigen presenting cells with subsequent activation of CD4+ T cells, whereas the *T. gondii* induced anti-inflammatory measures include IL-10 expression (reviewed by [13]). We showed earlier that the gut microbiota is essential for the initiation and progression of *T. gondii* induced ileitis and that small intestinal inflammation is accompanied by distinct changes in the commensal microbiota composition with Gram-negative species such as enterobacteria and *Bacteroides/Prevotella* spp. overgrowing the inflamed ileal lumen [11]. Toll-like receptor (TLR)-4 dependent signaling of lipopolysaccharide derived from Gram-negative intestinal commensals further perpetuates the hyper-inflammatory process [14, 15]. Hence, the Th1-type immunopathology underlying *T. gondii* induced ileitis and associated intestinal microbiota shifts resemble key features of acute episodes in patients suffering from Crohn's disease [10, 13]. In the present study we investigated whether acute small intestinal inflammation predisposes the vertebrate host with a human microbiota to infection with multidrug resistant (MDR) *P. aeruginosa*. To address this, mice harboring a complex human microbiota were challenged with high dose *T. gondii* infection for ileitis induction and subsequently infected with a MDR *P. aeruginosa* strain. The presented results shed further light onto the interplay between MDR *P. aeruginosa*, the host innate and adaptive immunity and human intestinal microbiota during acute small intestinal inflammation.

Methods

Ethical statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration numbers G0097/12 and G0039/15). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice. Mice suffering from weight loss >20% were euthanized by isoflurane treatment (Abbott, Germany) in accordance with the guidelines of the local authorities.

Generation of gnotobiotic (secondary abiotic) mice

Female C57BL/6j mice were bred under specific pathogen-free conditions in the Forschungsinstitute für Experimentelle Medizin (Charité-University Medicine, Berlin, Germany). Gnotobiotic (i.e. secondary abiotic) mice with a virtually depleted microbiota were generated as described previously [11]. In brief, 8 weeks old mice were transferred to sterile cages and subjected to

a broad-spectrum antibiotic treatment for 8–10 weeks by adding ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany), vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany) and metronidazole (1 g/L; Fresenius, Germany) to the drinking water (ad libitum).

Generation of human microbiota-associated mice

Fresh fecal samples free of enteropathogenic bacteria, viruses and parasites were collected from five individual healthy volunteers, dissolved in sterile phosphate buffered saline (PBS; Gibco, life technologies, UK), aliquoted and stored at −80 °C as described earlier [16]. Immediately before reconstitution experiments, individual fecal aliquots were thawed, pooled, and the main bacterial communities within the donor suspension quantitatively assessed by cultural and molecular methods [16]. To generate human intestinal microbiota-associated (hma) mice, gnotobiotic animals were subjected to peroral fecal transplantations with 0.3 mL of the donor suspension by gavage on 3 consecutive days. The total load of bacterial groups between independent experiments counted around 10^{10} colony forming units (CFU) and varied less than 0.5 logarithmic orders of magnitude. To assure proper establishment of the human microbiota in the murine host, mice were kept for at least 3 weeks until ileitis induction. Immediately before peroral *T. gondii* infection individual fecal samples were collected for quantitative cultural and molecular analyses of main intestinal bacterial communities.

Induction of acute ileitis

In order to induce acute ileitis mice harboring a human intestinal microbiota were infected perorally with 50 cysts of *T. gondii* (ME49 strain) by gavage as described previously [11, 14, 17].

Pseudomonas aeruginosa infection

Three days following ileitis induction mice were perorally infected with 10^9 CFU of a MDR *P. aeruginosa* strain by gavage in a total volume of 0.3 mL PBS. The *P. aeruginosa* isolate was cultured from respiratory material of a patient suffering from nosocomial pneumonia and kindly provided by Prof. Dr. Bastian Opitz (Charité-University Medicine, Berlin, Germany). Of note, the bacterial strain displayed antimicrobial sensitivity to fosfomycin and colistin only.

Cultural analysis of the intestinal loads of *P. aeruginosa*

On days 2, 3 and 4 post *P. aeruginosa* infection individual fecal samples were homogenized in sterile PBS, and serial dilutions streaked onto Columbia agar supplemented with 5% sheep blood (Oxoid, Germany) and Cetrimid agar (Oxoid) and incubated in an aerobic atmosphere at 37 °C for at least 48 h to assess intestinal *P. aeruginosa* loads.

Clinical conditions

Body weights as well as macroscopic and/or microscopic abundance of fecal blood were assessed in individual mice on a daily basis by the Guajac method using Haemoccult (Beckman Coulter/PCD, Germany).

Sampling procedures

Mice were sacrificed 7 days after ileitis induction by isoflurane treatment (Abbott, Germany). Cardiac blood and tissue samples from spleen, liver, lung, kidney, mesenteric lymph nodes (MLN), ileum and colon were removed under sterile conditions. Ileal and colonic samples from each mouse were collected in parallel for microbiological, immunological, immunohistochemical and histopathological analyses. Experiments were repeated at least twice.

Small intestinal lengths and histopathological scores

Small intestinal lengths were determined by measuring the distance from the duodenum leaving the stomach to the ileal-caecal transition. Ex vivo biopsies derived from the terminal ileum were immediately fixed in 5% formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E) and subjected to a standardized histopathological scoring system ranging from 0 to 6 as described earlier [11, 14].

Immunohistochemistry

5 µm thin paraffin sections of ileal ex vivo biopsies were used for in situ immunohistochemical analysis as described previously [18–20]. Primary antibodies against CD3 (Polycl.rabbit anti human, DAKO, Denmark; 1:10), FOXP3 (FJK-165, eBioscience, Germany; 1:100), B220 (eBioscience; 1:200) and F4/80 (biot. Clone BM 8 rat anti mouse, Life Technologies, USA; 1:100) were used to assess T lymphocytes, regulatory T cells (Treg), B lymphocytes and macrophages/monocytes, respectively. The average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2; 400× magnification) were determined by an independent blinded investigator.

Cultural survey of intestinal microbiota and bacterial translocation

For comprehensive quantitative survey of intestinal microbiota composition and translocation of viable bacteria to extra-intestinal compartments, colonic and ileal luminal contents as well as ex vivo biopsies

derived from MLN, spleen, liver and lung, respectively, were homogenized in sterile PBS and analyzed in serial dilutions on different solid culture media as described earlier [11, 14, 21]. Cardiac blood was incubated in thioglycolate enrichment broths (BD Bioscience, Germany) for 1 week at 37 °C and streaked onto solid media thereafter. Bacteria were grown at 37 °C for at least 2–3 days under aerobic, microaerobic and anaerobic conditions.

Molecular analysis of the ileal microbiota

DNA was extracted from fecal samples as described previously [11, 22]. In brief, DNA was quantified by using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng per µL. Then, main bacterial groups abundant in the murine and human intestinal microbiota were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) with species-, genera- or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as described previously [16, 18, 23] and numbers of 16S rRNA gene copies per ng DNA of each sample determined.

Cytokine detection in colon, ileum, mesenteric lymph nodes, liver, kidney, spleen and serum

Ex vivo biopsies of approximately 1 cm^2 (ileum cut longitudinally) were washed in PBS and placed in 24-flat-bottom well culture plates (Falcon, Germany) containing 500 mL serum-free RPMI 1640 medium (Gibco, life technologies) supplemented with penicillin (100 U/mL, Biochrom, Germany) and streptomycin (100 µg/mL; Biochrom). After 18 h at 37 °C, culture supernatants and serum samples were tested for IFN-γ, TNF-α, MCP, IL-12p10, IL-6 and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Bioscience) on a BD FACSCanto II flow cytometer (BD Bioscience). Nitric oxide was determined by the Griess reaction as described previously [11].

Statistical analysis

Mean values, medians, standard deviations (SD) and levels of significance were determined using appropriate tests as indicated (two-tailed Student's t test, Mann–Whitney U test, ordinary one-way ANOVA and Kruskal–Wallis test). Two-sided probability (p) values ≤ 0.05 were considered significant.

Results

Acute ileitis induction facilitates multidrug resistant *P. aeruginosa* infection of human microbiota-associated mice

The primary goal of our present study was to investigate the influence of acute intestinal inflammation on intestinal infection with MDR *P. aeruginosa* and its interplay with the intestinal commensal microbiota and the immune system in the vertebrate host. We first addressed whether acute ileitis impacted the intestinal colonization capacity of MDR *P. aeruginosa*. To accomplish this, we generated secondary abiotic mice with a virtually depleted microbiota by broad-spectrum antibiotic treatment and reconstituted these mice with a human intestinal microbiota by peroral transplantation of mixed fecal samples derived from five healthy individuals on 3 consecutive days. A comprehensive cultural survey of the main intestinal bacterial groups abundant in the human gut performed 3 weeks post fecal transplantation (and immediately before acute ileitis induction) revealed that the human microbiota had stably established within the murine host (Fig. 1). Furthermore, loads of respective bacterial groups did not differ between cohorts that were subsequently infected with MDR *P. aeruginosa* or remained uninfected (Fig. 1).

In order to induce acute ileitis, hma mice were perorally infected with 50 cysts of *T. gondii* ME 49 strain by

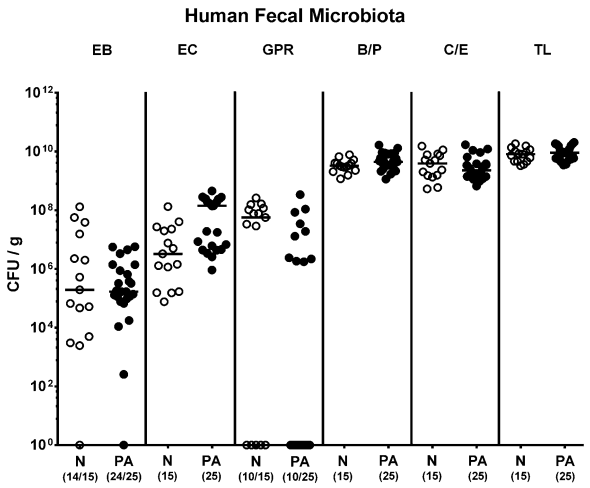

Fig. 1 Intestinal microbiota composition following human fecal transplantations. Gnotobiotic (secondary abiotic) mice were generated by broad-spectrum antibiotic treatment and subjected to peroral transplantations of human feces as described in "Methods" section. Three weeks following association with human microbiota and immediately before acute ileitis induction, a cultural survey of the intestinal microbiota composition was performed on fecal samples derived from mice of the *P. aeruginosa* infection cohort (PA; *filled circles*) as compared to the non-infected control group (N; *open circles*). Numbers of viable enterobacteria (EB), enterococci (EC), Gram-positive rods (GPR), *Bacteroides/Prevotella* spp. (B/P), *Clostridium/Eubacterium* spp. (C/E) and the total loads (TL) were expressed as colony forming units per gram feces (CFU/g). Numbers of animals harboring the respective bacterial groups are given in parentheses and medians are indicated. Data shown were pooled from three independent experiments

gavage. At day 3 following ileitis induction (a critical time point when initial histopathological changes within the ileal mucosa can be observed in peroral high dose *T. gondii* infection [11]), mice were perorally challenged with 10^9 CFU *P. aeruginosa* by gavage. Naive hma mice served as uninfected controls. In addition, one separate cohort of hma mice without induced ileitis was included into the *P. aeruginosa* infection studies. Interestingly, mice with ileitis could be infected by *P. aeruginosa* and displayed median fecal pathogenic loads of approximately 10^6 CFU per g that were two to three orders of magnitude higher as compared to those determined in hma mice without ileitis induction at days 3 and 4 post infection (p.i.) ($p < 0.01$ and $p < 0.0001$, respectively; Fig. 2). On day 4 p.i., 73.0% of hma mice without ileitis induction were carrying *P. aeruginosa*, whereas 95.0% of mice with an inflamed small intestine harbored the MDR bacteria (Fig. 2). Hence, acute small intestinal inflammation renders hma mice more susceptible to infection with MDR *P. aeruginosa*.

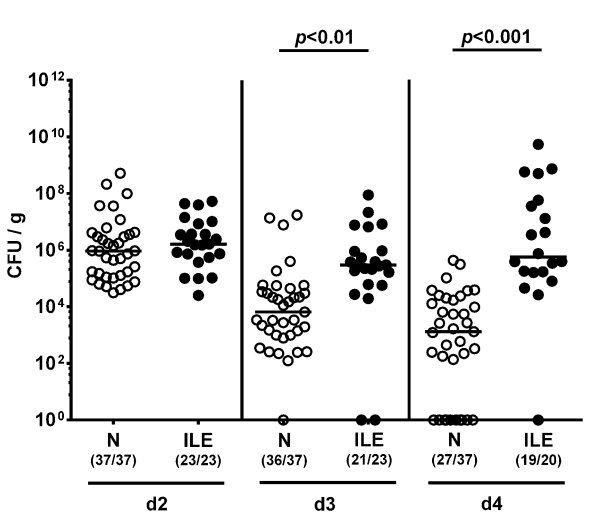

Fig. 2 Kinetic of fecal multidrug resistant *P. aeruginosa* loads in human microbiota-associated mice with and without acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis (ILE; *filled circles*) and additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction. *P. aeruginosa* infected human microbiota-associated mice without ileitis induction (N; *open circles*) served as controls. Intestinal colonization densities were determined in fecal samples at days (d) 2, 3 and 4 following *P. aeruginosa* infection by culture and expressed as colony forming units per gram feces (CFU/g). Numbers of mice harboring *P. aeruginosa* out of the total number of analyzed mice are given in parentheses. Medians (*black bars*) and significance levels (*p* values) determined by Kruskal–Wallis test are indicated. Data shown were pooled from three independent experiments

Intestinal microbiota composition in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis

We next assessed whether MDR *P. aeruginosa* infection of hma mice with induced acute ileitis was accompanied by distinct changes in intestinal microbiota composition. To address this, mice were sacrificed at day 7 following ileitis induction and cultural as well as culture-independent (i.e. molecular) analyses of intestinal luminal contents performed. Cultural analyses revealed total bacterial counts of up to 10^{11} CFU per g feces and increased loads of Gram-negative bacterial groups including enterobacteria and obligate anaerobic *Bacteroides/Prevotella* spp. (approximately 10^{10} CFU/g) as well as of enterococci (approximately 10^9–10^{10} CFU/g) and obligate anaerobic *Clostridium/Eubacterium* spp. (approximately 10^9–10^{10} CFU/g) in ileal and colonic luminal contents of mice suffering from acute ileitis (Fig. 3a). Aerobic Gram-positive rods, however, were virtually absent in small and large intestines of *T. gondii* infected mice (Fig. 3a). Notably, loads of the main cultivable bacterial groups did not differ between *P. aeruginosa* infected and uninfected mice with acute ileitis (Fig. 3a). In order to assess fastidious and non-cultivable bacteria we additionally performed quantitative molecular (i.e. 16S rRNA based) analyses of ileal contents. Interestingly, bifidobacterial gene numbers were more than two orders of magnitude lower in *P. aeruginosa* infected as compared to uninfected mice with acute ileitis ($p < 0.05$; Fig. 3b). The remaining bacterial groups, however, did not differ between the ilea of both cohorts (Fig. 3b), thus confirming results obtained from culture.

Clinical, macroscopic and microscopic sequelae of multidrug resistant *P. aeruginosa* infection of human microbiota-associated mice suffering from acute ileitis

We next investigated whether the clinical outcome of hma mice with acute ileitis was further deteriorated by MDR *P. aeruginosa* infection. Seven days post ileitis induction, blood could be detected microscopically or even macroscopically in fecal samples from any of *P. aeruginosa* infected hma mice, but only in 81.9% of hma mice without concomitant infection, whereas naive hma control animals were unaffected (Fig. 4a). Given that acute intestinal inflammation is associated with significant shortening of the inflamed gut [11, 16], we measured the small intestinal lengths of mice at necropsy. Both *P. aeruginosa* infected and uninfected hma mice with ileitis displayed comparably shorter small intestines than naive controls ($p < 0.001$; Fig. 4b). The comparable macroscopic sequelae of *P. aeruginosa* infected and uninfected hma mice with acute ileitis were paralleled by similar microscopic intestinal inflammatory

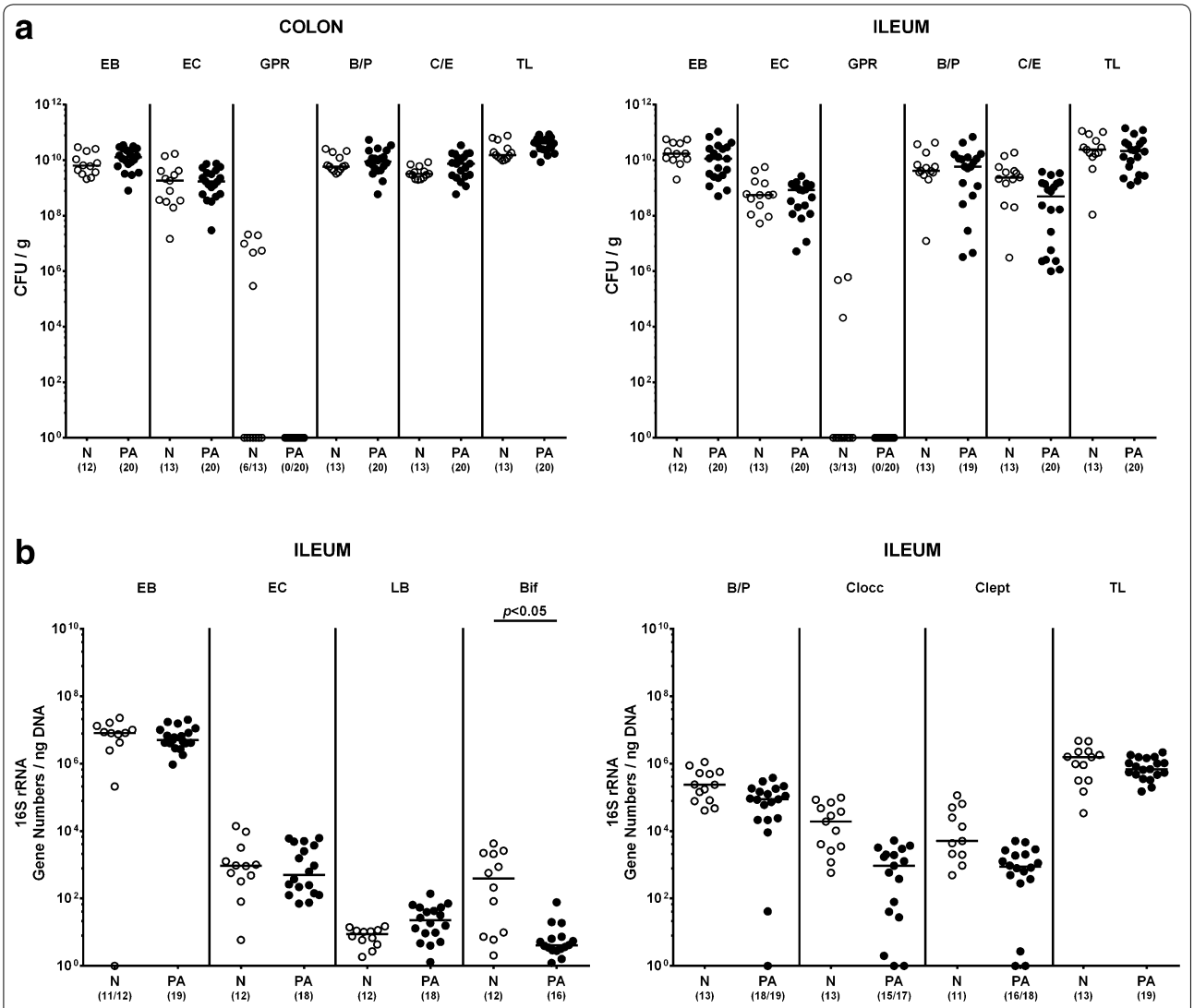

Fig. 3 Intestinal microbiota analysis in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis as described in "Methods" section. One group of mice (PA, *filled circles*) was additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction by gavage, whereas non-infected mice served as controls with acute ileitis only (N, *open circles*). Main intestinal bacterial groups were quantified by culture and molecular analysis of luminal colon and ileum samples at day 7 post *T. gondii* infection. **a** Numbers of viable enterobacteria (EB), enterococci (EC), Gram-positive rods (GPR), *Bacteroides/Prevotella* spp. (B/P), *Clostridium/Eubacterium* spp. (C/E) and the total bacterial loads (TL) were determined in colonic and ileal luminal contents by culture and expressed as colony forming units per gram (CFU/g). **b** 16SrRNA of the main ileal bacterial groups including enterobacteria (EB), enterococci (EC), lactobacilli (LB), bifidobacteria (Bif), *Bacteroides/Prevotella* spp. (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept) and total eubacterial loads (TL) are expressed as gene numbers per ng DNA. Numbers of animals harboring the respective bacterial group are given in parentheses. Medians and significance levels (*p* values) determined by Kruskal–Wallis test are indicated. Data shown were pooled from three independent experiments

changes. At day 7 post ileitis induction hma mice displayed destruction of villous architecture, cellular shedding into the lumen and massive necrosis of the ileal mucosa, irrespective of *P. aeruginosa* infection, as indicated by similarly elevated histopathological scores ($p < 0.001$ vs naive; Fig. 4c). In addition, ileal epithelial apoptotic cell numbers were comparably increased in *P. aeruginosa* infected and uninfected mice 7 days following ileitis induction ($p < 0.001$ vs naive; Fig. 4d). Taken together, *T. gondii* induced clinical, macroscopic and microscopic small intestinal changes did not further worsen upon MDR *P. aeruginosa* infection.

