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DISSERTATION

Die Mikrobiota-vermittelte Modulation intestinaler und extra-intestinaler Immunzellpopulationen in sekundär abiotischen Mäusen und im murinen *Campylobacter jejuni* Infektionsmodell

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Inhaltsverzeichnis

1. Zusammenfassung

1.1 Abstrakt

Es gibt überzeugende experimentelle und klinische Evidenz, die die Rolle der intestinalen Mikrobiota und der Antibiotika-induzierten Störungen ihrer Homöostase hervorhebt und mit zahlreichen physiologischen und pathologischen Prozessen in Verbindung bringt. Die spezifische Zusammensetzung der Darmmikrobiota ist außerdem wichtig für die Bestimmung von Kolonisationsresistenz vs. Suszeptibilität des Wirtes gegenüber enteropathogenen Bakterien, wie beispielsweise *Campylobacter jejuni*. Infektionen mit *C. jejuni* nehmen weltweit zu und bedürfen der Entwicklung neuartiger, u.a. Antibiotika-unabhängiger, Mikrobiota/Probiotika-vermittelter Therapiestrategien. Das Ziel der in dieser Arbeit zusammengefassten Untersuchungen war es, die komplizierte Interaktion zwischen dem murinen Immunsystem, der Darmmikrobiota und pathogenen Bakterien näher zu eruieren. Zu diesem Zweck wurden konventionelle C57BL/6j Mäuse einer Breitband-Antibiose für einen Zeitraum von 8 Wochen unterzogen, womit die Eradikation der Mikrobiota erreicht wurde. Ihre Wiederherstellung wurde mittels fäkaler Mikrobiota-Transplantation (FMT) durchgeführt. Die FMT führte zur Normalisierung der durch die antibiotische Behandlung reduzierten Zellzahl der CD4+ und CD8+ Lymphozyten im Dünndarm und CD4+ Lymphozyten im Kolon, während die CD8+ Lymphozyten des Dickdarms auch nach FMT erniedrigt blieben. Antibiotisch behandelte Mäuse wiesen außerdem eine geringere Produktion von IFN-γ im Dünn- und Dickdarm, sowie von IL-17, IL-22 und IL-10 im Dünn- und Dickdarm, in den mesenterialen Lymphknoten und in der Milz. Diesen Effekten konnte dennoch mittels FMT vollständig entgegengetreten werden. Im Anschluss wurde das Probiotikum VSL#3 (bestehend aus *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei* und *Lactobacillus delbrueckii subsp. Bulgaricus*) auf seine immunomodulatorischen Eigenschaften nach antibiotischer Therapie hin untersucht. Hier sahen wir, dass VSL#3 v.a. anti-inflammatorische Immunzellpopulationen induzierte (CD4+CD25+ sowie CD4+IL-10+ Zellen), ohne einen Einfluss auf die Expression von IFN-γ, IL-17 und IL-22 zu haben. Diese anti-inflammatorischen Eigenschaften kamen dann auch im murinen *C. jejuni* Infektionsmodell zur Geltung. So führte eine prophylaktische sowie therapeutische Gabe von VSL#3 bei mit *C. jejuni* infizierten Mäusen zu einer geringeren epithelialen Apoptoserate, und einem verminderten Einstrom zahlreicher Immunzellenpopulationen (T- und B-Lymphozyten, regulatorischen T Zellen und Makrophagen/Monozyten) in die Kolonmukosa.

Durch die prophylaktische Gabe von VSL#3 war außerdem die Produktion von IL-10 im Kolon erhöht, die MCP-1- und IL-6-Konzentrationen hingegen erniedrigt. Ebenfalls niedriger war unter diesen Bedingungen die TNF Produktion in der Milz.

Zusammenfassend konnten wir die erheblichen immunologischen Auswirkungen der kommensalen Mikrobiota und Probiotika und die vorteilhafte Verwendung letzterer als therapeutische oder prophylaktische Strategie im Kontext von Infektion und Entzündung zeigen.