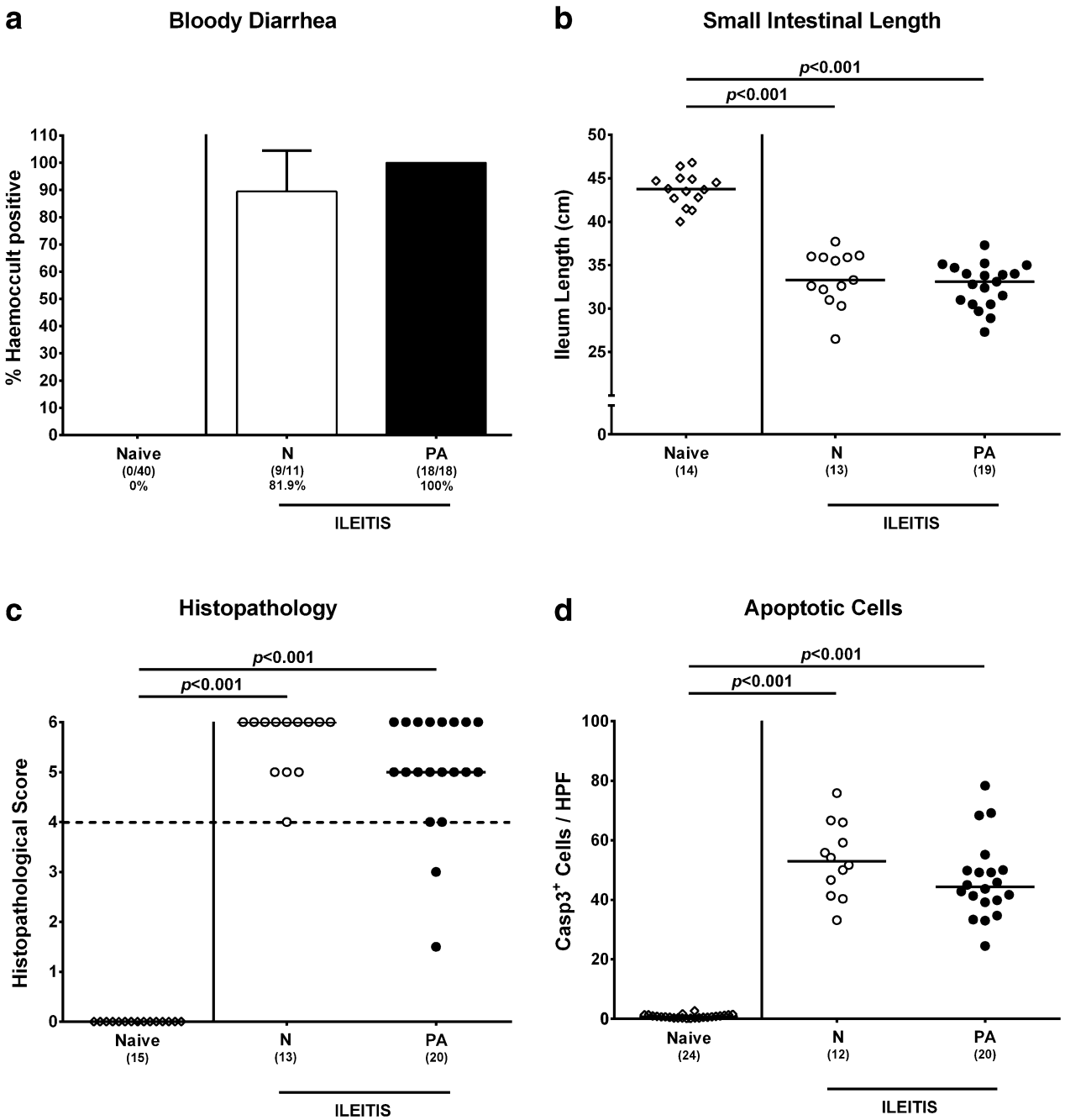

Fig. 4 Clinical, macroscopic and microscopic sequelae in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; *filled bar or circles*) or not (N; *open bar or circles*). Naive mice served as negative controls (*open diamonds*). Clinical, macroscopic and microscopic intestinal changes were assessed at day 7 post ileitis induction: **a** Abundance of bloody diarrhea was determined in fecal samples by the Guajac (Haemoccult) method (in %). Means, standard deviations and numbers of fecal blood positive mice out of the total numbers of animals (in *parentheses*) are indicated. **b** Absolute small intestinal lengths were measured (in cm) and **c** histopathological changes were determined in H&E stained ileal paraffin sections applying a standardized scoring system. Scores ≥4 (*dotted line*) indicate severe inflammation (with necrosis). **d** The average numbers of apoptotic cells (positive for caspase 3; $Casp3^{+}$) from at least six high power fields (HPF, ×400 magnification) per animal were determined microscopically in immunohistochemically stained ileal paraffin sections. Numbers of animals (in *parentheses*), medians and significance levels (*p* values) determined by Kruskal–Wallis test or ordinary one-way ANOVA are indicated. Data shown were pooled from three independent experiments

Intestinal pro-inflammatory immune responses in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice with acute ileitis

We next surveyed quantitative small intestinal immune cell responses upon MDR *P. aeruginosa* infection of hma mice suffering from acute ileitis applying in situ immunohistochemistry. At day 7 post ileitis induction, both *P. aeruginosa* infected and uninfected hma mice displayed multifold and comparably elevated numbers of T and B lymphocytes, Treg as well as of macrophages and monocytes within the ileal mucosa and lamina propria (Fig. 5). Hence, *T. gondii* infection resulted in a profound increase of innate and adaptive immune cells in the distal small intestines that was, however, not further enhanced by MDR *P. aeruginosa* infection. This was also true for intestinal pro-inflammatory cytokine levels as indicated by similarly elevated IFN-γ concentrations in ex vivo biopsies derived from ileum and MLN ($p < 0.001$) and additionally of IL-12p70 ($p < 0.05$ and $p < 0.01$, respectively) in the ileum of *P. aeruginosa* infected and uninfected hma mice 7 days following *T. gondii* infection (Fig. 6a, c). Furthermore, IL-12p70 levels were increased in the MLN of uninfected, but not *P. aeruginosa* infected hma mice with ileitis induction ($p < 0.05$; Fig. 6c). Interestingly, the anti-inflammatory cytokine IL-10 increased multifold upon ileitis induction in either mice ($p < 0.05$ and $p < 0.001$, respectively), but more distinctly in *P. aeruginosa* infected as compared to uninfected hma mice ($p < 0.05$; Fig. 6c). Given that upon peroral *T. gondii* infection the small, but not large intestinal tract has been described as exclusive intestinal predilection site of inflammation so far [13], we unexpectedly observed elevated IFN-γ secretion even in supernatants of colonic ex vivo biopsies at day 7 post ileitis induction in either mice ($p < 0.001$; Fig. 6b).

Extra-intestinal pro-inflammatory immune responses in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice with acute ileitis

We next addressed whether MDR *P. aeruginosa* infection worsened potential extra-intestinal inflammatory responses in hma mice with acute ileitis. Interestingly, IFN-γ and nitric oxide secretion increased in ex vivo biopsies taken from liver and kidney until day 7 following ileitis induction in both *P. aeruginosa* infected and uninfected hma mice ($p < 0.05$ to 0.001; Fig. 7), whereas nitric oxide levels were slightly lower in the liver of the latter as compared to the former ($p < 0.05$; Fig. 7a). Notably, hepatic TNF-α concentrations were elevated in *P. aeruginosa* infected hma mice only ($p < 0.01$; Fig. 7a), but renal MCP-1 levels increased exclusively in uninfected hma mice with ileitis ($p < 0.01$; Fig. 7b). Hence, ileitis induction is in fact accompanied by pro-inflammatory responses in extra-intestinal compartments. However, except for hepatic TNF-α levels MDR *P. aeruginosa* infection does not result in more distinct pro-inflammatory cytokine responses in liver and kidney of hma mice with acute ileitis.

Systemic sequelae of multidrug resistant *P. aeruginosa* infection in human microbiota-associated mice with acute ileitis

We further investigated whether MDR *P. aeruginosa* infection aggravated potential systemic responses upon ileitis induction. At day 7 following *T. gondii* infection, pro-inflammatory cytokines including IFN-γ, TNF-α, MCP-1, IL-12p70 and IL-6 were elevated in both *P. aeruginosa* infected and uninfected hma mice with ileitis as compared to naive controls ($p < 0.05$-0.001; Fig. 8a) that were accompanied by increased IFN-γ secretion in splenic ex vivo biopsies ($p < 0.001$; Fig. 8b). Upon ileitis induction, serum IL-10 concentrations increased in either hma mice, but less distinctly in *P. aeruginosa* infected mice as compared to uninfected controls ($p < 0.001$; Fig. 8a). Hence, MDR *P. aeruginosa* infection did not exacerbate systemic pro-inflammatory sequelae of acute ileitis, but resulted in lower anti-inflammatory responses.

Bacterial translocation following multidrug resistant *P. aeruginosa* infection of human microbiota-associated mice with acute ileitis

We next analyzed whether viable bacteria originating from the intestinal microbiota were abundant in extra-intestinal and systemic compartments of hma mice suffering from acute ileitis, and whether MDR *P. aeruginosa* infection facilitated bacterial translocation. In all MLN derived from *P. aeruginosa* infected and uninfected hma mice with ileitis, commensal intestinal bacteria such as enterobacteria, enterococci, lactobacilli, *Bacteroides/Prevotella* spp. and/or *Clostridium/Eubacterium* spp. could be cultured. In extra-intestinal compartments such as liver, however, mean bacterial translocation rates were 66.7% ± 9.4 and 85.0% ± 21.2 in *P. aeruginosa* infected and uninfected mice with ileitis, respectively, whereas commensal intestinal species could be cultured in 63.4% ± 4.8 and 85.0% ± 21.2 of lungs in respective cohorts (Fig. 9a). Interestingly, viable intestinal bacteria were more frequently detected in systemic compartments such as spleen and cardiac blood of *P. aeruginosa* infected as compared to uninfected mice at day 7 following ileitis induction (mean translocation rates spleen: 60.0% ± 28.3 vs 36.7% ± 4.7, respectively; cardiac blood: 40.0% ± 28.3 vs 10.0% ± 14.1, respectively) (Fig. 9a). *P. aeruginosa* could be isolated on average from approximately 25–40% of respective organ homogenates and blood (Fig. 9b; Additional file 1: Figure S1). Notably, no

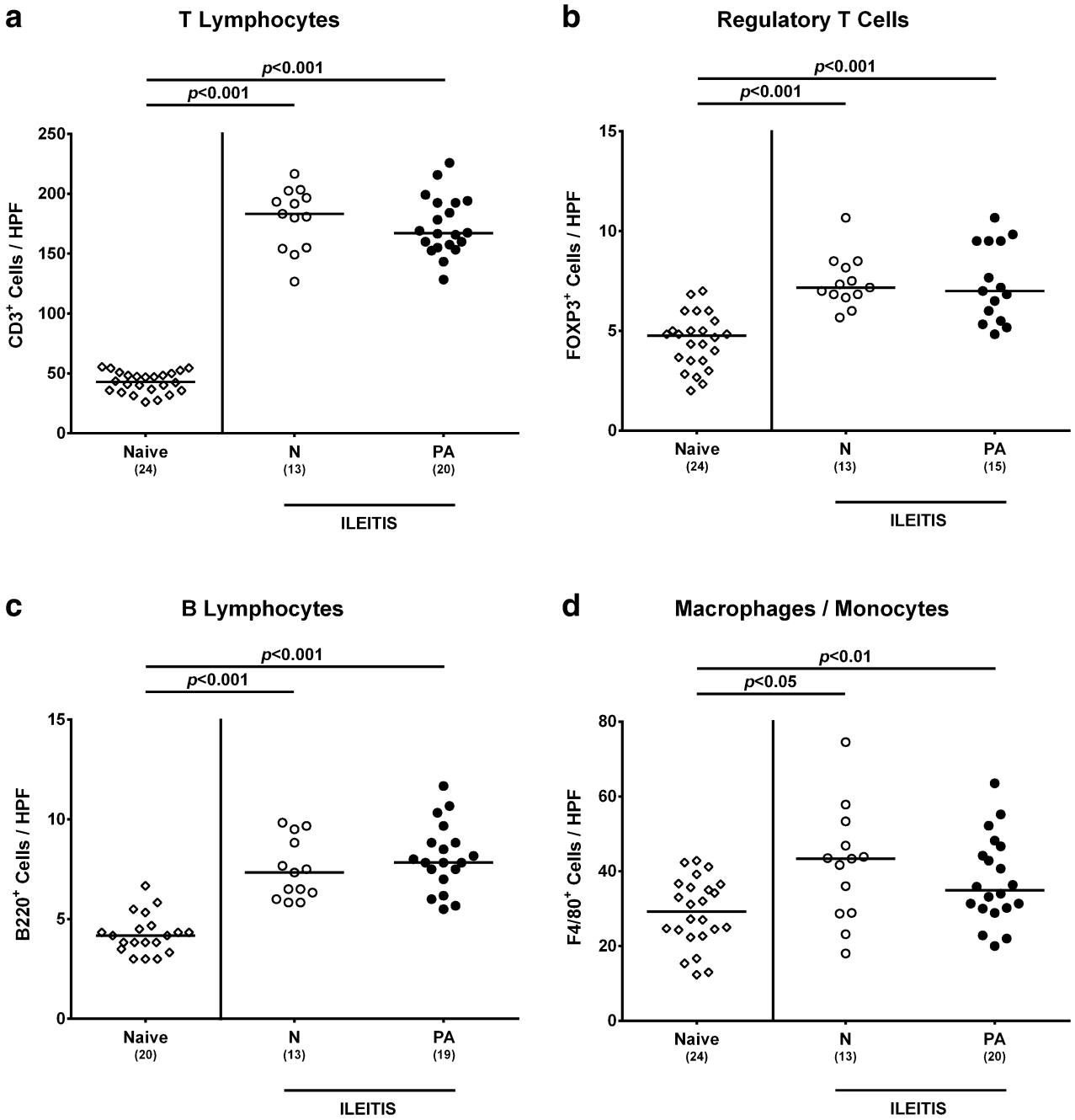

Fig. 5 Small intestinal immune cell responses in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; *filled circles*) or not (N; *open circles*). The average number of ileal **a** T lymphocytes (positive for CD3), **b** regulatory T cells (positive for FOXP3), **c** B lymphocytes (positive for B220), and **d** macrophages and monocytes (positive for F4/80) from six representative high power fields (HPF, ×400 magnification) per animal was determined microscopically in immunohistochemically stained ileal paraffin sections at day 7 post ileitis induction. Naive mice served as negative controls (*open diamonds*). Numbers of mice (in *parentheses*), medians (*black bars*) and significance levels (*p* values) determined by Kruskal–Wallis test or ordinary one-way ANOVA are indicated. Data shown were pooled from three independent experiments

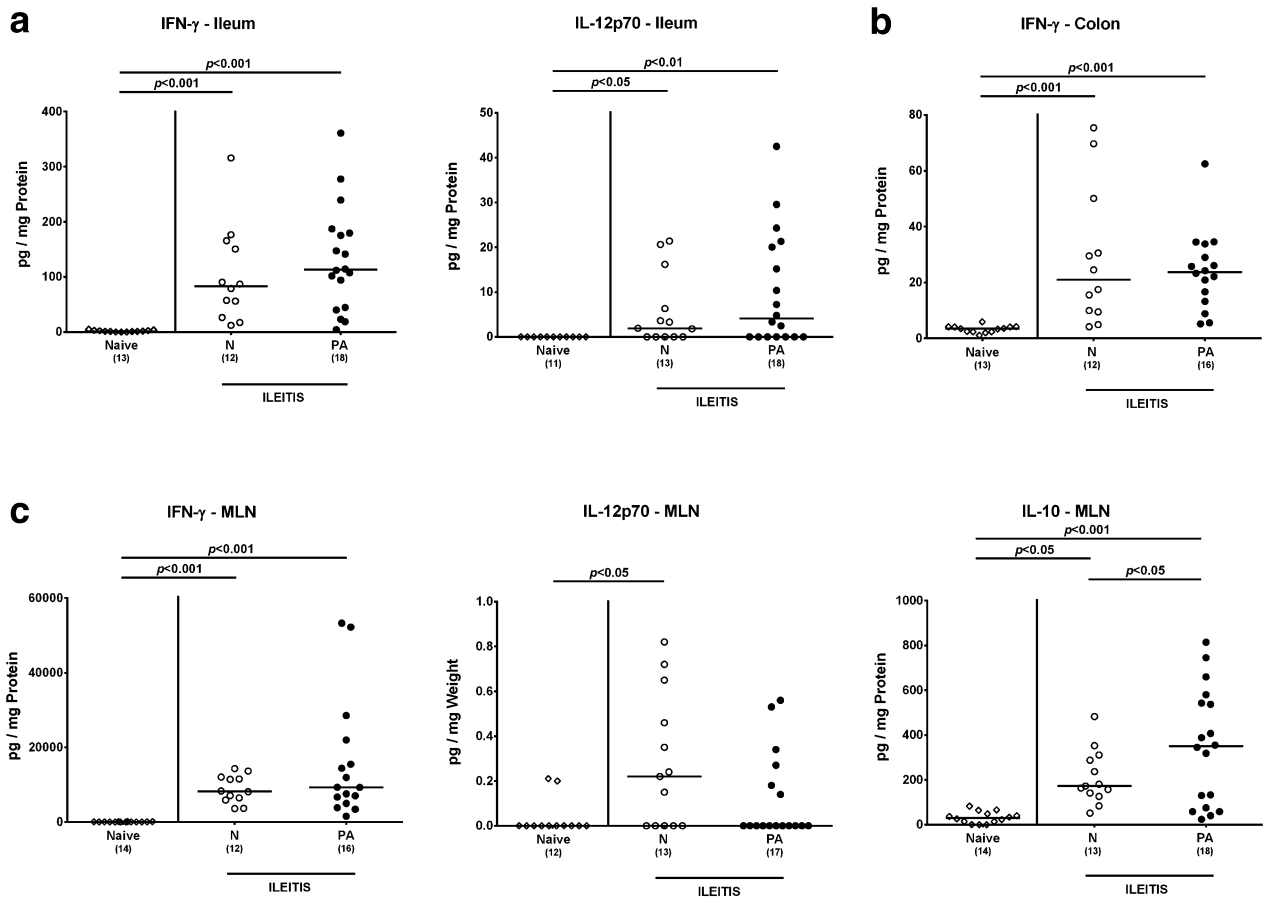

Fig. 6 Intestinal cytokine responses in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; *filled circles*) or not (N; *open circles*). At day 7 post ileitis induction secretion of distinct pro- and anti-inflammatory cytokines were determined in ex vivo biopsies derived from different intestinal compartments including **a** ileum (IFN-γ and IL-12p70), **b** colon (IFN-γ) and **c** MLN (IFN-γ, IL-12p70, IL-10). Naive mice served as negative controls (*open diamonds*). Numbers of mice (in *parentheses*), medians (*black bars*) and significance levels (*p* values) determined by Kruskal–Wallis test or ordinary one-way ANOVA are indicated. Data shown were pooled from three independent experiments

translocating intestinal bacterial species could be cultured from respective extra-intestinal and systemic compartments of naive hma mice (not shown).

Discussion

In the present study we were able to show that an acute intestinal inflammatory condition facilitates MDR *P. aeruginosa* infection of the vertebrate host. Whereas (already fatal) ileal inflammatory changes were similar, extra-intestinal sequelae of high dose *T. gondii* infection were further aggravated by subsequent *P. aeruginosa* infection.

Applying a well-known infection model of acute *T. gondii* induced ileitis in (with respect to their microbiota) "humanized" mice, we first verified that prior to ileitis induction, human microbiota had stably established within the murine host and that microbiota composition was comparable in experimental groups that were subsequently infected with MDR *P. aeruginosa* or remained uninfected. These results are in accordance with our previous studies, where gnotobiotic (i.e. secondary abiotic) mice were replenished with human microbiota in order to overcome colonization resistance against *Campylobacter jejuni*. Quantitative molecular analysis further revealed that following peroral fecal transplantation human microbiota could stably establish within the intestinal tract for more than 6 weeks [16]. Moreover, we showed previously that following peroral infection with high doses (i.e. >50 cysts) of *T. gondii* mice developed a distinct Th1-type immunopathology and exhibited an overgrowth of the inflamed ileal lumen with commensal Gram-negative bacterial species such as *Escherichia coli*

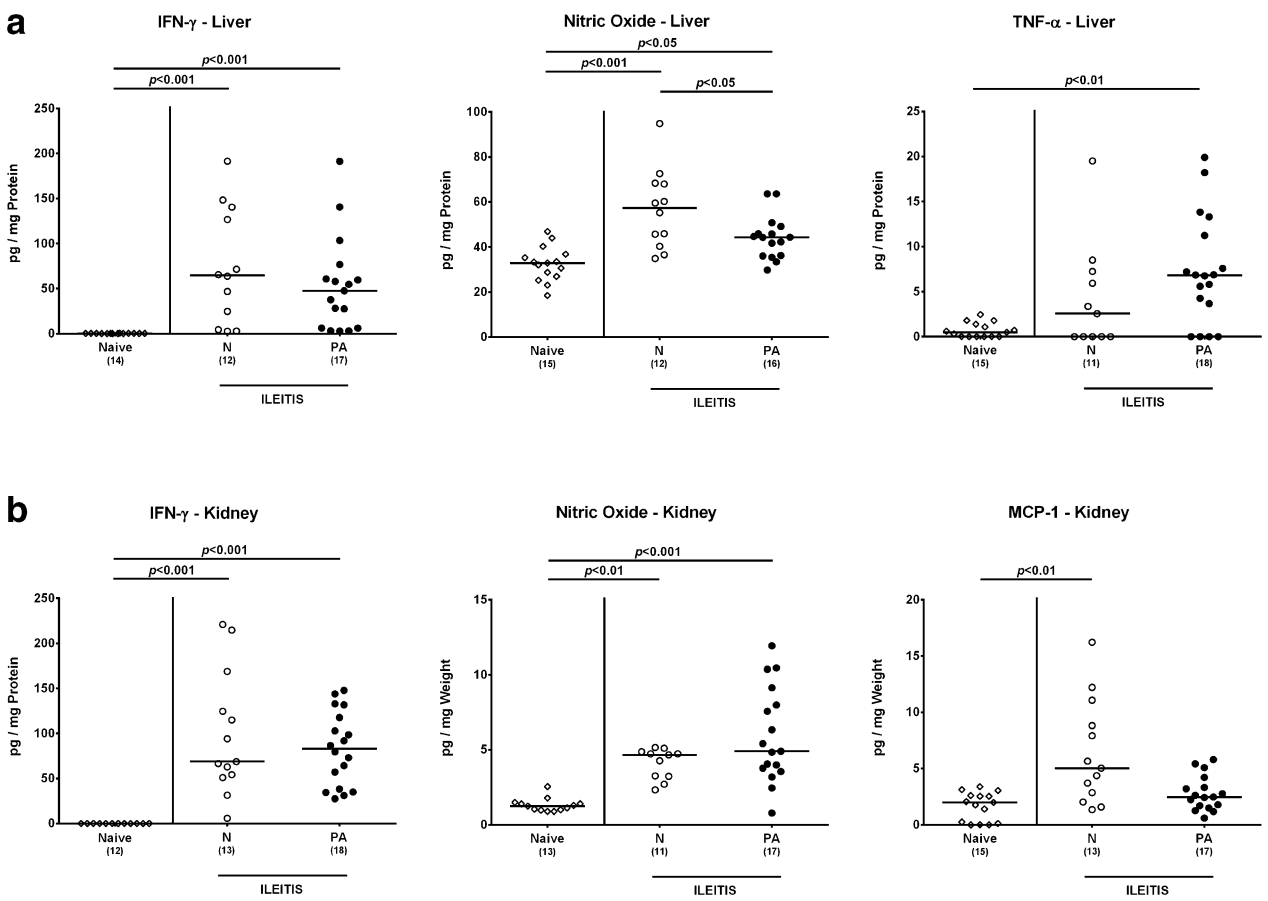

Fig. 7 Extra-intestinal cytokine responses in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; *filled circles*) or not (N; *open circles*). At day 7 post ileitis induction secretion of distinct pro-inflammatory cytokines were determined in ex vivo biopsies derived from different extra-intestinal compartments including **a** liver (IFN-γ, nitric oxide, TNF-α) and **b** kidney (IFN-γ, nitric oxide, MCP-1). Naive mice served as negative controls (*open diamonds*). Numbers of mice (in *parentheses*), medians (*black bars*) and significance levels (*p* values) determined by Kruskal–Wallis test or ordinary one-way ANOVA are indicated. Data shown were pooled from three independent experiments

(*E. coli*) and *Bacteroides/Prevotella* spp. [11]. Due to progressive virulence of the applied *T. gondii* ME49 strain by repeated passages in NMRI "bank" mice for several generations, we also subjected hma mice to 50 cysts in the present study instead of 100 cysts as in our previous reports [10–12, 14, 22, 24–29].

Remarkably, acute ileitis induced by 50 cysts of *T. gondii* rendered hma mice susceptible to MDR *P. aeruginosa* infection. This is in accordance with our previous studies demonstrating that hma mice suffering from small intestinal inflammation could be stably colonized by *C. jejuni* [27]. It is hence tempting to speculate that inflammation induced changes of the intraluminal milieu within the intestinal tract predisposes the host for infection with obligate enteropathogenic species such as *C. jejuni*, but also opportunistic pathogens including *P. aeruginosa*. Our hypothesis is further supported by a study showing that a nonpathogenic intestinal *E. coli* isolate as well as the pathogen *Salmonella typhimurium* were able to outcompete the endogenous microbiota of IL-10$^{-/-}$ mice, a murine model of chronic colitis, suggesting that growth of Gram-negative enterobacteria is enhanced by host mediated intestinal inflammation and following modification of the composition of intestinal microbiota [30, 31]. Furthermore, in a murine co-infection model intestinal colonization capacity of *S. typhimurium* was enhanced by inflammatory changes in the intestinal mucosa and dysbiosis elicited by *Plasmodium yoelii* infection [32].

Given that the intestinal intraluminal milieu is determined by a plethora of factors including the bacteria residing in the intestinal compartments, we performed a

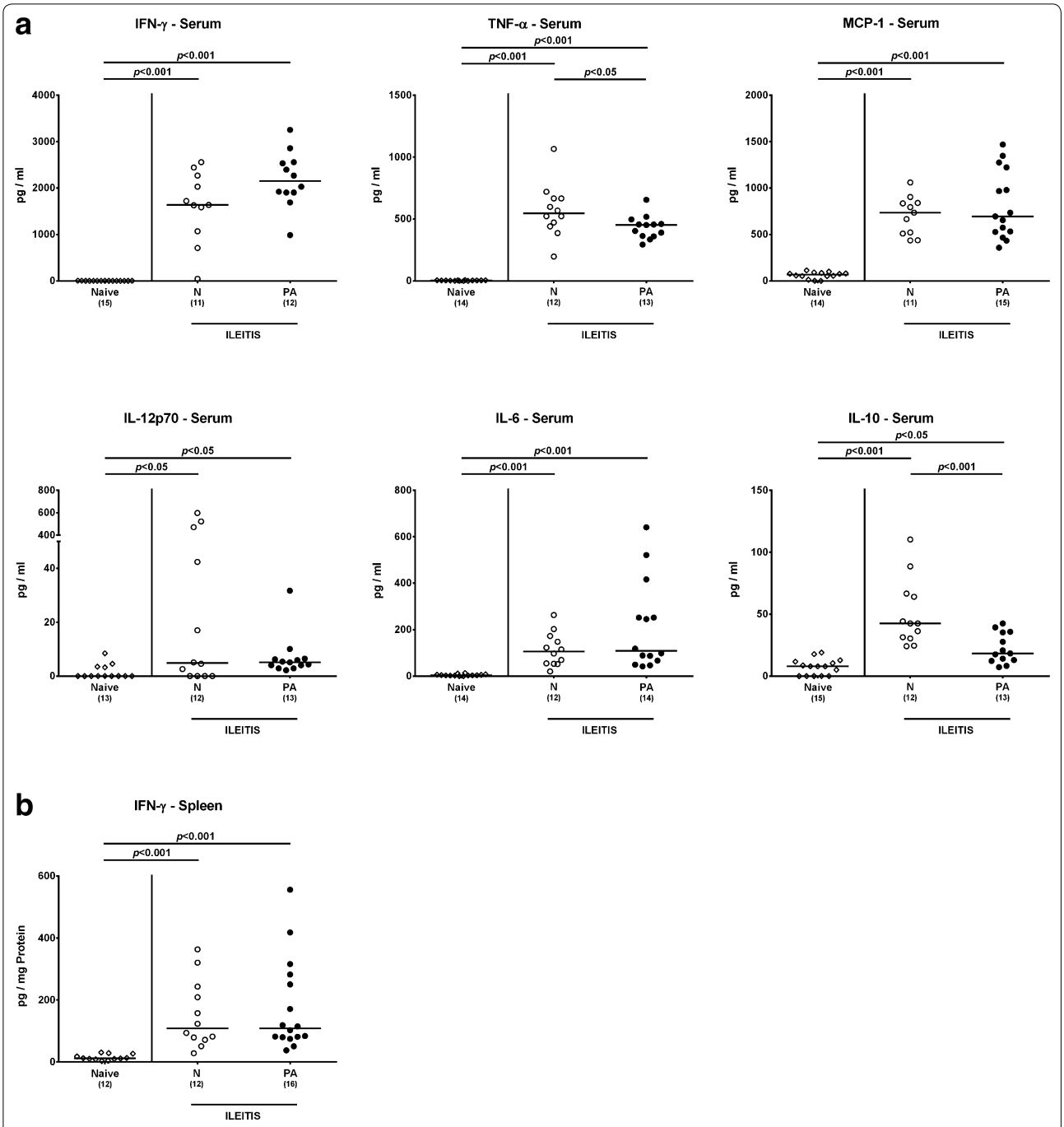

Fig. 8 Systemic cytokine responses in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; *filled circles*) or not (N; *open circles*). At day 7 post ileitis induction secretion of distinct pro- and anti-inflammatory cytokines were determined in systemic compartments such as **a** serum (IFN-γ, TNF-α, MCP-1, IL-12p70, IL-6 and IL-10) and **b** spleen (IFN-γ). Naive mice served as negative controls (*open diamonds*). Numbers of mice (in *parentheses*), medians (*black bars*) and significance levels (*p* values) determined by Kruskal–Wallis test or ordinary one-way ANOVA are indicated. Data shown were pooled from three independent experiments

comprehensive quantitative survey of the intestinal microbiota composition. Cultural and molecular analyses of the predominant intestinal bacterial groups revealed that differences in the overall microbiota composition of *P. aeruginosa* infected and uninfected hma mice with induced ileitis were rather subtle at the first glance. Ileal loads of

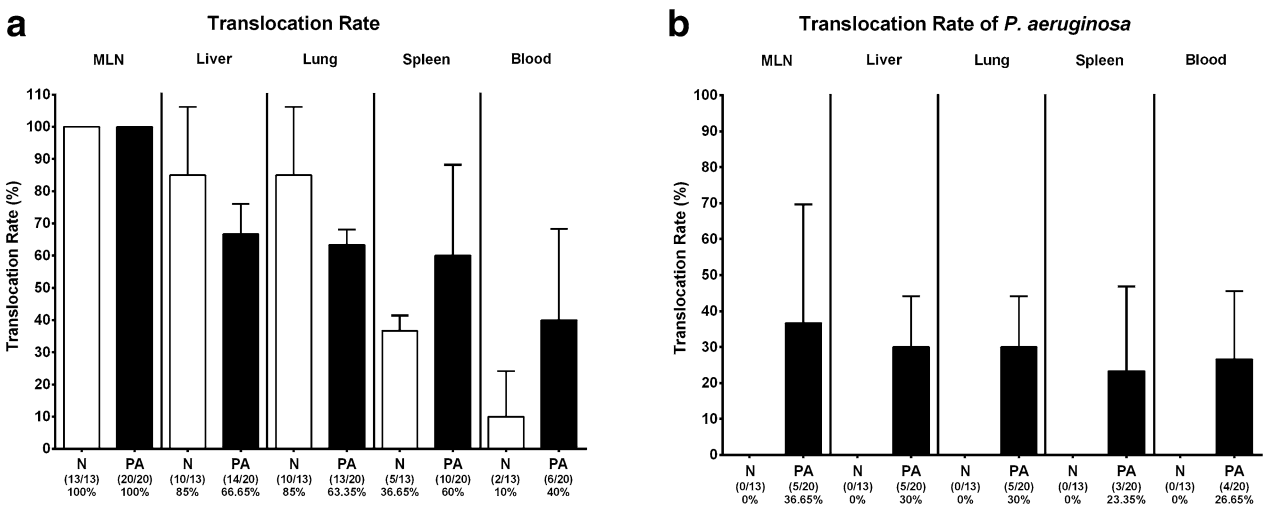

Fig. 9 Translocating intestinal bacteria in multidrug resistant *P. aeruginosa* infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; *filled bars*) or not (N; *open bars*). At day 7 post ileitis induction translocation rates of **a** intestinal bacterial species and of **b** *P. aeruginosa* to extra-intestinal and systemic compartments were determined by cultivation of homogenated ex vivo biopsies derived from mesenteric lymph nodes (MLN), liver, lung and spleen (direct plating on solid media) and of cardiac blood (in thioglycolate enrichment broths) with subsequent subcultivation and species identification. Mean translocation rates (%) ±SD and absolute numbers of positive samples out of total number analyzed (in *parentheses*) are indicated. Data shown were pooled from three independent experiments

bifidobacteria, however, were more than two orders of magnitude lower following *P. aeruginosa* infection. As commensal residents with beneficial effects for intestinal homeostasis, bifidobacteria contribute to mucosal barrier functions directed against pathogenic colonization of the vertebrate host [33, 34]. Two bifidobacterial strains that were isolated from resident infant human gastrointestinal microbiota exerted antibacterial activity against several pathogens such as *S. typhimurium, E. coli, Klebsiella pneumoniae, Yersinia pseudotuberculosis, Staphylococcus aureus* and *P. aeruginosa* in vitro [34]. In addition, in vitro as well as in vivo studies revealed that *Bifidobacterium animalis* AHC7, for instance, exerts an anti-inflammatory effect through the attenuation of NF-κB activation in response to murine *S. typhimurium* infection and that stimulation of dendritic cells with *B. animalis* AHC7 significantly increased CD25+Foxp3+ T cell (Treg) numbers [35]. We could further show previously that conventionally colonized mice deficient in the innate immune-receptor nucleotide-oligomerization-domaine-2 (NOD2) were virtually lacking the bifidobacterial population in their intestines and (at least in part) exhibited compromised host resistance, reduced local anti-inflammatory and increased systemic pro-inflammatory immune response upon peroral high dose *T. gondii* infection [29].

Given that acute *T. gondii* induced ileitis is highly dependent on Toll-like Receptor (TLR)-4 mediated signaling of lipopolysaccharide derived from Gram-negative intestinal commensals [11, 15], one could speculate that additional infection with a Gram-negative bacterium such as *P. aeruginosa* might further exacerbate the induced inflammatory process. Our study, however, revealed that clinical and ileal changes, the profound influx of innate and adaptive immune cells into the distal small intestines and increased secretion of pro-inflammatory cytokines in ileum and MLN following *T. gondii* infection were not further aggravated by MDR *P. aeruginosa* infection. This is surprising considering the plethora of virulence factors of *P. aeruginosa*. For instance, *Pseudomonas* lipid A, a core component of bacterial lipopeptide, has been shown to activate NFκB signaling through TLR-4 and subsequent pro-inflammatory cytokine secretion [36]. In turn, neutrophils are recruited to the infection site and contribute to the inflammatory host response to *P. aeruginosa* [1]. One needs to take into consideration, however, that peroral high dose *T. gondii* infection (irrespective whether performed with 50 or 100 cysts) results in a profound Th1-driven hyper-inflammatory scenario ("cytokine tsunami") [13] that cannot further be deteriorated by additional MDR *P. aeruginosa* infection. Strikingly, we were able to observe elevated IFN-γ secretion not only in ileal, but also colonic ex vivo biopsies 7 days post ileitis induction. To our best knowledge, the terminal ileum has been reported as exclusive predilection site following peroral high dose *T. gondii* infection of conventionally colonized mice so far [13].

Remarkably, extra-intestinal sequelae of acute ileitis induction were further amplified by *P. aeruginosa* challenge as indicated by even more pronounced increases in hepatic TNF-α concentrations following *P. aeruginosa* infection of *T. gondii* pre-infected mice. Potential inflammatory effects of MDR *P. aeruginosa* infection in the small intestines (and beyond) should therefore be more distinctly deciphered either in a less acute (i.e. less severe) or more chronic infection model following peroral low dose infection with less than 10 cysts of *T. gondii*, for instance. To the best of our knowledge, however, a chronic *T. gondii* ileitis model has not been established so far. Alternatively, experimental models of large intestinal inflammation could be applied to investigate host susceptibility and the pro-inflammatory potential of peroral MDR *P. aeruginosa* infection during intestinal inflammation in a better discriminatory way than with the model used here. To address this, the murine dextran sulfate sodium induced colitis model would be a promising candidate, for instance.

In the present study we were further able to demonstrate that anti-inflammatory IL-10 levels were multifold increased in the MLN following ileitis induction, but even more distinctly upon additional *P. aeruginosa* infection, whereas conversely, serum IL-10 concentrations were lower in *P. aeruginosa* infected as compared to non-infected hma mice suffering from acute ileitis. Hence, the more pronounced intestinal anti-inflammatory response upon *P. aeruginosa* application was not sufficient to counteract the pro-inflammatory sequelae caused by *T. gondii* and *P. aeruginosa* co-infection. One might have also expected comparable increases in systemic IL-10 levels, given that translocation of viable bacteria originating from the intestinal microbiota to systemic compartments such as spleen and blood had occurred more frequently in *P. aeruginosa* co-infected versus non-infected hma mice with acute ileitis.