Abstract

Compelling experimental and clinical evidence highlights the outstanding role of the microbiota in host physiology and links its antibiotics induced perturbations with various pathologies. Moreover, the host specific microbiota composition is one of the most important determinants regarding colonization resistance against vs. susceptibility to enteropathogenic bacteria, such as *Campylobacter jejuni*. The incidence of *C. jejuni* infections is progressively increasing worldwide and requires the development of novel, antibiotics independent intervention strategies. In the present work, results of three separate *in vivo* studies addressing the interactions between host immunity, intestinal microbiota and pathogens have been summarized. Conventionally colonized C57BL/6j mice were subjected to broad-spectrum antibiotic treatment to virtually deplete the intestinal microbiota. Subsequently, fecal microbiota transplantation (FMT) was carried out perorally *via* gavage. FMT led to reestablishment of small intestinal CD4+ and CD8+ as well as colonic CD4+ cell numbers. Furthermore, CD4+ lymphocytes of with antibiotics treated mice revealed reduced expressions of IFN-γ in the small and large intestines, as well as of IL-17, IL-22 and IL-10 in all analyzed lymphoid compartments, including the small and large intestinal lamina propria, mesenteric lymph nodes and spleen. These effects were, however, completely restored upon FMT. Subsequently, we analyzed the immunomodulatory properties of the probiotic compound VSL#3 (consisting of *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei,* and *Lactobacillus delbrueckii subsp. Bulgaricus*) following antibiotic treatment. We could observe that VSL#3 induced mainly regulatory, anti-inflammatory immune responses (CD4+CD25+, as well as CD4+IL10+ cells) without impacting pro-inflammatory cytokines like IFN-γ, IL-17 and IL-22. These anti-inflammatory properties of VSL#3 seemed to play a role in the limitation of intestinal and extra-intestinal sequelae of *C. jejuni* infection *in vivo*. For instance, prophylactic as well as therapeutic VSL#3 treatment of *C. jejuni* infected mice resulted in decreased epithelial apoptosis and attenuated influx of immune cells (T- and B lymphocytes, regulatory T cells and Macrophages/Monocytes) into the colonic mucosa. In

prophylactically treated mice, higher levels of colonic IL-10, lower levels of colonic MCP-1 and IL-6, as well as lower levels of splenic TNF could be observed.
Taken together, both FMT and VSL#3 recolonization display extensive impacts on mucosal, peripheral and systemic immunity, while VSL#3 induces mainly anti-inflammatory immune responses. This latter property might open up for valuable therapeutic strategies directed against inflammatory and infectious diseases, including *C. jejuni* infections and its sequelae.

1.2 Einführung

1.2.1 Die Darmmikrobiota und ihre Rolle für die Physiologie und Pathologie des Wirtes

Der menschliche Gastrointestinaltrakt (GIT) beherbergt eine außerordentlich große Anzahl von Mikroorganismen (Bakterien, Viren, Pilzen, Würmer und Protozen), die in ihrer Gesamtheit als kommensale Mikrobiota bekannt sind. Den größten Anteil der Darmmikrobiota machen jedoch Bakterien aus (1), wobei die Gesamtbakterienlast von proximal nach distal zunimmt (2). Neure Schätzungen legen nahe, dass sich ca. 10^{13} Bakterien im GIT befinden, womit das Verhältnis zwischen menschlichen Zellen und Bakterien 1:1 beträgt (3). Zahlreiche Faktoren, u.a. Genetik (4), Geburtsvorgang (5), Alter (6), Ernährungsgewohnheiten (7), Medikamente (v.a. Antibiotika) (8), Hospitalisierung (v.a. im frühen Lebensalter) (9), sowie Exposition gegenüber pathogenen Bakterien (10) nehmen Einfluss auf die Zusammensetzung der Mikrobiota. Unter homöostatischen Bedingungen ist die Mikrobiota-Wirts-Interaktion durch Mutualismus geprägt. Einerseits stellt der Wirt eine physiologische, nährstoffreiche Nische für die Mikrobiota dar, andererseits ist diese an zahlreichen, für den Wirt wichtigen physiologischen Prozessen, einschließlich Vitaminsynthese (11), Verdauung von Nahrungsbestandteilen (12), intestinaler Angiogenese (13) und Schutz vor pathogenen Bakterien (14, 15), beteiligt. Von entscheidender Bedeutung ist außerdem der Einfluss der Mikrobiota auf die Entwicklung, Differenzierung und Regulation des Immunsystems (16-18). Dies wurde zunächst durch Studien an keimfreien Mäusen, die in Isolatoren unter strikt keimfreien Bedingungen generiert und gehalten wurden, festgestellt. In diesem Mausmodell wurden zahlreiche Beeinträchtigungen des Immunsystems beobachtet, wie z.B. eine geringere Anzahl von IgA produzierenden Plasmazellen im Epithel (19) und CD4+ T-Lymphozyten in der Lamina propria (LP) (20) des Kolons, hypoplastische Keimzentren in den mesenterialen Lymphknoten (mesenteric lymph nodes; MLN), und schwach ausgebildete B- und T-Zell-Zonen in der Milz (18, 21). Darüber hinaus gibt es Belege bezüglich des Potenzials verschiedener Bakterienstämme, spezifische Subpopulationen des Immunsystems zu induzieren. So wurde im Mausmodell gezeigt, dass segmentierte filamentöse Bakterien die Entwicklung von IL-17-produzierenden T-Helfer

(Th-)17-Zellen verstärken (22), während definierte *Clostridium*-Arten die Akkumulation von regulatorischen T-Zellen (Treg) in der LP des Kolons fördern (23).