We were finally able to show that viable intestinal bacteria were more frequently detected in systemic compartments such as spleen and blood of *P. aeruginosa* infected as compared to uninfected hma mice with induced ileitis indicating that (even though not otherwise determined in our study) epithelial barrier leakage was supposably even more pronounced by *P. aeruginosa* co-challenge than by acute ileitis induction alone, further facilitating bacterial translocation to extra-intestinal including systemic sites. Notably, several studies illustrate a link between the etiology of inflammatory bowel diseases (IBD) including Crohn's disease and the abundance of *Pseudomonas* species such as *P. fluorescens* within the intestinal mucosa [37–39]. Solid data regarding the role of *P. aeruginosa* in IBD pathogenesis, however, are scarce. One study applying molecular analysis of *Pseudomonas* specific 16S RNA in ileal tissue samples derived from children suffering from Crohn's disease revealed a higher prevalence of several *Pseudomonas* species including *P. proteolytica* and *P. brenneri* in pediatric Crohn's disease patients as compared to control individuals without IBD, whereas interestingly *P. aeruginosa* could be detected in non-IBD patients only [40]. In patients suffering from irritable bowel disease, however, detection rates of *P. aeruginosa* specific 16S RNA were increased in duodenal mucosa-associated biopsies and fecal samples [8]. Furthermore, a case report of a child suffering from ulcerative colitis revealed that *P. aeruginosa* 16S rRNA could be identified in colonic biopsies [9]. Already an older study from 1966 suggested a temporal correlation between identical *P. aeruginosa* strains isolated from patients' lesions and feces pointing towards a spread of viable *P. aeruginosa* via the blood stream [7]. Meanwhile, there is evidence that the risk of developing a clinically manifest *P. aeruginosa* infection is significantly higher upon rectal colonization of patients in intensive care units [41].

Conclusion

Taken together, acute small intestinal inflammation renders hma mice susceptible to infection with MDR *P. aeruginosa*, but *P. aeruginosa* infection does neither further deteriorate ileal, nor extra-intestinal sequelae of ileitis induction. We conclude that intestinal inflammation might also pose particularly hospitalized patients at risk for acquisition of MDR Gram-negative bacteria including *P. aeruginosa*. Given the importance of the interaction between *P. aeruginosa*, the intestinal microbiota and the host immune system as shown here, future studies applying less acute and/or more chronic in vivo infection models are needed for a better understanding of the underlying molecular mechanisms.

Additional file

Additional file 1: Figure S1. Translocating multidrug resistant *P. aeruginosa* in infected human microbiota-associated mice suffering from acute ileitis. Human microbiota-associated mice were perorally challenged with *T. gondii* ME49 to induce acute ileitis and either additionally infected with MDR *P. aeruginosa* 3 days following ileitis induction (PA; filled bars) or not (N; open bars). At day 7 following ileitis induction *P. aeruginosa* loads were quantitatively assessed in extra-intestinal and systemic compartments such as mesenteric lymph nodes (MLN), liver, lung, spleen and cardiac blood by direct plating. Absolute numbers of positive samples out of total number analyzed are indicated in parentheses. Data shown were pooled from three independent experiments.

Abbreviations

CFU: colony forming units; *E. coli*: *Escherichia coli*; hma: human microbiota-associated; IBD: inflammatory bowel diseases; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein; MLN: mesenteric lymph nodes; MDR: multidrug resistant; *P. aeruginosa*: *Pseudomonas aeruginosa*; PBS:

phosphate buffered saline; p.i.: post infection; qRT-PCR: quantitative real-time polymerase chain reaction; *T. gondii*: *Toxoplasma gondii*; Th1: T helper cell 1; TNF-α: tumor necrosis factor-α; TLR: toll-like receptor; Treg: regulatory T cells.

Authors' contributions
Conceived and designed the experiments: EVK, MMH. Performed the experiments: EVK, IE, MMH. Analyzed the data: EVK, IE, MMH. Wrote the paper: EVK, SB, MMH. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Please contact author for data requests.

Ethics approval
All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration numbers G0097/12 and G0039/15). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice. Mice suffering from weight loss >20% were euthanized by isoflurane treatment (Abbott, Germany) in accordance with the guidelines of the local authorities.

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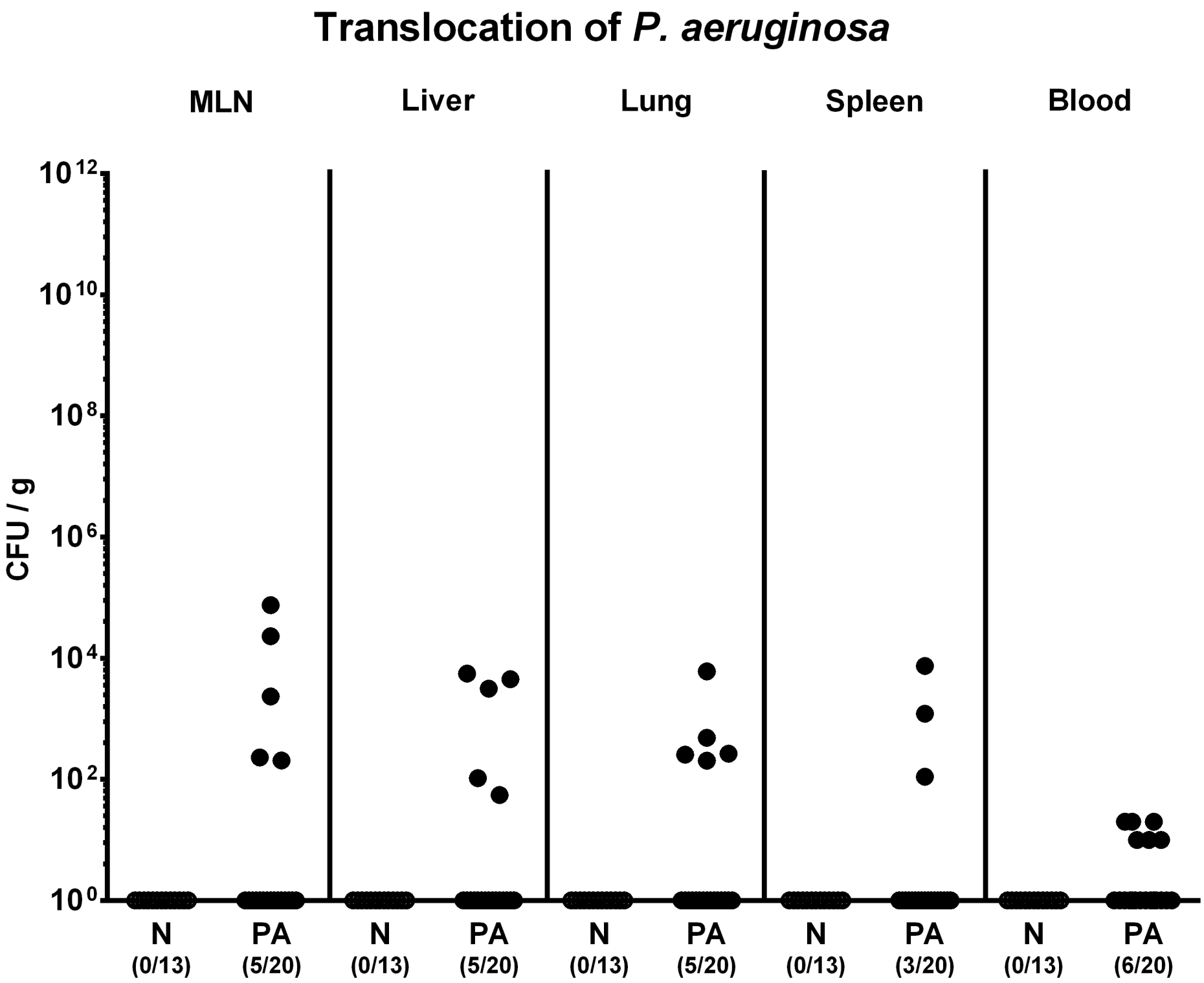
References

1. Gellatly SL, Hancock RE. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159–73.
2. Vincent JL. Nosocomial infections in adult intensive-care units. Lancet. 2003;361(9374):2068–77.
3. Oliver A, Mulet X, Lopez-Causape C, Juan C. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updates. 2015;21–22:41–59.
4. Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. Int J Antimicrob Agents. 2015;45(6):568–85.
5. Driscoll JA, Brody SL, Kollef MH. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs. 2007;67(3):351–68.
6. Buck AC, Cooke EM. The fate of ingested *Pseudomonas aeruginosa* in normal persons. J Med Microbiol. 1969;2(4):521–5.
7. Shooter RA, Walker KA, Williams VR, Horgan GM, Parker MT, Asheshov EH, et al. Faecal carriage of *Pseudomonas aeruginosa* in hospital patients. Possible spread from patient to patient. Lancet. 1966;2(7477):1331–4.
8. Kerckhoffs AP, Ben-Amor K, Samsom M, van der Rest ME, de Vogel J, Knol J, et al. Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in irritable bowel syndrome. J Med Microbiol. 2011;60(Pt 2):236–45.
9. Wang M, Molin G, Ahrne S, Adawi D, Jeppsson B. High proportions of proinflammatory bacteria on the colonic mucosa in a young patient with ulcerative colitis as revealed by cloning and sequencing of 16S rRNA genes. Dig Dis Sci. 2007;52(3):620–7.
10. Liesenfeld O. Oral infection of C57BL/6 mice with *Toxoplasma gondii*: a new model of inflammatory bowel disease? J Infect Dis. 2002;185(Suppl 1):S96–101.
11. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, et al. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. J Immunol. 2006;177(12):8785–95.
12. Liesenfeld O, Kang H, Park D, Nguyen TA, Parkhe CV, Watanabe H, et al. TNF-alpha, nitric oxide and IFN-gamma are all critical for development of necrosis in the small intestine and early mortality in genetically susceptible mice infected perorally with *Toxoplasma gondii*. Parasite Immunol. 1999;21(7):365–76.
13. Munoz M, Liesenfeld O, Heimesaat MM. Immunology of *Toxoplasma gondii*. Immunol Rev. 2011;240(1):269–85.
14. Heimesaat MM, Fischer A, Jahn HK, Niebergall J, Freudenberg M, Blaut M, et al. Exacerbation of murine ileitis by Toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. Gut. 2007;56(7):941–8.
15. Erridge C, Duncan SH, Bereswill S, Heimesaat MM. The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5. PLoS ONE. 2010;5(2):e9125.
16. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kühl AA, et al. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. PLoS ONE. 2011;6(6):e20953.
17. Struck D, Frank I, Enders S, Steinhoff U, Schmidt C, Stallmach A, et al. Treatment with interleukin-18 binding protein ameliorates *Toxoplasma gondii*-induced small intestinal pathology that is induced by bone marrow cell-derived interleukin-18. Eur J Microbiol Immunol. 2012;2(3):249–57.
18. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut. 2010;59(8):1079–87.
19. Gölz G, Karadas G, Alutis ME, Fischer A, Kuhl AA, Breithaupt A, et al. *Arcobacter butzleri* induce colonic, extra-intestinal and systemic inflammatory responses in gnotobiotic IL-10 deficient mice in a strain-dependent manner. PLoS ONE. 2015;10(9):e0139402.
20. Alutis ME, Grundmann U, Fischer A, Hagen U, Kühl AA, Göbel UB, et al. The role of gelatinases in *Campylobacter jejuni* infection of gnotobiotic mice. Eur J Microbiol Immunol. 2015;5(4):256–67.
21. Heimesaat MM, Fischer A, Siegmund B, Kupz A, Niebergall J, Fuchs D, et al. Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like receptors 2 and 4. PLoS ONE. 2007;2(7):e662.
22. Bereswill S, Kühl AA, Alutis M, Fischer A, Möhle L, Struck D, et al. The impact of Toll-like-receptor-9 on intestinal microbiota composition and extra-intestinal sequelae in experimental *Toxoplasma gondii* induced ileitis. Gut Pathog. 2014;6:19.
23. Rausch S, Held J, Fischer A, Heimesaat MM, Kühl AA, Bereswill S, et al. Small intestinal nematode infection of mice is associated with increased enterobacterial loads alongside the intestinal tract. PLoS ONE. 2013;8(9):e74026.
24. Munoz M, Heimesaat MM, Danker K, Struck D, Lohmann U, Plickert R, et al. Interleukin (IL)-23 mediates *Toxoplasma gondii*-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. J Exp Med. 2009;206(13):3047–59.
25. Munoz M, Eidenschenk C, Ota N, Wong K, Lohmann U, Kühl AA, et al. Interleukin-22 induces interleukin-18 expression from epithelial cells during intestinal infection. Immunity. 2015;42(2):321–31.
26. Bereswill S, Munoz M, Fischer A, Plickert R, Haag LM, Otto B, et al. Anti-inflammatory effects of resveratrol, curcumin and simvastatin in acute small intestinal inflammation. PLoS ONE. 2010;5(12):e15099.
27. Haag LM, Fischer A, Otto B, Plickert R, Kühl AA, Göbel UB, et al. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. PLoS ONE. 2012;7(5):e35988.
28. Heimesaat MM, Dunay IR, Schulze S, Fischer A, Grundmann U, Alutis M, et al. Pituitary adenylate cyclase-activating polypeptide ameliorates experimental acute ileitis and extra-intestinal sequelae. PLoS ONE. 2014;9(9):e108389.

29. Heimesaat MM, Dunay IR, Alutis M, Fischer A, Möhle L, Göbel UB, et al. Nucleotide-oligomerization-domain-2 affects commensal gut microbiota composition and intracerebral immunopathology in acute *Toxoplasma gondii* induced murine ileitis. PLoS ONE. 2014;9(8):e105120.
30. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*. Cell Host Microbe. 2007;2(2):119–29.
31. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, et al. *Salmonella enterica* serovar *typhimurium* exploits inflammation to compete with the intestinal microbiota. PLoS Biol. 2007;5(10):2177–89.
32. Mooney JP, Lokken KL, Byndloss MX, George MD, Velazquez EM, Faber F, et al. Inflammation-associated alterations to the intestinal microbiota reduce colonization resistance against non-typhoidal *Salmonella* during concurrent malaria parasite infection. Sci Rep. 2015;5:14603.
33. Coudeyras S, Forestier C. Microbiota and probiotics: effects on human health. Can J Microbiol. 2010;56(8):611–50.
34. Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR, et al. *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut. 2000;47(5):646–52.
35. O'Mahony D, Murphy S, Boileau T, Park J, O'Brien F, Groeger D, et al. *Bifidobacterium animalis* AHC7 protects against pathogen-induced NF-κB activation in vivo. BMC Immunol. 2010;11:63.
36. Korneev KV, Arbatsky NP, Molinaro A, Palmigiano A, Shaikhutdinova RZ, Shneider MM, et al. Structural relationship of the lipid A acyl groups to activation of murine toll-like receptor 4 by lipopolysaccharides from pathogenic strains of *Burkholderia mallei*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Front Immunol. 2015;6:595.
37. Wei B, Huang T, Dalwadi H, Sutton CL, Bruckner D, Braun J. *Pseudomonas fluorescens* encodes the Crohn's disease-associated I2 sequence and T-cell superantigen. Infect Immun. 2002;70(12):6567–75.
38. Forbes JD, Van Domselaar G, Bernstein CN. Microbiome survey of the inflamed and noninflamed gut at different compartments within the gastrointestinal tract of inflammatory bowel disease patients. Inflamm Bowel Dis. 2016;22(4):817–25.
39. Eckburg PB, Relman DA. The role of microbes in Crohn's disease. Clin Infect Dis. 2007;44(2):256–62.
40. Wagner J, Short K, Catto-Smith AG, Cameron DJ, Bishop RF, Kirkwood CD. Identification and characterisation of *Pseudomonas* 16S ribosomal DNA from ileal biopsies of children with Crohn's disease. PLoS ONE. 2008;3(10):e3578.
41. Gomez-Zorrilla S, Camoez M, Tubau F, Canizares R, Periche E, Dominguez MA, et al. Prospective observational study of prior rectal colonization status as a predictor for subsequent development of *Pseudomonas aeruginosa* clinical infections. Antimicrob Agents Chemother. 2015;59(9):5213–9.


Translocation of P. aeruginosa
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Spleen
Blood
CFU / g
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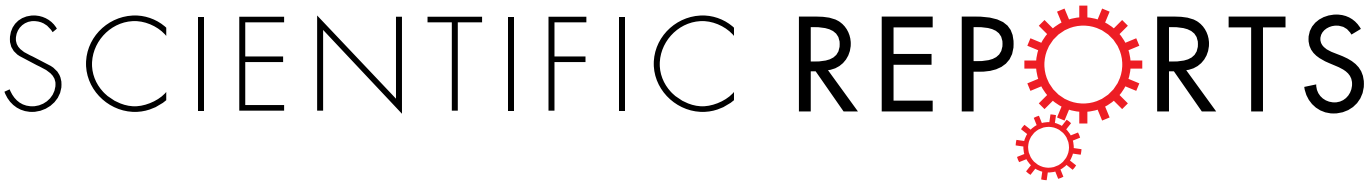

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Multidrug-resistant *Pseudomonas aeruginosa* aggravates inflammatory responses in murine chronic colitis

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The World Health Organization has rated multidrug-resistant (MDR) Gram-negative bacteria including *Pseudomonas aeruginosa* (*Psae*) as serious threat to human health. We here addressed whether chronic murine gut inflammation facilitates intestinal MDR *Psae* colonization and whether bacterial infection subsequently worsens colonic immunopathology. Converse to wildtype counterparts, *Psae* colonized the intestines of IL-10$^{-/-}$ mice with chronic colitis following peroral challenge, but did not lead to changes in intestinal microbiota composition. *Psae* infection accelerated both macroscopic (i.e. clinical) and microscopic disease (i.e. colonic epithelial apoptosis), that were accompanied by increased intestinal pro-inflammatory immune responses as indicated by elevated colonic numbers of innate and adaptive immune cell subsets and enhanced secretion of pro-inflammatory cytokines such as TNF and IFN-γ in mesenteric lymph nodes of *Psae*-infected as compared to unchallenged IL-10$^{-/-}$ mice. Remarkably, *Psae*-induced pro-inflammatory immune responses were not restricted to the gut, but could also be observed systemically as indicated by increased TNF and IFN-γ concentrations in sera upon *Psae*-infection. Furthermore, viable commensals originating from the intestinal microbiota translocated to extra-intestinal compartments such as liver, kidney and spleen of *Psae*-infected IL-10$^{-/-}$ mice with chronic colitis only. Hence, peroral MDR *Psae*-infection results in exacerbated colonic as well as systemic pro-inflammatory immune responses during chronic murine colitis.

Pseudomonas aeruginosa (*Psae*), constitute opportunistic pathogens, that may cause a variety of nosocomial infections particularly in immunocompromised patients or patients with chronic pulmonary diseases such as chronic obstructive pulmonary disease or cystic fibrosis[1–4]. Patients suffering from ventilator-associated pneumonia or burn wound infections face high mortality rates of over 30%[1]. As Gram-negative bacteria with emerging antimicrobial resistance *Psae* are among several multi-drug resistant (MDR) bacterial species that can be found in a global priority pathogen list issued by the World Health Organization (WHO) in order to help prioritizing the development of novel antimicrobial strategies[5]. Already early studies suggest that besides contaminated respirators and other medical equipment the human gastrointestinal tract might be an important internal source of *Psae* infection in hospitals[6,7]. Furthermore, a recent study revealed that a majority of intensive care unit (ICU) patients with a *Psae* infection displayed prior rectal colonization with the bacterial opportunistic pathogen[8]. Particularly a preceding antimicrobial treatment had been shown to disturb the complex intestinal microbiota composition and therefore to compromise the physiological colonization resistance[9–12] which, in turn, may enable invading (opportunistic) pathogens including MDR *Psae* to establish within the human gastrointestinal ecosystem[13]. However, valid scientific data concerning the pathogenic potential of *Psae* infection of the intestinal tract are scarce. Our very recent study revealed for the first time that mere intestinal carriage of a clinical MDR *Psae* isolate by otherwise healthy microbiota-depleted wildtype (WT) mice resulted in distinct local as well as systemic pro-inflammatory immune responses[13]. We were further able to demonstrate that with intestinal inflammation

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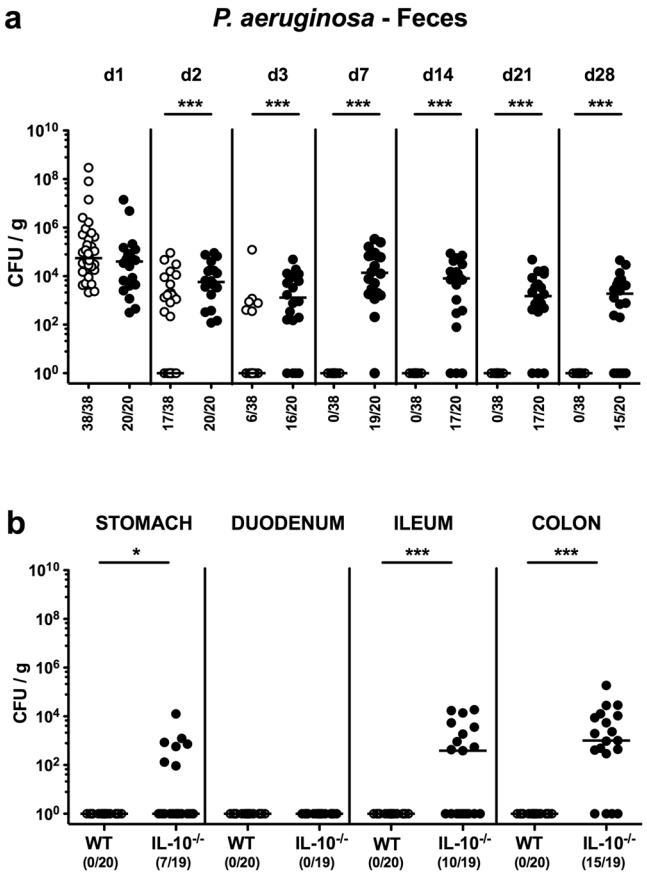

Figure 1. Fecal *P. aeruginosa* loads over time following peroral infection of mice suffering from chronic colitis. Conventionally colonized IL-10$^{-/-}$ mice with chronic colitis (black circles) were perorally infected with a multidrug-resistant *P. aeruginosa* strain on day (d) 0. Wildtype mice without large intestinal inflammation (white circles) served as (infected) controls. (**a**) Intestinal colonization densities were determined in fecal samples until d28 postinfection by culture and expressed as colony forming units per gram (CFU/g). (**b**) Upon necropsy at day 42 postinfection, pathogenic loads were cultured from distinct compartments of the gastrointestinal tract (i.e. stomach, duodenum, ileum and colon). Numbers of mice harboring *P. aeruginosa* out of the total number of analyzed mice, medians (black bars) and significance levels (p-values) determined by Mann Whitney U test are indicated (*$p < 0.05$; ***$p < 0.001$). Data shown were pooled from at least three independent experiments.

associated gut microbiota shifts facilitated murine infection with the enteropathogen *Campylobacter jejuni*[14–17]. Information whether intestinal inflammation may also facilitate stable *Psae* colonization and may even worsen the outcome of the underlying disease are scarce, however. In the present study, we therefore perorally challenged conventionally colonized IL-10$^{-/-}$ suffering from chronic colitis with a clinical MDR *Psae* isolate and assessed intestinal colonization properties of the opportunistic pathogen, shifts in gut microbiota composition, *Psae*-induced intestinal and systemic pro-inflammatory infectious sequelae, and translocation of viable bacteria to extra-intestinal including systemic compartments. In summary, our data indicate that peroral MDR *Psae* infection exacerbates macroscopic and colonic apoptotic sequelae as well as systemic pro-inflammatory immune responses during chronic murine colitis.

Results

Chronic colitis facilitates intestinal MDR *P. aeruginosa* infection of mice. Conventional IL-10$^{-/-}$ mice with chronic colitis and healthy WT control animals were perorally infected with 10^9 colony-forming units (CFU) of a clinical MDR *Psae* strain. Whereas WT mice had expelled the pathogen within one week p.i., IL-10$^{-/-}$ mice displayed higher fecal *Psae* loads than their WT counterparts as early as 48 hours p.i. ($p < 0.001$; Fig. 1a). Even six weeks thereafter, *Psae* could be isolated from the gastrointestinal tract of IL-10$^{-/-}$, but not WT mice ($p < 0.05$–0.001; Fig. 1b) with most frequent abundances of approximately 79% in the large intestinal tract of IL-10$^{-/-}$ mice at day 42 p.i. (Fig. 1b).

Given potential crosstalk between (opportunistic) pathogens and commensals, we furthermore addressed whether *Psae* association of IL-10$^{-/-}$ mice was accompanied by shifts in intestinal microbiota composition. Both cultural and culture-independent (i.e. molecular 16S rRNA based) analyses of fecal samples revealed that IL-10$^{-/-}$ mice with chronic colitis harbored comparable intestinal loads of the main intestinal bacterial groups

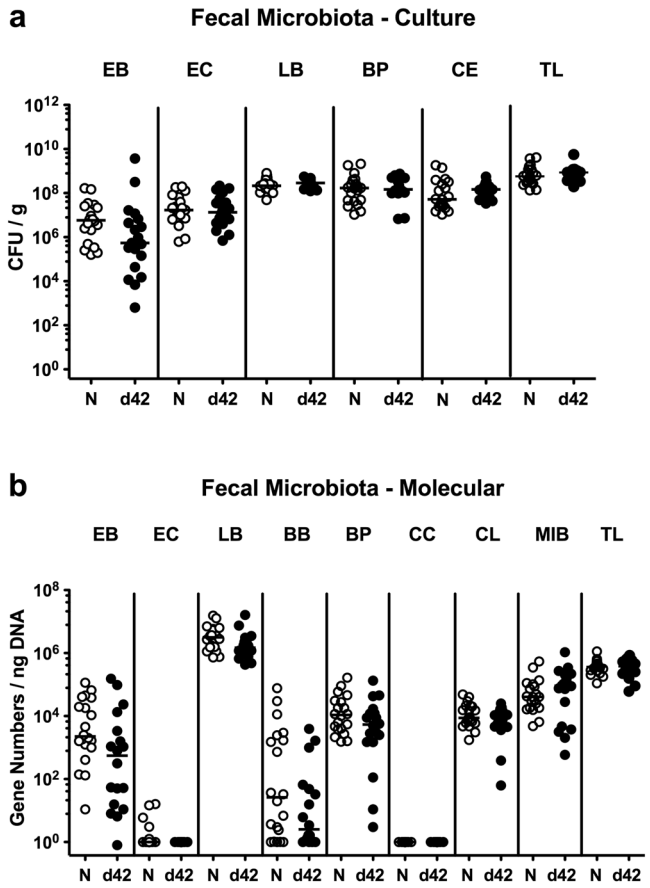

Figure 2. Intestinal microbiota composition following multidrug-resistant *P. aeruginosa* infection of mice suffering from chronic colitis. IL-10$^{-/-}$ mice with chronic colitis (n = 19) were perorally infected with a multidrug-resistant *P. aeruginosa* strain on day (d) 0. Before (N, naive; white circles) and six weeks thereafter (d42 postinfection; black circles) a comprehensive survey of the intestinal microbiota composition was performed on fecal samples applying both (**a**) culture (expressed as colony forming units per gram, CFU/g) and (**b**) 16S rRNA based molecular analyses (expressed as gene numbers per ng DNA) of the total bacterial load (TL) and main intestinal bacterial groups including enterobacteria (EB), enterococci (EC), lactobacilli (LB), *Bacteroides*/*Prevotella* spp. (BP), *Clostridium*/*Eubacterium* spp. (CE), bifidobacteria (BB), *Clostridium coccoides* group, *Clostridium leptum* group (CL) and *Mouse Intestinal Bacteroides* (MIB). Medians are indicated. Data shown were pooled from three independent experiments.

before and 42 days after *Psae* challenge (Fig. 2). Hence, chronic IL-10$^{-/-}$ colitis facilitates intestinal infection with MDR *Psae* that does not lead to changes in gut microbiota composition.

Macroscopic and microscopic sequelae in MDR *P. aeruginosa* infected mice with chronic colitis. We next surveyed macroscopic (i.e. clinical) and microscopic (i.e. histopathological and immunohistochemical) sequelae in *Psae* infected IL-10$^{-/-}$ mice with chronic colitis. Whereas 20% of mice from both the infected and naive cohort of IL-10$^{-/-}$ mice displayed macroscopic and/or microscopic abundances of blood in their feces at day 0 (i.e. immediately before *Psae* infection), overall fecal blood positivity rates were higher in infected as compared to uninfected mice when surveyed until day 42 following bacterial challenge (Supplementary Information). Of note, mean body weights of mice in the *Psae* infected and uninfected cohort did neither differ at day 0 nor at day 42 p.i. (Supplementary Information). Given that intestinal inflammation is accompanied by significant shortening of the inflamed intestine[18], we measured colonic lengths upon necropsy. A trend towards shorter large intestines could be observed in *Psae* infected as compared to uninfected mice at day 42 p.i.; the difference, however, did not reach statistical significance due to high standard deviations in respective groups (Supplementary Information). We further quantitatively assessed colonic histomorphological sequelae of *Psae* infection in IL-10$^{-/-}$ mice applying a standardized histopathological scoring system[19]. *Psae* infected and uninfected mice with chronic colitis displayed comparable histopathological scores at day 42 p.i., indicative for mild inflammatory cell infiltrates in the mucosa with mild hyperplasia and goblet cell loss (Supplementary Information).

Given that apoptosis constitutes an established parameter for histopathological evaluation and grading of intestinal inflammation[11], we next quantitatively assessed apoptotic colonic epithelial cells applying *in situ* immunohistochemistry. Six weeks following *Psae* infection IL-10$^{-/-}$ mice displayed more two times higher apoptotic cell numbers in their large intestines as compared to uninfected counterparts ($p < 0.001$; Fig. 3a, Supplementary

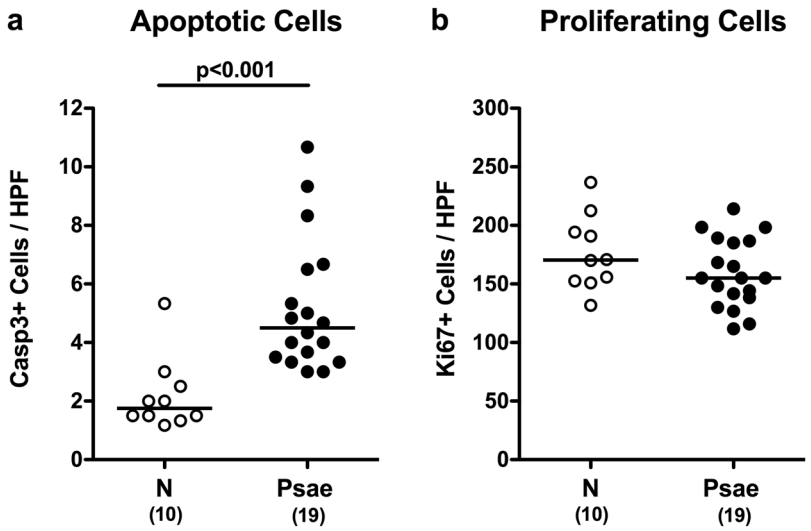

Figure 3. Apoptotic and proliferating colonic epithelial cells in multidrug-resistant *P. aeruginosa* infected mice suffering from chronic colitis. IL-10$^{-/-}$ mice with chronic colitis were perorally infected with a multidrug-resistant *P. aeruginosa* (Psae) strain on day (d) 0. Six weeks thereafter (on d42 postinfection; black circles) the average numbers of colonic epithelial (**a**) apoptotic (positive for caspase 3, Casp3) and (**b**) proliferating cells (positive for Ki67) were determined from six high power fields (HPF, 400x magnification) per animal in immunohistochemically stained large intestinal paraffin sections. Naive (N) IL-10$^{-/-}$ mice served as negative controls (open circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the Mann Whitney U test are indicated. Data shown were pooled from three independent experiments.

Information). Numbers of Ki67+ colonic epithelial cells, however, did not differ in infected and naive mice (n.s.; Fig. 3b, Supplementary Information), thus indicating comparable proliferative/regenerative properties of the large intestinal epithelia in either murine cohort. Hence, *Psae* infection accelerates both macroscopic (i.e. clinical) and microscopic (i.e. colonic apoptotic) sequelae in IL-10$^{-/-}$ mice suffering from chronic colitis.

Intestinal immune responses in MDR *P. aeruginosa* infected mice with chronic colitis. In the following we quantitatively assessed large intestinal immune cell responses upon *Psae* infection of IL-10$^{-/-}$ mice with chronic colitis applying *in situ* immunohistochemistry. Within 42 day p.i., numbers of distinct innate as well as of adaptive immune cell subsets including macrophages and monocytes as well as T lymphocytes, regulatory T cells (Treg) and B lymphocytes, respectively, increased in the large intestinal mucosa and lamina propria of IL-10$^{-/-}$ mice ($p < 0.005$–0.001; Fig. 4, Supplementary Information). *Psae*-induced increases in large intestinal immune responses were accompanied by elevated secretion of pro-inflammatory cytokines such as TNF and IFN-γ in mesenteric lymph nodes (MLN) of IL-10$^{-/-}$ mice ($p < 0.05$; Fig. 5a,b), whereas MCP-1 and IL-6 concentration were comparable in *Psae* infected and uninfected mice at day 42 p.i. (Fig. 5c,d). Hence, MDR *Psae* colonization of IL-10$^{-/-}$ mice with chronic colitis is accompanied by increased intestinal pro-inflammatory cytokine responses.

Systemic immune responses in MDR *P. aeruginosa* infected mice with chronic colitis. We next assessed whether *Psae*-induced intestinal pro-inflammatory immune responses in mice suffering from chronic colitis could also be observed in systemic compartments such as spleen and serum. Whereas a trend towards lower TNF and IFN-γ levels could be observed in spleens of infected versus uninfected mice with colitis (n.s.; Fig. 6a,b), lower MCP-1 and IL-6 concentrations could be measured in splenic *ex vivo* biopsies at day 42 p.i. as compared to naive IL-10$^{-/-}$ mice ($p < 0.01$; Fig. 6c,d), Conversely, *Psae* infection resulted in more pronounced secretion of pro-inflammatory cytokines into the circulation as indicated by higher TNF and IFN-γ ($p < 0.05$; Fig. 7a,b), but comparable MCP-1 and IL-6 concentrations in serum samples derived from infected as compared to uninfected mice with chronic colitis (n.s.; Fig. 7c,d). Hence, MDR *Psae*-induced pro-inflammatory immune responses were not restricted to the intestinal tract, but could also be observed systemically.

Bacterial translocation in MDR *P. aeruginosa* infected mice with chronic colitis. We finally surveyed whether bacteria originating from the commensal intestinal microbiota had translocated to extra-intestinal and systemic compartments of *Psae* colonized IL-10$^{-/-}$ mice. Whereas no bacterial translocation could be observed in uninfected mice with chronic colitis at all, viable intestinal commensal bacteria such as lactobacilli, enterococci, *E. coli*, *Bacteroides/Prevotella* species and *Clostridium/Eubacterium* species could be cultured from MLN, spleen, liver and kidney in 31.6%, 10.5%, 26.3% and 15.8% of infected IL-10$^{-/-}$ mice, respectively (Supplementary Information). Notably, neither in blood of naive nor *Psae* infected mice with colitis any bacteria could be detected. Hence, viable commensals originating from the intestinal microbiota translocated to extra-intestinal compartments of MDR *Psae* colonized IL-10$^{-/-}$ mice with chronic colitis only.

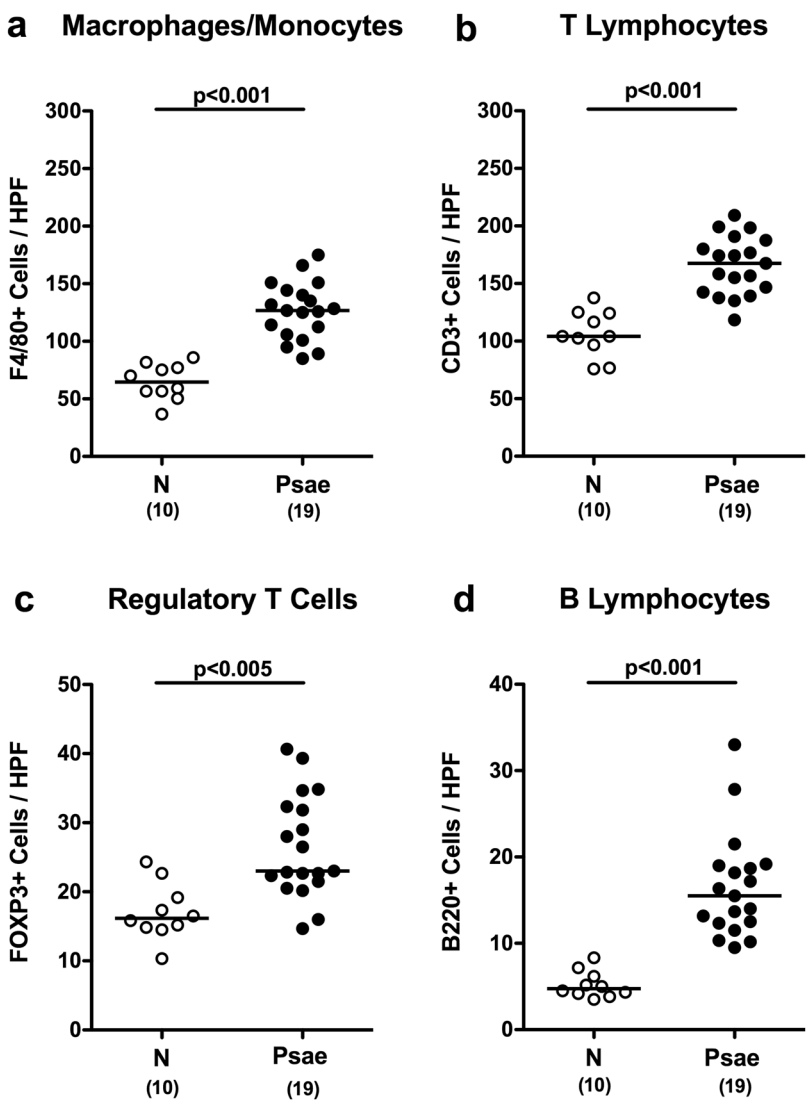

Figure 4. Colonic immune cell responses in multidrug-resistant *P. aeruginosa* infected mice suffering from chronic colitis. IL-10$^{-/-}$ mice with chronic colitis were perorally infected with a multidrug-resistant *P. aeruginosa* (Psae) strain on day (d) 0. Six weeks thereafter (on d42 postinfection; black circles) the average numbers of colonic (**a**) macrophages and monocytes (positive for F4/80), (**b**) T lymphocytes (positive for CD3), (**c**) regulatory T cells (positive for FOXP3), and (**d**) B lymphocytes (positive for B220) were determined from six high power fields (HPF, 400x magnification) per animal in immunohistochemically stained large intestinal paraffin sections. Naive (N) IL-10$^{-/-}$ mice served as negative controls (open circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the Mann Whitney U test are indicated. Data shown were pooled from three independent experiments.

Taken together, peroral MDR *Psae* infection exacerbates macroscopic and colonic apoptotic sequelae as well as intestinal and systemic pro-inflammatory cytokine responses in IL-10$^{-/-}$ mice with chronic colitis.

Discussion

The rising increases in incidences of MDR bacterial Gram-negative strains including *Psae* colonizing and infecting humans as well as live stock animals all over the world have resulted in an increased awareness not only of health care professionals, but also of the general public and the political institutions for this global threat of human health and for the actually very limited strategies preventing morbidity and mortality[2,5,20]. It is thus rather surprising, however, that studies addressing the immunopathological consequences of intestinal colonization and infection of vertebrates with *Psae* in health and disease including intestinal inflammation are scare to date.