1.2.2 Antibiotika als Störfaktor des intestinalen Ökosystems und deren Folgen für den Wirt

Antibiotische Substanzen haben die Medizin revolutioniert und einen enormen Beitrag zur Verlängerung der Lebenserwartung des Menschen geleistet. Jedoch stellen sie einen wichtigen Störfaktor des Gleichgewichts des mikrobiellen Ökosystems im GIT dar, und verursachen eine sogenannte Dysbiose. In letzter Zeit wurde deshalb den schädlichen Folgen des Antibiotikagebrauchs zunehmende Beachtung geschenkt. So können antibiotische Substanzen die Anfälligkeit des Organismus für pathogene Bakterien, u.a. *Salmonella enterica* (14), Vancomycin-resistente *Enterokokken* und *Clostridium difficile* (25), erhöhen. Eine kurzzeitige Therapie mit Clindamycin ist ausreichend, um die Diversität der intestinalen Mikrobiota nachhaltig zu reduzieren und folglich behandelte Mäuse anfälliger für eine *C. difficile*-Infektion zu machen (8). Die antibiotische Therapie und die mit ihr einhergehende Dysbiose wurden außerdem mit zahlreichen intra- und extraintestinalen Pathologien in Verbindung gebracht (26), darunter chronisch-entzündlichen Darmerkrankungen (CED, (27)), Reizdarmsyndrom (28), Zöliakie (29), Typ 1 Diabetes mellitus (30), Arthritis (31), Allergien (32) und Asthma bronchiale (33). Weitere Ergebnisse weisen auf die immunmodulierenden Eigenschaften der Antibiotika hin. So wiesen in einer Studie Mäuse, die von Geburt an mit Vancomycin oder Colistin behandelt worden waren, eine geringere Anzahl von Lymphfollikeln im Dünn- und Dickdarm auf (34). Die Behandlung mit Vancomycin führte außerdem zu einer reduzierten Anzahl von Treg im murinen Kolon (23), während die Eradikation der Darmflora mittels Breitband-Antibiose eine Reduktion der Treg-Population in den MLN und Peyer'schen Platten (PP) zur Folge hatte (35). Ob die beobachteten Effekte die Konsequenz der veränderten Zusammensetzung der intestinalen Mikrobiota oder aber auf substanzspezifische Eigenschaften zurückzuführen waren, ist jedoch nicht abschließend geklärt.

1.2.3 Probiotika als neuartige, immunomodulierende Therapiestrategie in intestinalen Entzüdungsmodellen

Laut Weltgesundheitsorganisation sind Probiotika als lebende Mikroorganismen die, in ausreichender Menge aufgenommen, einen gesundheitsfördernden Einfluss auf den Wirtsorganismus haben, definiert (36). Ziel ihrer Anwendung ist die Verschiebung und Modulation der Zusammensetzung der Mikrobiota in Richtung „nutzbringender" Spezies. Intensiv wurde die Rolle der Probiotika besonders bei Darmentzündung erforscht. Beispielsweise konnte