In an earlier study, ingested *Psae* were detectable in three healthy volunteers up to 6 days thereafter, but without any reported adverse effects[7]. Very recently we were able to show that upon peroral challenge with the same clinical MDR *Psae* isolate used in the present survey, healthy WT mice with a virtually depleted gut microbiota following broad-spectrum antibiotic treatment harbored the opportunistic pathogen at high loads in their gastrointestinal tract, but were clinically uncompromised[13]. This observation is well in line with a previous report

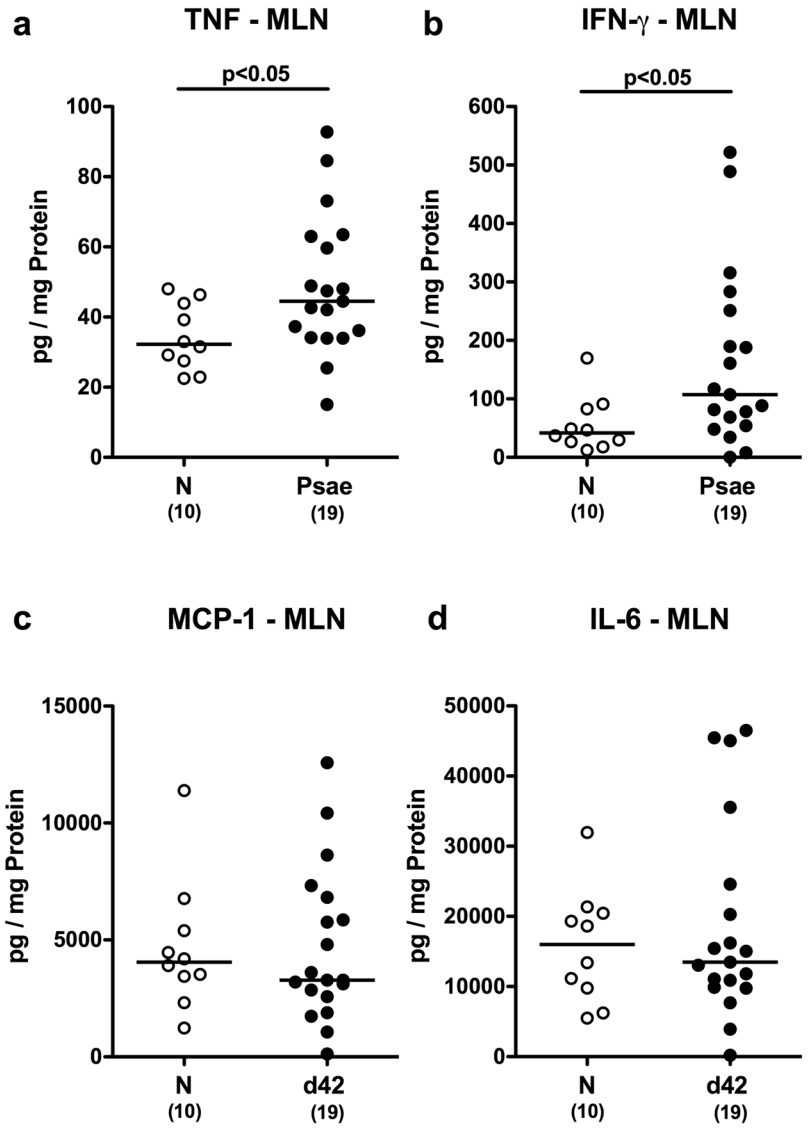

Figure 5. Intestinal cytokine responses in multidrug-resistant *P. aeruginosa* infected mice suffering from chronic colitis. IL-10$^{-/-}$ mice with chronic colitis were perorally infected with a multidrug-resistant *P. aeruginosa* (Psae) strain on day (d) 0. Six weeks thereafter (on d42 postinfection; black circles) pro-inflammatory cytokines including (**a**) TNF, (**b**) IFN-γ, (**c**) MCP-1 and (**d**) IL-6 were measured in supernatants of *ex vivo* biopsies derived from mesenteric lymph nodes (MLN). Naive (N) IL-10$^{-/-}$ mice served as negative controls (open circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the Mann Whitney U test are indicated. Data shown were pooled from three independent experiments.

by Hentgens *et al*., given that following antibiotic treatment and subsequent *Psae* infection mice were clinically unaffected[9].

Despite the well-known arsenal of virulence factors and hence pathogenic properties of *Psae*, its potential in initiating and perpetuating pre-existing immunopathological morbidities, particularly in the gastrointestinal tract, is only incompletely understood[13]. Recent surveys revealed that the opportunistic pathogen was more abundant in the gastrointestinal tract of patients suffering from underlying intestinal morbidities such as ulcerative colitis[21] or irritable bowel syndrome[22]. Remarkably, in our recent study MDR *Psae* induced pronounced pro-inflammatory sequelae in both intestinal and extra-intestinal including systemic compartments in antibiotics-pretreated, but otherwise healthy mice[13]. In addition, we were able to show in another recent survey that acute small intestinal inflammation facilitated MDR *Psae* acquisition by mice harboring a human gut microbiota[23]. The applied inflammation model was, however, too acute to decipher additional inflammatory sequelae of the applied *Psae* strain.

In the present study we further showed that chronic colitis in conventionally colonized IL-10$^{-/-}$ mice (that had not been subjected to antibiotic pretreatment) did not only facilitate murine MDR *Psae* carriage, but also worsened distinct features of the underlying disease as shown on both macroscopic (i.e. clinical) and microscopic

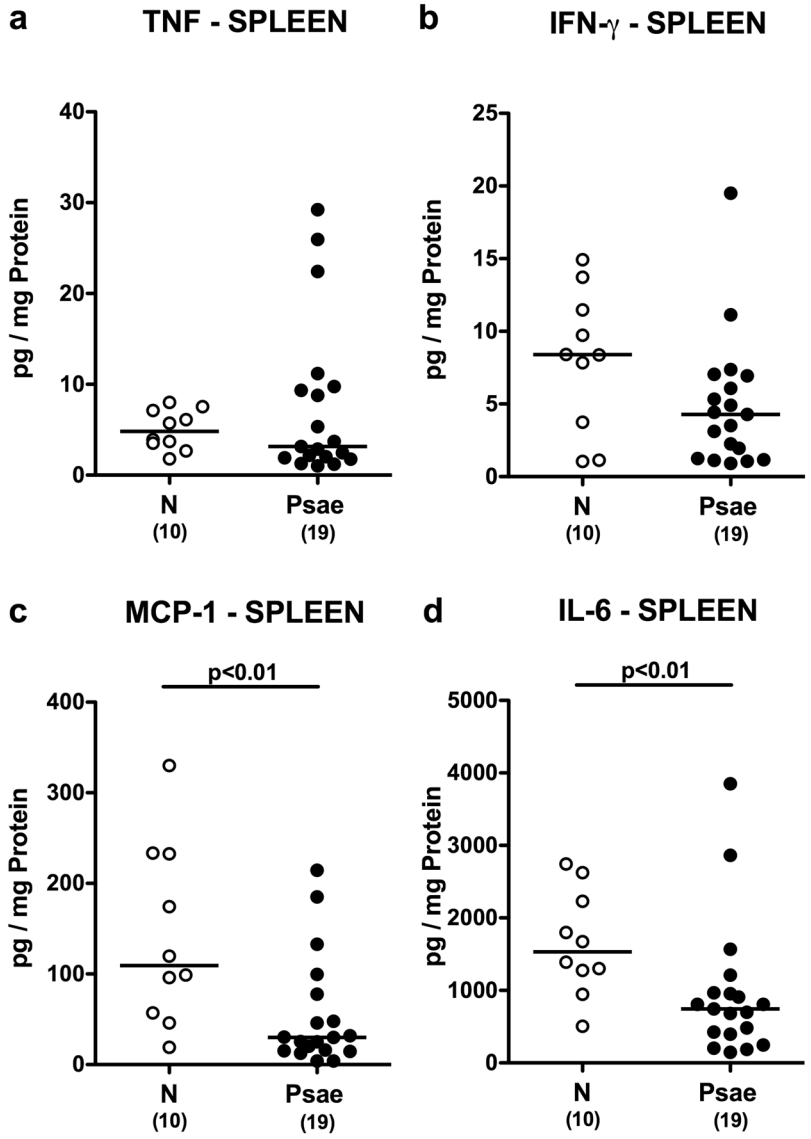

Figure 6. Splenic cytokine responses in multidrug-resistant *P. aeruginosa* infected mice suffering from chronic colitis. IL-10$^{-/-}$ mice with chronic colitis were perorally infected with a multidrug-resistant *P. aeruginosa* (Psae) strain on day (d) 0. Six weeks thereafter (on d42 postinfection; black circles) proinflammatory cytokines including (**a**) TNF, (**b**) IFN-γ, (**c**) MCP-1 and (**d**) IL-6 were determined in supernatants of splenic *ex vivo* biopsies. Naive (N) IL-10$^{-/-}$ mice served as negative controls (open circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the Mann Whitney U test are indicated. Data shown were pooled from three independent experiments.

levels. In fact, *Psae*-colonized IL-10$^{-/-}$ mice more frequently presented with blood in their feces and exhibited more pronounced colonic apoptosis that were accompanied by higher abundances of innate as well as adaptive immune cell populations in the inflamed colonic mucosa and lamina propria and increased secretion of the pro-inflammatory cytokines TNF and IFN-γ in MLN. Remarkably, accelerated disease was not restricted to the intestinal tract but could also be observed systemically, given that *Psae*-colonized IL-10$^{-/-}$ mice displayed elevated TNF and IFN-γ serum levels. Increased systemic pro-inflammatory cytokines were accompanied by translocation of viable commensal bacteria originating from the intestinal microbiota to extra-intestinal compartments of MDR *Psae*-colonized IL-10$^{-/-}$ mice. This indicates that additional acquisition of the opportunistic pathogen by colitic mice compromised intestinal barrier function subsequently facilitating bacterial spread from the leaky gut. Surprisingly, pro-inflammatory cytokine concentrations were lower in the spleen of *Psae* carrying IL-10$^{-/-}$ mice as compared to non-carriers. This might be explained by increased recruitment of leukocytes from the spleen to the inflamed intestinal tract.

In our present survey, *Psae* could be isolated from the intestines in up to 80% of IL-10$^{-/-}$ mice suffering from chronic colitis, even though mice harbored a conventional gut microbiota that usually protects mice from invading (opportunistic) pathogens[11]. One needs to take into consideration, however, that intestinal inflammatory

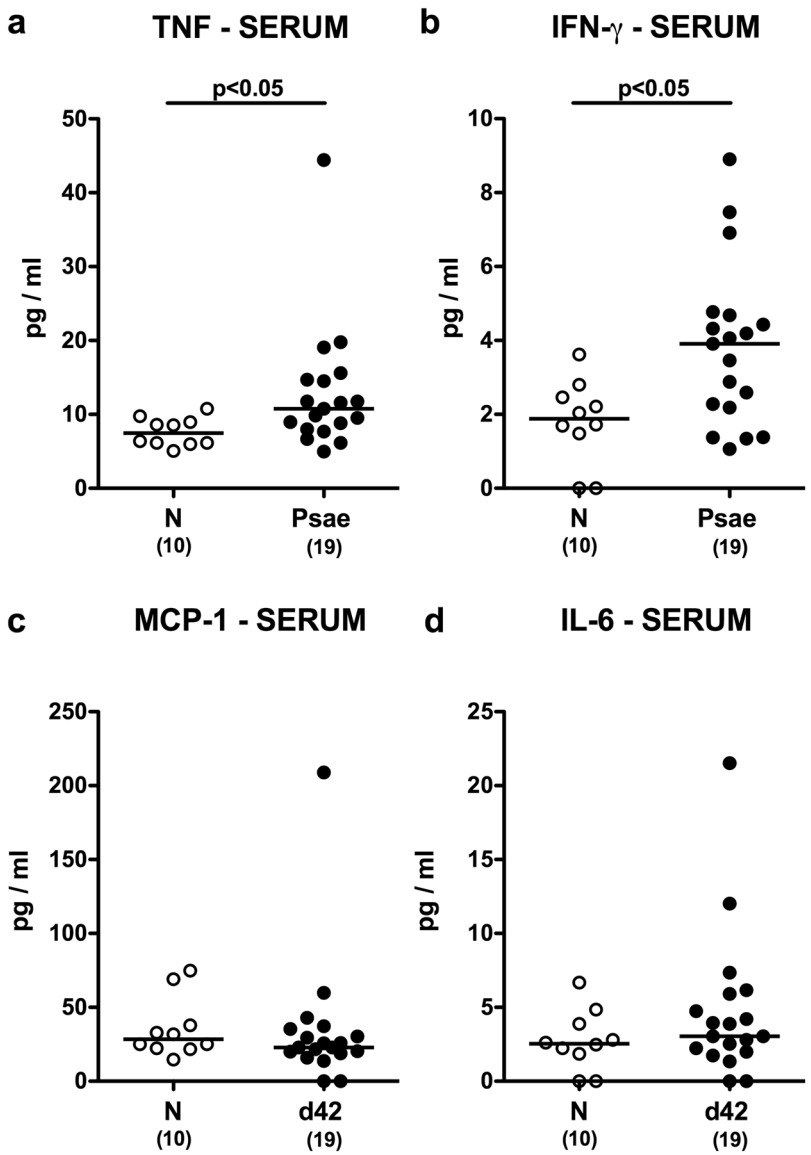

Figure 7. Systemic cytokine responses in multidrug-resistant *P. aeruginosa* infected mice suffering from chronic colitis. IL-10$^{-/-}$ mice with chronic colitis were perorally infected with a multidrug-resistant *P. aeruginosa* (Psae) strain on day (d) 0. Six weeks thereafter (on d42 postinfection; black circles) pro-inflammatory cytokines including (**a**) TNF, (**b**) IFN-γ, (**c**) MCP-1 and (**d**) IL-6 were determined in serum. Naive (N) IL-10$^{-/-}$ mice served as negative controls (open circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the Mann Whitney U test are indicated. Data shown were pooled from three independent experiments.

conditions *per se* might facilitate pathogenic colonization, given that the increased intraluminal abundance of cell debris, for instance, may serve as valuable nutrient sources for specific invading bacteria and may thus provide growth advantages in direct competition with the complex intestinal microbiota for nutrients and niches[16,17,23,24]. Furthermore, both acute and chronic inflammation of the small and large intestines are accompanied by distinct changes in murine microbiota composition providing growth benefits for certain intestinal commensals such as enterobacteria[24–27], which in turn might change the intraluminal milieu in favor of distinct (opportunistic) pathogenic species. In line, our previous studies revealed that elevated intestinal loads of commensal *Escherichia coli* were sufficient to override physiological colonization resistance and to facilitate murine infection with the enteropathogen *Campylobacter jejuni*[14–16,28].

Given a potential (bi-directional) crosstalk between (opportunistic) pathogens and intestinal commensals we performed a comprehensive survey of the large intestinal microbiota composition before and after MDR *Psae* challenge applying both culture and culture-independent (molecular) methods. Interestingly, a 6-week intestinal MDR *Psae* carriage by IL-10$^{-/-}$ with chronic colitis did not alter the microbiota within the inflamed colon lumen.

In conclusion, we here show that peroral MDR *Psae* challenge results in intestinal infection that exacerbates chronic colitis as indicated by macroscopic and colonic apoptotic sequelae as well as intestinal and systemic

pro-inflammatory cytokine responses in IL-10$^{-/-}$ mice. In ongoing studies we are currently unraveling the distinct intraluminal factors favoring and preventing intestinal MDR *Psae* establishment in health and disease. This may provide novel antibiotics-independent approaches to prevent intestinal colonization with MDR Gram-negative species including *Psae* in vertebrates and to combat subsequent infections of the susceptible host at risk including immunocompromized patients or individuals suffering from intestinal immunopathological morbidities.

Material and Methods

Ethical statement. In accordance with the European Guidelines for animal welfare (2010/63/EU), mice were reared, maintained and twice daily monitored assessing clinical conditions and weight loss. The commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration number G0097/12 and G0039/15) had given approval before start of animal experiments.

Mice and *P. aeruginosa* infection. Approximately 4 months old conventionally colonized, female IL-10$^{-/-}$ mice with chronic colitis (in C57BL/6j background) and corresponding WT mice were raised in the specific pathogen free unit of the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité – University Medicine Berlin) and perorally infected with 10^9 CFU of a MDR *Psae* strain as described earlier[13,23,29] In brief, the *Psae* isolate had been initially isolated from respiratory fluid of a patient with nosocomial pneumonia and exhibited antimicrobial sensitivity towards colistin and fosfomycin only (provided by Prof. Dr. Bastian Opitz, Charité – University Medicine, Berlin, Germany). The MDR bacterial strain was applied in a total volume of 0.3 mL phosphate buffered saline (PBS; Gibco, life technologies, UK) to mice by oral gavage.

Cultural analysis of *P. aeruginosa*. Fecal and luminal samples from stomach, duodenum, ileum and colon were plated in serial dilutions onto Columbia agar supplemented with 5% sheep blood (Oxoid, Germany) and Cetrimide agar (Oxoid, Germany) after homogenization in sterile PBS[13,23,29]. Inoculated agar plates were then incubated in an aerobic atmosphere at 37 °C for 48 h to assess *Psae* loads after infection as described earlier[13,23,29].

Clinical conditions. On a daily basis, fecal samples derived from each mouse were screened for abundance of fecal blood by the Guajac method (Haemoccult, Beckman Coulter/PCD, Germany) as reported previously[18,29].

Sampling procedures. Six weeks following *Psae* infection mice were sacrifized by isoflurane inhalation (Abott, Germany). Cardiac blood and tissue samples from spleen, liver, lung, kidney, MLN, ileum and colon were collected under sterile conditions as described earlier[13,23,29]. After measuring the large intestinal lengths, colonic tissue samples were collected in parallel for histopathological and immunohistochemical analyses as well as for cytokine measurements.

Histopathology and immunohistochemistry. Histopathological changes observed in 5 µm thin hematoxylin and eosin stained colonic paraffin sections were quantitated applying a standardized histopathological scoring system ranging from 0 to 4 as described by Erben *et al.*[19]. In brief: Score 0: unaffected histomorphology. Score 1: minimal inflammatory cell infiltrates in the mucosa with intact epithelium. Score 2: mild inflammatory cell infiltrates in the mucosa with mild hyperplasia and mild goblet cell loss. Score 3: moderate inflammatory cell infiltrates in mucosa and submucosa with moderate goblet cell loss. Score 4: marked inflammatory cell infiltration into mucosa and submucosa with marked hyperplasia and marked goblet cell loss, multiple crypt abscesses and crypt loss[19].

To display immune cell response in colonic *ex vivo* biopsies, immunohistochemical staining was performed on five-µm paraffin sections as described earlier[13,23,27,29–32]. In short, primary antibodies against cleaved caspase-3 (Asp175, #9661, Cell Signaling, Netherlands; 1:200) for apoptitic cells, Ki67 (clone 16A8, #652401, BioLegend/Biozol, Germany; 1:200) for proliferating/regenerating cells, F4/80 (clone BM8, #MF48015, Life Technologies, Germany; 1:100) for macrophages/monocytes, CD3 (#IR50361-2, Dako, USA; 1:5) for T lymphocytes, FOXP3 (clone FJK-165, #14–5773, eBioscience, Germany; 1:100) for Tregs and B220 (#14-0452-81, eBioscience; 1:200) for B lymphocytes were used. The average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2; 400x magnification) were determined by a blinded independent investigator.

Cultural survey of intestinal microbiota and bacterial translocation. To survey the composition of the commensal colonic microbiota and the abundance of viable commensals of intestinal origin that had translocated to extra-intestinal including systemic sites, colonic luminal contents and tissue samples derived from MLN, spleen, liver, kidney and lung were homogenized in sterile PBS, streaked out in serial dilutions on agar plates and incubated under aerobic, microaerobic and anaerobic conditions at 37 °C for two to three days as reported earlier[23,24,32,33]. The detection limit of viable bacteria was approximately 100 CFU per g. To assess the appearance of bacteremia, thioglycolate enrichment broths (BD Bioscience, Germany) were inoculated with cardiac blood of individual mice and incubated for seven days at 37 °C and subsequently streaked onto respective agares for further identification as described[23,32].