Escherichia coli Nissle 1917 die mukosale Barrierefunktion in Mäusen verbessern (37) und eine akute wie chronische Kolitis verhindern (38). Die Behandlung von IL-10-gendefizienten Mäusen mit *Lactobacillus plantarium* führte zu einer reduzierten mukosalen Produktion von IL-12p40 und IFN-γ und somit einem milderen Verlauf der Entzündung im Dickdarm (39). Auch das probiotische Gemisch VSL#3 (bestehend aus *Streptococcus thermophilus, Bifidobacterium breve, B. longum, B. infantis, Lactobacillus acidophilus, L. plantarum, L. paracasei,* und *L. delbrueckii subsp. Bulgaricus*) zeigte sich bei der Therapie sowohl der murinen IL-$10^{-/-}$ Kolitis (40) als auch der Trinitrobenzolsulfonsäure (TNBS) induzierten Kolitis (41) als effektiv. Positive Auswirkungen einer probiotischen Therapie konnten auch in klinischen Studien an CED-Patienten gezeigt werden. Bemerkenswerterweise sind *E. coli* Nissle 1917 und Mesalazin z.B. gleichwertige Therapiestrategien bei der Remissionserhaltung nach einem Colitis ulcerosa-Schub (42). Laut einer Metaanalyse ist auch VSL#3 in der Lage, eine Remission eines Colitis ulcerosa-Schubes zu induzieren (43). Hingegen konnten die Probiotika keinen positiven Effekt bei der Therapie von Patienten mit Morbus Crohn entfalten (44). Als Erklärung der Wirkungsweise der Probiotika sind verschiedene Mechanismen vorgeschlagen worden, u.a. die Verstärkung der intestinalen Barrierefunktion (37) und der Kolonisationsresistenz (45), die Erhöhung der Diversität der Darmmikrobiota, sowie die Modulation des angeborenen und adaptiven Immunsystems (46). Denkbar ist auch ein synergistischer Effekt mehrerer Mechanismen.

1.2.4 Das murine *Campylobacter jejuni* Infektionsmodell und das therapeutische Potenzial probiotischer Interventionen

Die Inzidenzraten von *Campylobacter jejuni*-Infektionen weisen weltweit eine zunehmende Häufigkeit auf (47, 48). Der Mensch infiziert sich mit dem gram-negativen Pathogen hauptsächlich über die Nahrungskette und leidet daraufhin an einer Durchfallerkrankung unterschiedlichen Schweregrades (15, 49). In den meisten Fällen verläuft die Erkrankung selbstlimitierend, es sind aber auch protrahierte Verläufe mit postinfektiösen Spätschäden wie Guillain-Barré-Syndrom und reaktiver Arthritis beschrieben worden (50, 51). Für lange Zeit war die Entwicklung muriner Modelle zur Untersuchung der Mechanismen von *C. jejuni*-Infektionen dadurch erschwert, dass Mäuse dank der Zusammensetzung ihrer intestinalen Mikrobiota eine ausgeprägte physiologische Kolonisationsresistenz aufweisen. In früheren Arbeiten unserer Arbeitsgruppe konnte gezeigt werden, dass die Modifizierung der Darmmikrobiota eine Infektion mit *C. jejuni* begünstigt (15, 52). Nach kompletter Eradikation der Mikrobiota mittels Breitband-Antibiose konnte sich das Pathogen im murinen GIT ansiedeln und dort eine inflammatorische Immunantwort auslösen (15). Somit scheint das sekundär abiotische Mausmodell für die Erforschung der Interaktion zwischen

dem Pathogen, den kommensalen Bakterien und dem Immunsystem des Wirts geeignet zu sein. Probiotika bergen das Potenzial, eine für den Wirt „nutzbringende“ Modifizierung der Mikrobiota-Zusammensetzung herbeizuführen. Ergebnisse aus *in vitro* und *in vivo* Studien zeigten indessen die Wirksamkeit der Probiotika bei der Prophylaxe und Therapie von Infektionen mit enteropathogenen Bakterien. Zahlreiche probiotische Stämme wie *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. gasseri*, können beispielsweise das Wachstum, die Adhäsion und den Stoffwechsel enteropathogener Keime verhindern (53-56). Auch klinische Studien bestätigen entsprechend positive Effekte. So konnte durch die Anwendung von VSL#3 (57) oder *Saccharomyces boulardii* (58) die Inzidenz der Antibiotika-assoziierten Durchfallerkrankung signifikant erniedrigt, und die Dauer von infektiösen Durchfallepisoden kann durch die Anwendung von Probiotika sowohl bei Kindern als auch bei Erwachsenen reduziert werden (59). In einer Metaanalyse, die 84 klinische Studien mit über 10.000 Patienten einschloss, wurde die Wirksamkeit von Probiotika in der Therapie der Antibiotika-assoziierten Durchfallerkrankung, *C. difficile* induzierten Gastroenteritis, Pouchitis und des Reizdarmsyndroms bestätigt (60). Die Rolle der Probiotika für die Prophylaxe und/oder Therapie von *C. jejuni* ist noch nicht abschließend geklärt, stellt aber, aufgrund der Rolle der intestinalen Mikrobiota für die Entstehung von Kolonisationsresistenz und Suszeptibilität, einen sehr attraktiven Lösungsansatz dar.

1.3 Zielsetzung

Das Ziel der vorliegenden Arbeit war es, das Wechselspiel zwischen der Darmmikrobiota, dem Immunsystem des Wirtes und pathogenen Darmbakterien (am Beispiel von *C. jejuni*) näher zu erforschen. Zunächst untersuchten wir die Auswirkungen der Eradikation der Darmmikrobiota mittels Fünffach-Antibiose sowie der anschließenden fäkalen Mikrobiota-Transplantation (FMT) auf die mukosale und systemische Immunität in konventionellen Mäusen. Anschließend wurde überprüft, ob auch das Probiotikum VSL#3 immunomodulierende Eigenschaften nach antibiotischer Therapie entfalten kann und ob diese Eigenschaften eine Rolle bei der Prophylaxe und Therapie einer murinen *C. jejuni* Infektion spielen. Diese Arbeit stellt Ausschnitte aus drei bereits veröffentlichten Artikeln dar und soll einen Überblick über die bisher erlangten Erkenntnisse schaffen.

1.4 Methodik

1.4.1 Ethische Erklärung

Sämtliche Tierversuche wurden im Einklang mit dem europäischen Tierschutzgesetz durchgeführt (2010/63/EU) und durch das Landesamt für Gesundheit und Soziales (LaGeSo, Berlin) genehmigt (Registrierungsnummer: G0184/12 and G0097/12). Der klinische Gesundheitszustand der Tiere wurde täglich überprüft.

1.4.2 Versuchstiere und Tierhaltung

Alle Tiere wurden in den Räumlichkeiten der Forschungseinrichtung für Experimentelle Medizin der Charité Berlin gezüchtet und unter spezifisch-pathogenfreien (SPF) Bedingungen gehalten. Für die vorliegende Arbeit wurden weibliche, C57BL/6j Wildtyp Mäuse verwendet. Zur Eradikation der Darmmikrobiota wurden 8-10 Wochen alte Mäuse in sterile Käfige transferiert, und mit einem Antibiotika-Cocktail, wie früher beschrieben, behandelt (61). Bei Abwesenheit kultivierbarer Bakterien in Faecesproben (bei Bebrüten der jeweiligen Proben in Thioglykolat-Bouillon (Oxoid, Deutschland) bei 37°C) für drei aufeinander folgende Wochen, wurden die Tiere als sekundär abiotisch (gnotobiotisch; ABx) angesehen.

1.4.3 Bakterielle Rekolonisierung und *Campylobacter jejuni* Infektion sekundär abiotischer Mäuse

Drei Tage vor Rekolonisierung und Infektion wurde die Anbtibiotikalösung mit autoklaviertem Leitungswasser ersetzt. Für die Durchführung der FMT, wurden Faecesproben von 10 gesunden SPF Kontrollmäusen gesammelt, gepoolt und in 10 ml steriler phosphat-gepufferter Salzlösung (phosphate buffered saline, PBS, Gibco, Life Technologies, GB) gelöst. Die zu rekolonisierenden Mäuse erhielten 0.3 ml dieser Suspension peroral mittels Gavage.

Für die Rekolonisierung mit dem probiotischen Gemisch VSL#3 wurde dieses kommerziell erworben (SIIT S.r.l. Trezzano sul Naviglio, Italien). 4.5×10^{11} Baktieren wurden in 50 ml PBS gelöst, und 0.3 ml dieser Lösung wurde den Mäusen per Gavage appliziert. Via derselben Route erfolgte auch die Infektion mit 10^9 Kolonie-bildenden Einheiten (KBE) *C. jejuni* Stamm 81-176 in 0.3 ml PBS. Bei den Ko-Kolonisierungsexperimenten erfolgte die Gabe von VSL#3 fünf Tage vor (prophylaktische Gabe) oder nach (therapeutische Gabe) der *C. jejuni* Infektion.

1.4.4 Probengewinnung

Die Mäuse wurden zu definierten Zeitpunkten mittels Isofluran (Abott, Deutschland) Inhalation getötet. Proben des luminalen Inhaltes von Ileum und Kolon sowie Gewebeproben aus Milz, MLN,

Ileum und Kolon wurden steril entnommen. *Ex vivo* Biopsien von Ileum und Kolon wurden parallel für mikrobiologische, immunologische und immunhistologische Analysen aufbereitet.