Molecular analysis of the intestinal microbiota. The numbers of 16S rRNA gene copies per ng DNA of main bacterial groups abundant in the murine and human gut microbiota were quantified applying quantitative real-time polymerase chain reaction with species-, genera- or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as stated elsewhere[11,27,34]. In brief, DNA was extracted from individual fecal samples, quantified by using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng per µl[24,35,36].

Cytokine detection. Tissue samples of MLN and spleen were transferred to 24-well-flat-bottom culture plates (Falcon, Germany) and incubated in 500 µL serum-free RPMI 1640 medium (Gibco, life technologies) supplemented with penicillin (100 U/mL, Biochrom, Germany) and streptomycin (100 µg/mL; Biochrom) for 18 h at 37 °C. TNF, IFN-γ, MCP-1, and IL-6 concentrations were measured in supernatants and serum samples applying the Mouse Inflammation Cytometric Bead Assay (CBA; BD Bioscience) on a BD FACSCanto II flow cytometer (BD Bioscience) as stated elsewhere[11,23].

Statistical analysis. Mean values, medians, and levels of significance were determined using Mann-Whitney-U Test. Two-sided probability (p) values ≤ 0.05 were considered significant. Experiments were repeated at least twice.

References

1. Vincent, J. L. Nosocomial infections in adult intensive-care units. *Lancet.* **361**, 2068–2077, https://doi.org/10.1016/s0140-6736(03)13644-6 (2003).
2. Oliver, A., Mulet, X., Lopez-Causape, C. & Juan, C. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat.* **21-22**, 41–59, https://doi.org/10.1016/j.drup.2015.08.002 (2015).
3. Lyczak, J. B., Cannon, C. L. & Pier, G. B. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect.* **2**, 1051–1060 (2000).
4. Potron, A., Poirel, L. & Nordmann, P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. *Int J Antimicrob Agents.* **45**, 568–585, https://doi.org/10.1016/j.ijantimicag.2015.03.001 (2015).
5. Tacconelli, E. & Magrini, N. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *WHO*. http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1 (2017).
6. Shooter, R. A. *et al*. Faecal carriage of *Pseudomonas aeruginosa* in hospital patients. Possible spread from patient to patient. *Lancet.* **2**, 1331–1334 (1966).
7. Buck, A. C. & Cooke, E. M. The fate of ingested *Pseudomonas aeruginosa* in normal persons. *J Med Microbiol.* **2**, 521–525, https://doi.org/10.1099/00222615-2-4-521 (1969).
8. Gomez-Zorrilla, S. *et al*. Prospective observational study of prior rectal colonization status as a predictor for subsequent development of *Pseudomonas aeruginosa* clinical infections. *Antimicrob Agents Chemother.* **59**, 5213–5219, https://doi.org/10.1128/aac.04636-14 (2015).
9. Hentges, D. J., Stein, A. J., Casey, S. W. & Que, J. U. Protective role of intestinal flora against infection with *Pseudomonas aeruginosa* in mice: influence of antibiotics on colonization resistance. *Infect Immun.* **47**, 118–122 (1985).
10. Marshall, J. C., Christou, N. V. & Meakins, J. L. The gastrointestinal tract. *The "undrained abscess" of multiple organ failure. Ann Surg.* **218**, 111–119, https://doi.org/10.1097/00000658-199308000-00001 (1993).
11. Bereswill, S. *et al*. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. *PloS One.* **6**, e20953, https://doi.org/10.1371/journal.pone.0020953 (2011).
12. Fiebiger, U., Bereswill, S. & Heimesaat, M. M. Dissecting the interplay between intestinal microbiota and host immunity in health and disease: Lessons learned from germfree and gnotobiotic animal models. *Eur J Microbiol Immunol (Bp).* **6**, 253–271, https://doi.org/10.1556/1886.2016.00036 (2016).
13. Von Klitzing, E., Bereswill, S. & Heimesaat, M. Multidrug-resistant *Pseudomonas aeruginosa* induce systemic pro-inflammatory immune responses in colonized mice. *Eur J Microbiol Immunol (Bp).* **7**, 200–209, https://doi.org/10.1556/1886.2017.00022 (2017).
14. Haag, L. M. *et al*. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. *PloS One.* **7**, e35988, https://doi.org/10.1371/journal.pone.0035988 (2012).
15. Haag, L. M. *et al*. *Campylobacter jejuni* infection of infant mice: acute enterocolitis is followed by asymptomatic intestinal and extra-intestinal immune responses. *Eur J Microbiol Immunol (Bp).* **2**, 2–11, https://doi.org/10.1556/EuJMI.2.2012.1.2 (2012).
16. Otto, B. *et al*. *Campylobacter jejuni* induces extra-intestinal immune responses via Toll-like-receptor-4 signaling in conventional IL-10 deficient mice with chronic colitis. *Eur J Microbiol Immunol (Bp).* **2**, 210–219, https://doi.org/10.1556/EuJMI.2.2012.3.7 (2012).
17. Heimesaat, M. M., Grundmann, U., Alutis, M. E., Fischer, A. & Bereswill, S. Microbiota composition and immune responses during *Campylobacter jejuni* infection in conventionally colonized IL-$10^{-/-}$ mice lacking Nucleotide oligomerization domain 2. *Eur J Microbiol Immunol (Bp).* **7**, 1–14, https://doi.org/10.1556/1886.2016.00038 (2017).
18. Haag, L. M. *et al*. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-$10^{-/-}$ mice via Toll-like-receptor-2 and -4 signaling. *PloS One.* **7**, e40761, https://doi.org/10.1371/journal.pone.0040761 (2012).
19. Erben, U. *et al*. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int J Clin Exp Pathol.* **7**, 4557–4576 (2014).
20. Livermore, D. M. Has the era of untreatable infections arrived? *Antimicrob Chemother.* **64**(Suppl 1), i29–36, https://doi.org/10.1093/jac/dkp255 (2009).
21. Wang, M., Molin, G., Ahrne, S., Adawi, D. & Jeppsson, B. High proportions of proinflammatory bacteria on the colonic mucosa in a young patient with ulcerative colitis as revealed by cloning and sequencing of 16S rRNA genes. *Dig Dis Sci.* **52**, 620–627, https://doi.org/10.1007/s10620-006-9461-1 (2007).
22. Kerckhoffs, A. P. *et al*. Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in irritable bowel syndrome. *J Med Microbiol.* **60**, 236–245, https://doi.org/10.1099/jmm.0.022848-0 (2011).
23. von Klitzing, E., Ekmekciu, I., Bereswill, S. & Heimesaat, M. M. Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. *Gut Pathog.* **9**, 4, https://doi.org/10.1186/s13099-017-0154-4 (2017).
24. Heimesaat, M. M. *et al*. Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii*. *J Immunol.* **177**, 8785–8795, https://doi.org/10.4049/jimmunol.177.12.8785 (2006).
25. Heimesaat, M. M. *et al*. Exacerbation of murine ileitis by Toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. *Gut.* **56**, 941–948, https://doi.org/10.1136/gut.2006.104497 (2007).
26. Erridge, C., Duncan, S. H., Bereswill, S. & Heimesaat, M. M. The induction of colitis and ileitis in mice is associated with marked increases in intestinal concentrations of stimulants of TLRs 2, 4, and 5. *PloS One.* **5**, e9125, https://doi.org/10.1371/journal.pone.0009125 (2010).
27. Heimesaat, M. M. *et al*. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut.* **59**, 1079–1087, https://doi.org/10.1136/gut.2009.197434 (2010).
28. Heimesaat, M. M. *et al*. Survey of extra-intestinal immune responses in asymptomatic long-term *Campylobacter jejuni*-infected mice. *Eur J Microbiol Immunol (Bp).* **3**, 174–182, https://doi.org/10.1556/EuJMI.3.2013.3.4 (2013).

29. Eliane, V. K., Ekmekciu, I., Bereswill, S. & Heimesaat, M. M. Intestinal and systemic immune responses upon multi-drug resistant *Pseudomonas aeruginosa* colonization of mice harboring a human gut microbiota. *Front Microbiol.* **8**, 2590, https://doi.org/10.3389/fmicb.2017.02590 (2017).
30. Ekmekciu, I. *et al.* Immune responses to broad-spectrum antibiotic treatment and fecal microbiota transplantation in mice. *Front Immunol.* **8**, 397, https://doi.org/10.3389/fimmu.2017.00397 (2017).
31. Ekmekciu, I. *et al.* The probiotic compound VSL#3 modulates mucosal, peripheral, and systemic immunity following murine broad-spectrum antibiotic treatment. *Front Cell Infect Microbiol.* **7**, 167, https://doi.org/10.3389/fcimb.2017.00167 (2017).
32. von Klitzing, E., Ekmekciu, I., Kuhl, A. A., Bereswill, S. & Heimesaat, M. M. Intestinal, extra-intestinal and systemic sequelae of *Toxoplasma gondii* induced acute ileitis in mice harboring a human gut microbiota. *PloS One.* **12**, e0176144, https://doi.org/10.1371/journal.pone.0176144 (2017).
33. Heimesaat, M. M. *et al.* Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like receptors 2 and 4. *PloS One.* **2**, e662, https://doi.org/10.1371/journal.pone.0000662 (2007).
34. Rausch, S. *et al.* Small intestinal nematode infection of mice is associated with increased enterobacterial loads alongside the intestinal tract. *PloS One.* **8**, e74026, https://doi.org/10.1371/journal.pone.0074026 (2013).
35. Bereswill, S. *et al.* The impact of Toll-like-receptor-9 on intestinal microbiota composition and extra-intestinal sequelae in experimental *Toxoplasma gondii* induced ileitis. *Gut Pathog.* **6**, 19, https://doi.org/10.1186/1757-4749-6-19 (2014).
36. von Klitzing, E. *et al.* Comprehensive survey of intestinal microbiota changes in offspring of human microbiota-associated mice. *Eur J Microbiol Immunol (Bp).* **7**, 65–75, https://doi.org/10.1556/1886.2017.00002 (2017).

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Author Contributions

E.V.K. Performed the experiments, analyzed data, co-edited the paper I.E.: Performed the experiments, co-edited the paper A.A.K.: Provided reagents and analysis tools, analyzed data, co-edited the paper S.B.: Proved advice in design, performance of experiments, co-edited the paper M.M.H.: Designed and performed the experiments, analyzed data, wrote the paper.

Additional Information

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Multidrug-resistant *Pseudomonas aeruginosa* aggravate intestinal and systemic pro-inflammatory cytokine responses in murine chronic colitis

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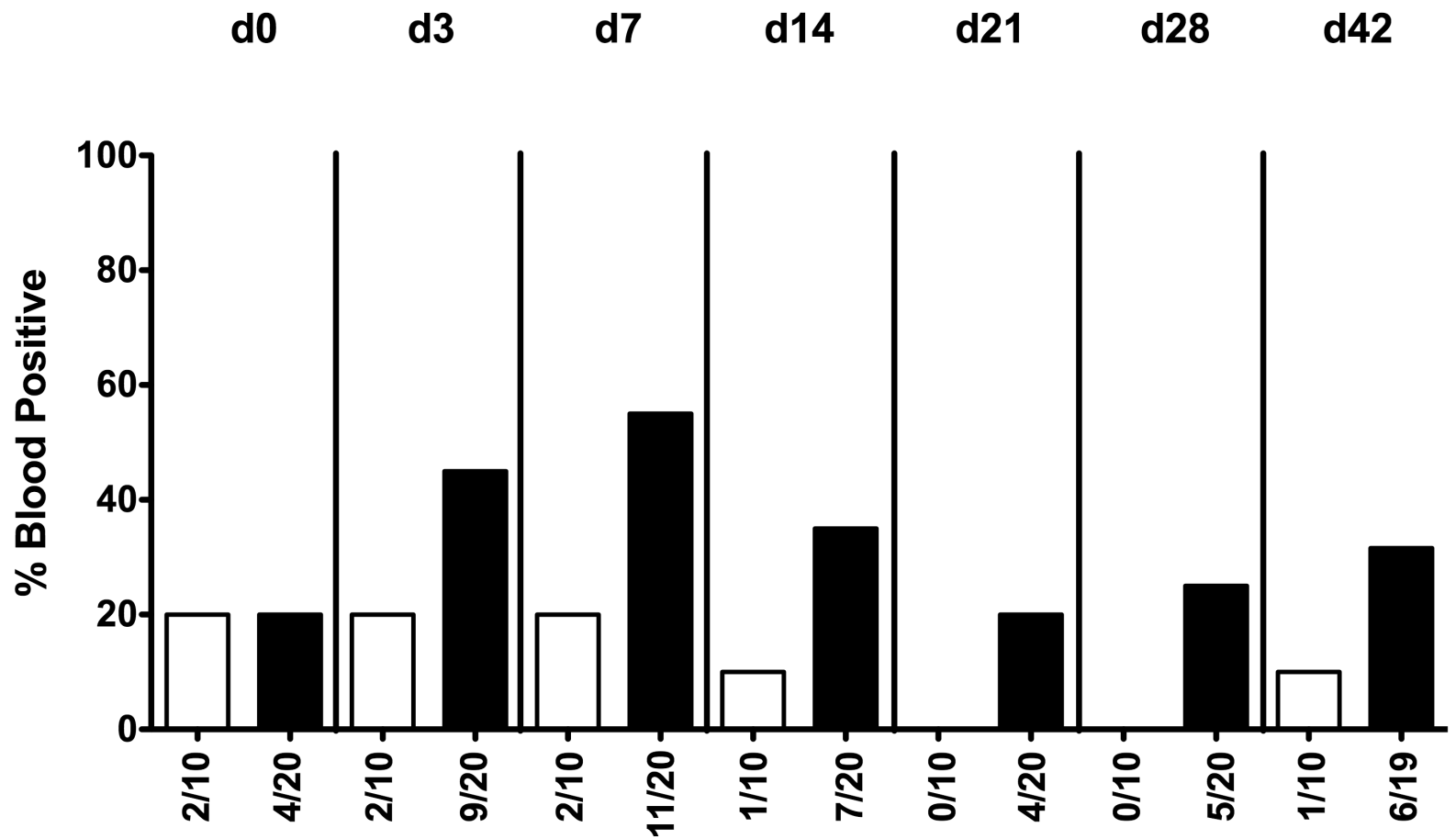
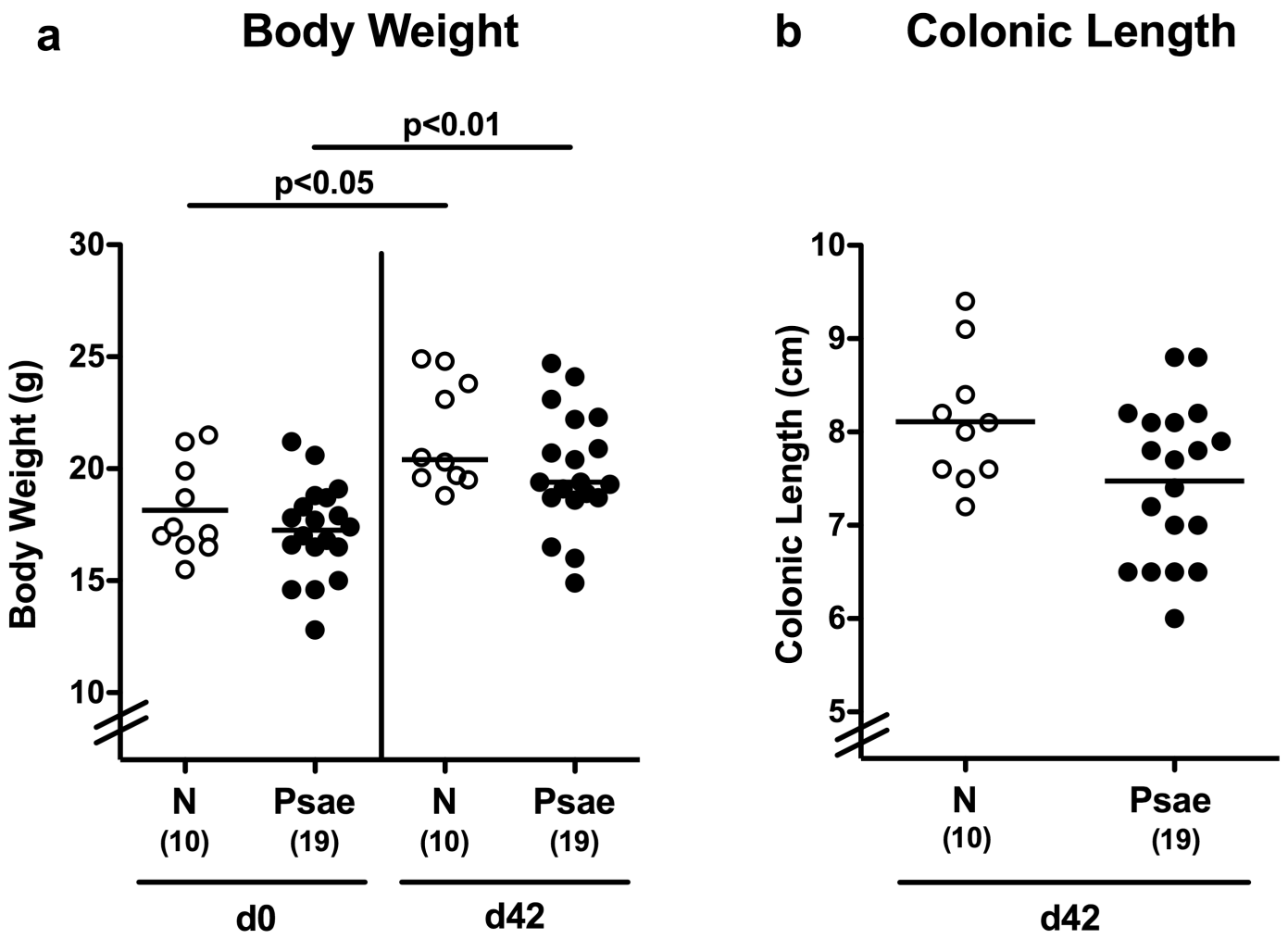
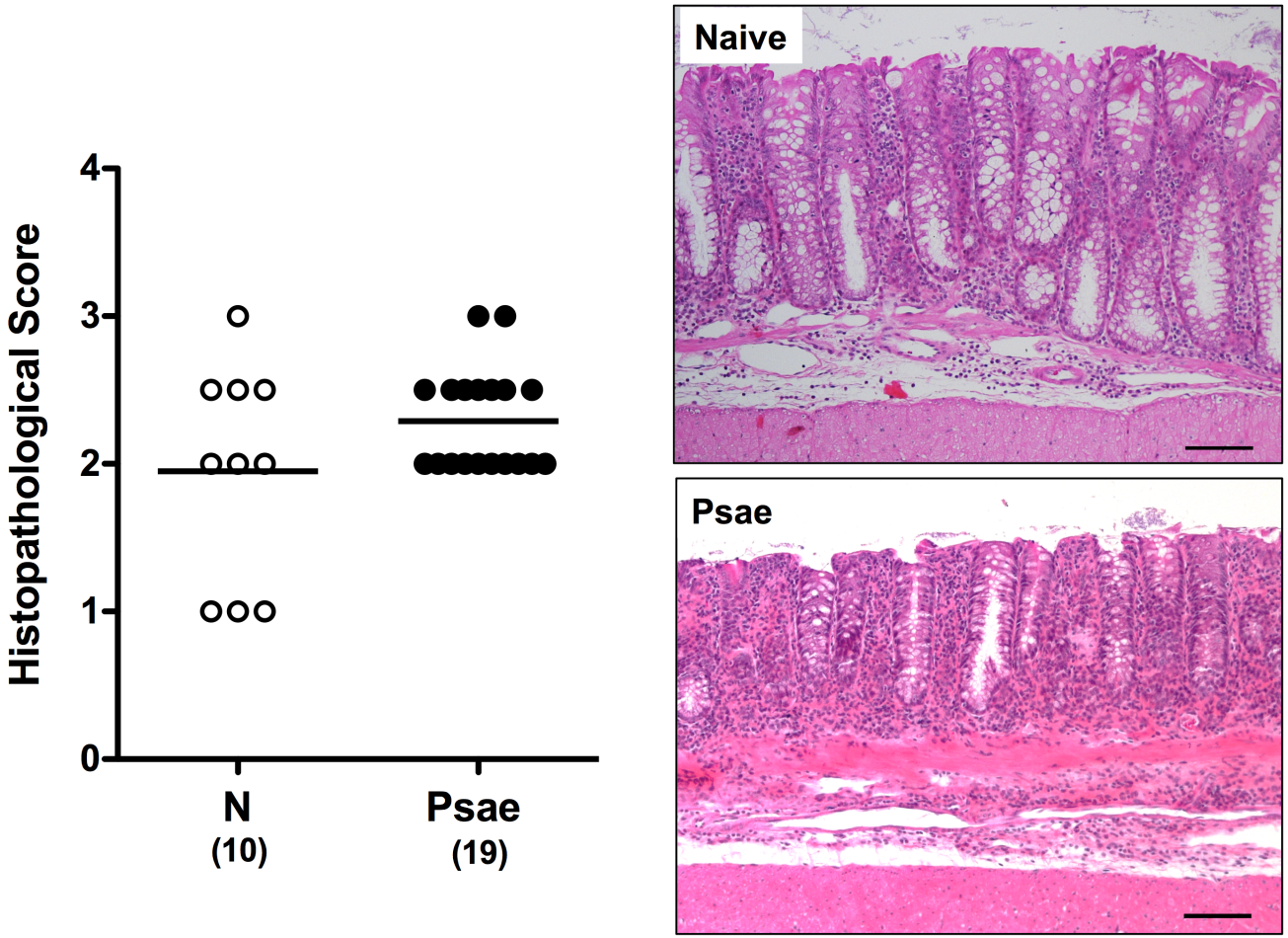


Abundance of blood in fecal samples derived from multidrug resistant *P. aeruginosa* infected mice with chronic colitis. IL-10$^{-/-}$ mice suffering from chronic colitis were perorally infected with a multidrug resistant *P. aeruginosa* strain on day (d) 0 (black bars). Subsequently, the abundance of blood was assessed in fecal samples until necropsy (d42 postinfection) by the Guajac (Haemoccult) method. Naive IL-10$^{-/-}$ mice served as negative (uninfected) controls (white bars). Cumulative blood-positivity rates of three independent experiments (in %) and absolute numbers of mice with bloody feces out of the total number of analyzed animals (on x-axis) are indicated.



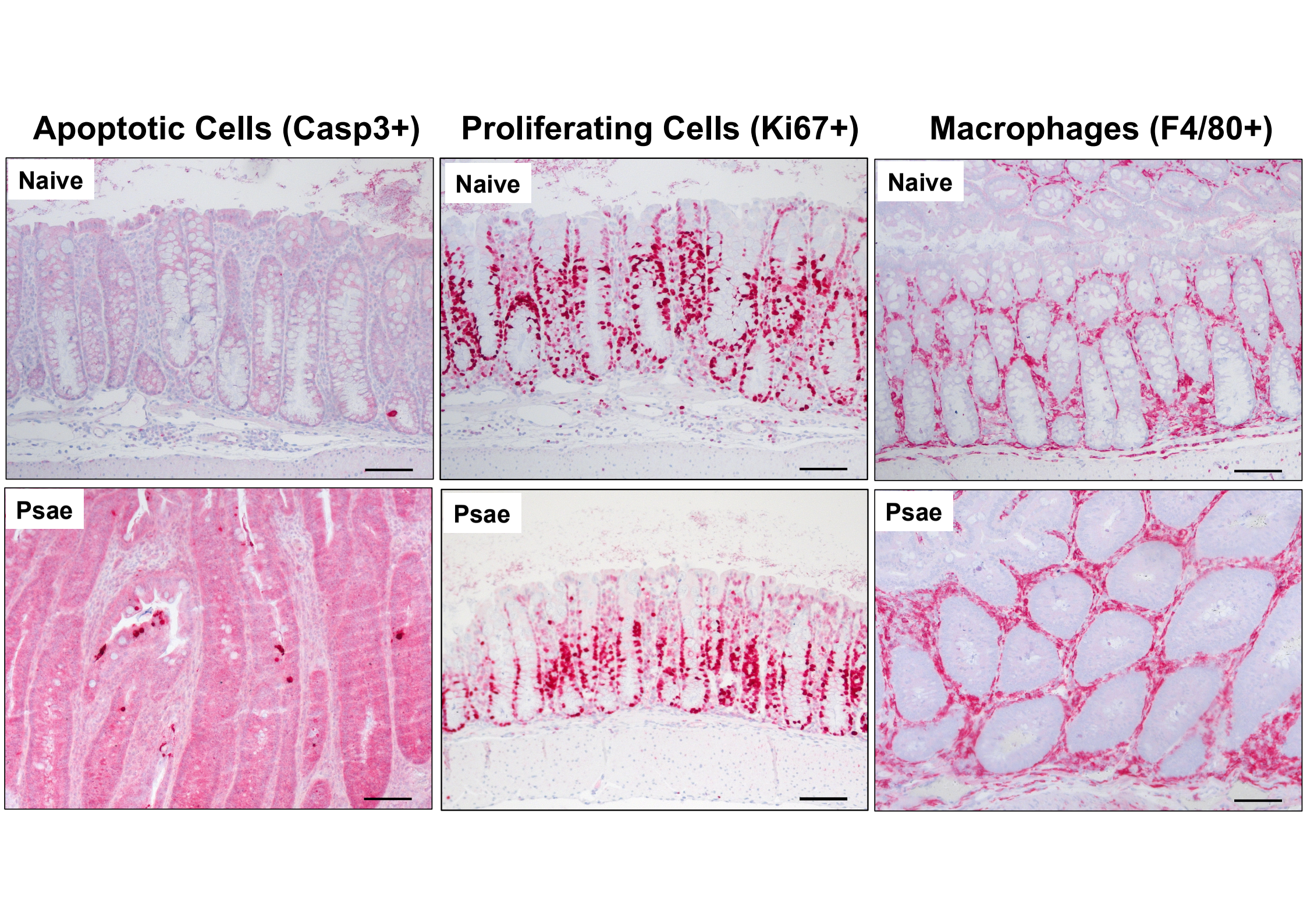
Macroscopic changes in multidrug resistant *P. aeruginosa* infected mice with chronic colitis. IL-10$^{-/-}$ mice suffering from chronic colitis were perorally infected with a multidrug resistant *P. aeruginosa* (Psae) strain on day (d) 0. To assess individual macroscopic changes **(a)** body weights of mice were obtained immediately before (day (d) 0) and six weeks after (d42) Psae infection (black circles) and compared to uninfected, naive (N) controls at respective time points (white circles). Upon necropsy (i.e. at d42 postinfection) colonic lengths (in cm) were measured in Psae infected (black circles) and naive mice (white circles). Numbers of mice (in parentheses), medians (black bars) and significance levels (p-values) determined by the Mann Whitney U test are indicated. Data shown were pooled from three independent experiments.

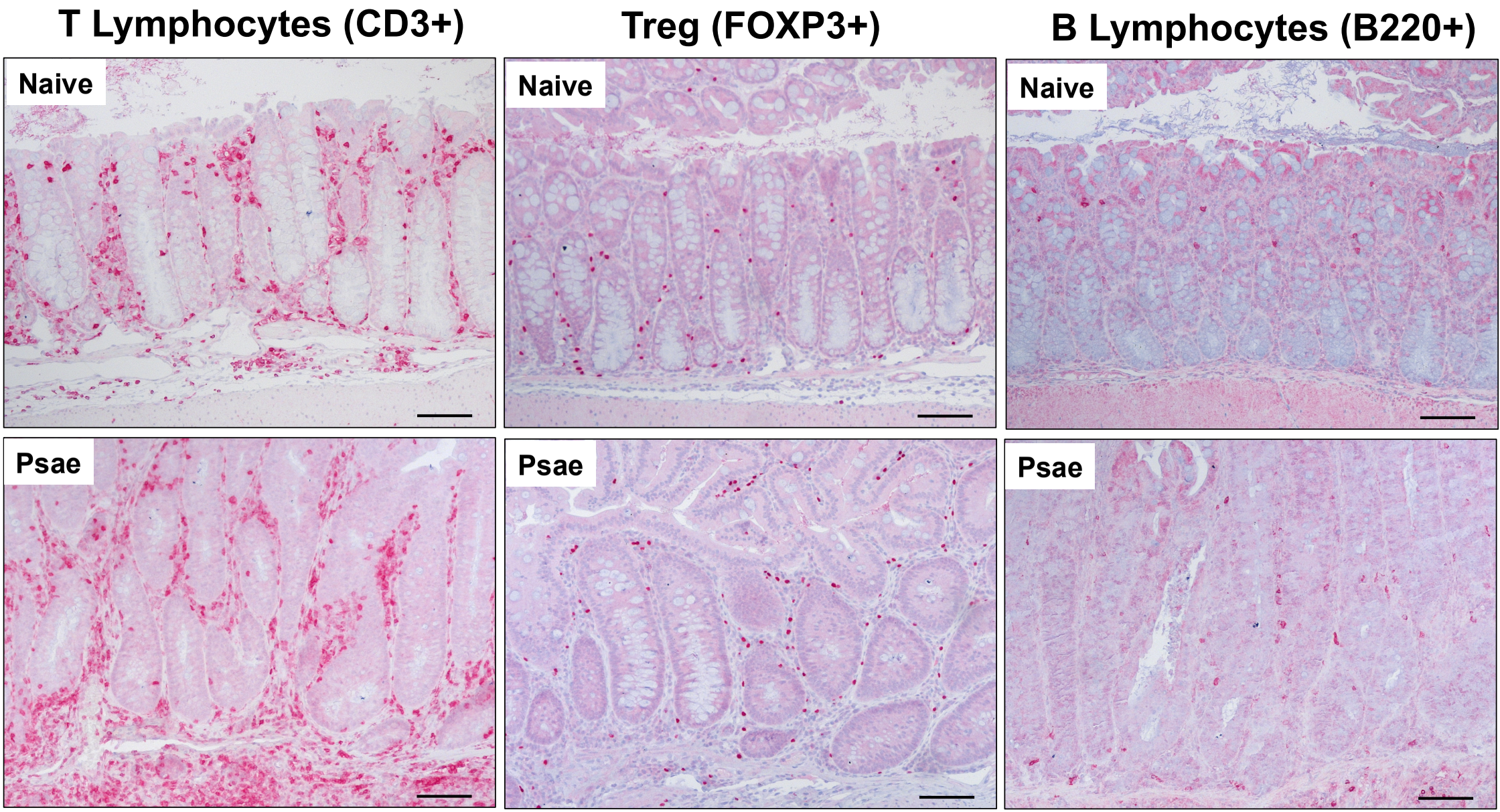
Histopathology



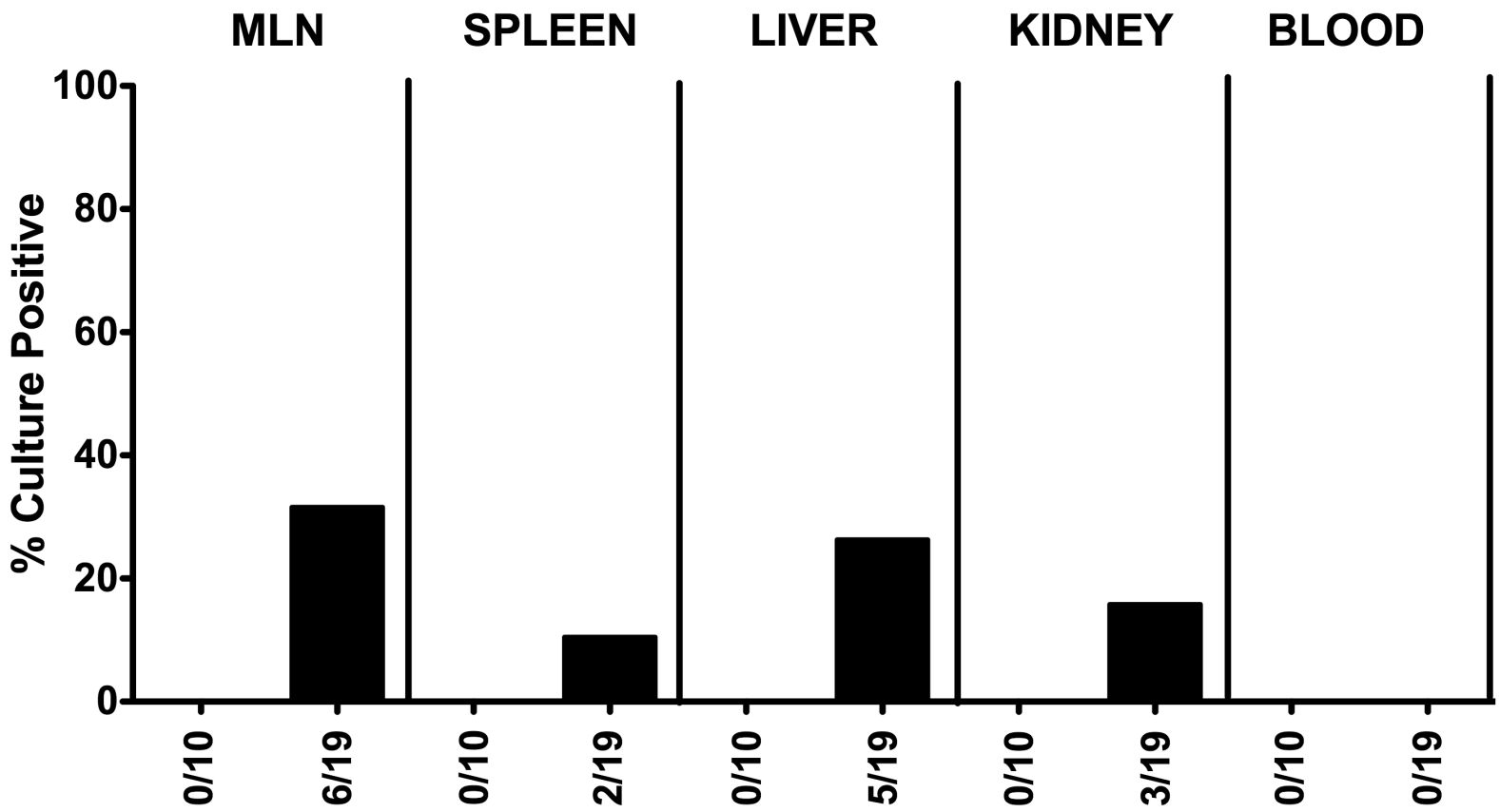
Histopathological changes in large intestines of multidrug resistant *P. aeruginosa* infected mice with chronic colitis. IL-10$^{-/-}$ mice suffering from chronic colitis were perorally infected with a multidrug resistant *P. aeruginosa* (Psae) strain on day 0. Six weeks thereafter histopathological changes were assessed in hematoxylin and eosin stained colonic paraffin sections applying a standardized histopathological scoring system (left). Naive, uninfected mice served as negative controls (N). Numbers of mice (in parentheses) and medians (black bars) are indicated. Data shown were pooled from three independent experiments. Right: Representative photomicrographs illustrate respective histomorphological changes (100x magnification, scale bar 100 µm).

Representative photomicrographs of apoptotic and proliferating epithelial cells, macrophages/monocytes, T lymphocytes, regulatory T cells (Treg) and B lymphocytes in colonic paraffin sections derived from multidrug resistant *P. aeruginosa* infected mice with chronic colitis. IL-10$^{-/-}$ mice suffering from chronic colitis were perorally infected with a multidrug resistant *P. aeruginosa* (Psae) strain on day (d) 0 and sacrifized six weeks thereafter (d42 postinfection; lower panel). Representative photomicrographs illustrate epithelial apoptotic (positive for caspase 3, Casp3) and proliferating cells (positive for Ki67), macrophages and monocytes (positive for F4/80), T lymphocytes (positive for CD3), regulatory T cells (Treg, positive for FOXP3) and B lymphocytes (positive for B220) in immuno-histochemically stained large intestinal paraffin sections (100x magnification, scale bar 100 µm). Naive, uninfected IL-10$^{-/-}$ mice served as negative controls (upper panel).


Apoptotic Cells (Casp3+)
Proliferating Cells (Ki67+)
Macrophages (F4/80+)
Naive
Naive
Naive
Psae
Psae
Psae


T Lymphocytes (CD3+)
Treg (FOXP3+)
B Lymphocytes (B220+)
Naive
Naive
Naive
Psae
Psae
Psae

Bacterial Translocation



Bacterial translocation in multidrug resistant *P. aeruginosa* infected mice with chronic colitis. IL-10$^{-/-}$ mice suffering from chronic colitis were perorally infected with a multidrug resistant *P. aeruginosa* strain on day (d) 0. Six weeks thereafter (d42 postinfection; black circles) translocation of intestinal bacteria to extra-intestinal and systemic compartments were determined by cultivation of homogenated *ex vivo* biopsies such as MLN, spleen, liver and kidney (direct plating on solid media) as well as of cardiac blood (in thioglycolate enrichment broths) with subsequent subcultivation and species identification. Cumulative relative rates of positive samples (%) out of three independent experiments are indicated.

4 Lebenslauf Eliane von Klitzing

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

5 Publikationsliste

1. von Klitzing E, Bereswill S, Heimesaat MM. Multidrug-resistant *Pseudomonas aeruginosa* induce systemic pro-inflammatory immune responses in colonized mice. Eur J Microbiol Immunol (Bp). 2017;7(3):200-9. (Aktuell noch kein Impact Factor, laut Research Gate taxiert auf: 2.03)

2. von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM. Intestinal and systemic immune responses upon multi-drug resistant *Pseudomonas aeruginosa* colonization of mice harboring a human gut microbiota. Front Microbiol. 2017;8:2590. (2017 Journal Impact Factor: 4.019)

3. von Klitzing E, Ekmekciu I, Bereswill S, Heimesaat MM. Acute ileitis facilitates infection with multidrug resistant *Pseudomonas aeruginosa* in human microbiota-associated mice. Gut Pathog. 2017;9:4. (2017 Journal Impact Factor: 2.809)

4. von Klitzing E, Ekmekciu I, Kuhl AA, Bereswill S, Heimesaat MM. Multidrug-resistant *Pseudomonas aeruginosa* aggravates inflammatory responses in murine chronic colitis. Sci Rep. 2018;8(1):6685. (2017 Journal Impact Factor: 4.122)

5. von Klitzing E, Ekmekciu I, Kuhl AA, Bereswill S, Heimesaat MM. Intestinal, extra-intestinal and systemic sequelae of *Toxoplasma gondii* induced acute ileitis in mice harboring a human gut microbiota. PLoS One. 2017;12(4):e0176144. (2017 Journal Impact Factor: 2.766)

6. von Klitzing E, Oz F, Ekmekciu I, Escher U, Bereswill S, Heimesaat MM. Comprehensive survey of intestinal microbiota changes in offspring of human microbiota-associated mice. Eur J Microbiol Immunol (Bp). 2017;7(1):65-75. (Aktuell noch kein Impact Factor, laut Research Gate taxiert auf: 2.03)

7. Ekmekciu I, von Klitzing E, Fiebiger U, Escher U, Neumann C, Bacher P, Scheffold A, Kuhl AA, Bereswill S, Heimesaat MM. Immune responses to broad-spectrum antibiotic treatment and fecal microbiota transplantation in mice. Front Immunol. 2017;8:397. (2017 Journal Impact Factor: 5.511)

8. Ekmekciu I, von Klitzing E, Fiebiger U, Neumann C, Bacher P, Scheffold A, Bereswill S, Heimesaat MM. The probiotic compound vsl#3 modulates mucosal, peripheral, and systemic immunity following murine broad-spectrum antibiotic treatment. Front Cell Infect Microbiol. 2017;7:167. (2017 Journal Impact Factor: 3.520)

9. Ekmekciu I, von Klitzing E, Neumann C, Bacher P, Scheffold A, Bereswill S, Heimesaat MM. Fecal microbiota transplantation, commensal *Escherichia coli* and *Lactobacillus johnsonii* strains differentially restore intestinal and systemic adaptive immune cell populations following broad-spectrum antibiotic treatment. Front Microbiol. 2017;8:2430. (2017 Journal Impact Factor: 4.019)

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