1.4.5 Quantitative Analyse der Rekolonisierungs- und Infektionseffizienz

Zur Bestimmung der Zusammensetzung und Menge der intestinalen lebenden Bakterien wurden zu definierten Zeitpunkten Faecesproben sowie zum Zeitpunkt der Sektion Proben des luminalen Inhalts von Ileum und Kolon entnommen und mittels kultureller und molekularer Methoden untersucht. Die Zusammensetzung der intestinalen Flora nach FMT wurde mittels quantitativer 16S rRNA basierter Realtime-PCR, wie früher beschrieben (62, 63), ermittelt. Die Rekolonisierungseffizienz von VSL#3 wurde sowohl kulturell als auch molekular überprüft. Die kulturellen Analysen erfolgten mittels Homogenisierung der Faecesproben in PBS sowie Bebrütung auf entsprechenden Agar-Nährmedien (15, 61, 64).

1.4.6 Immunhistochemie

Für die immunhistochemischen Untersuchungen wurden die *ex vivo* Biopsate des Darms in 5%-igem Formalin fixiert, in Paraffin eingebettet und in 5 µm dünne Schichten geschnitten, und mit spezifischen Antikörpern gefärbt (15, 64, 65). Für jedes Tier wurde die durchschnittliche Anzahl der jeweiligen Zellen *in situ* ermittelt, indem die positiv gefärbten Zellen in jeweils sechs hochauflösenden Feldern („High Power Fields“, HPF; 0,287 mm2; 400-fache Vergrößerung) von einem geblindetem Untersucher gezählt wurden. Verwendet wurden Antikörper gegen Caspase-3 für apoptotische Zellen (Asp175, Cell Signaling, USA, 1:200), Ki67 für proliferierende Zellen (TEC3, Dako, Dänemark, 1:100), CD3 für T-Lymphozyten (#N1580, Dako, 1:10) FOXP3 für regulatorische T-Zellen (FJK-16s, eBioscience, USA, 1:100), B220 für B-Lymphozyten (eBioscience,1:200) und F4/80 für Monozyten/Makrophagen (#14-1801, clone BM8, eBioscience, 1:50).

1.4.7 Lymphozytenisolierung

Milz- und MLN-Proben wurden vorsichtig mit einem Spitzenstempel durch ein 100 µm Nylonsieb gedrückt und mit PBS/ 0.5% Rinderserumalbumin (Bovine Serum Albumin, BSA, Sigma-Aldrich, USA) gespült. Bei den Milzproben wurde zusätzlich eine Erythrozytenlyse mit 1.66%-igem Ammoniumchlorid (Carl Roth, Deutschland) durchgeführt. Alle Proben wurden in definierten Volumina resuspendiert und entsprechend weiter verarbeitet (35).

Die jeweiligen Segmente des murinen Darms wurden entnommen, vom Fett- und Bindegewebe, sowie den PP befreit, longitudinal aufgeteilt und mit eiskaltem PBS gespült, um den luminalen

Inhalt und den Schleim zu entfernen. Die weitere Aufbereitung zur Isolierung der Lamina propria Lymphozyten (LPL) des Ileums und Kolons erfolgte nach einem Standardprotokoll (66). Die Verdauungslösung enthielt 0,5 mg/ml Kollagenase A (Roche, Deutschland), 0.5 mg/ml DNAse I (Roche), 10% FKS und jeweils 1 mM CaCl2 und MgCl2 (beide Carl Roth) in RPMI 1640. Anschließend wurden die Zellen gewaschen, in 5ml 44%-igem Percoll (GE Healthcare, Schweden) resuspendiert und auf 5 ml 67%-igem Percoll überschichtet. Die Separation mittels Percoll-Gradient erfolgte durch Zentrifugation bei 600g und Raumtemperatur für 20 min. Die LPL wurden aus der Interphase des Percoll-Gradienten akquiriert, gewaschen, und in PBS/0.5% BSA resuspendiert.

1.4.8 Oberflächen- und intrazelluläre Färbungen und Durchflusszytometrie

Oberflächenfärbungen wurden mit den folgenden Antikörpern durchgeführt: FITC-anti-CD4 (Klon RM4-5; 1:200), PerCP-anti-CD8 (Klon 53-6.7; 1:100), PacBlue-anti-B220 (Klon RA3-6B2, 1:200), APC-Cy7-anti-CD25 (Klon PC61, 1:200), PE-anti-CD44 (Klon IM7, 1:200), APC-anti-CD86 (Klon B7-2, 1:200) (alle von BD Biosciences, USA). Für die intrazellulären Färbungen wurden die Zellen für 5 Stunden mit 10 ng/ml Phorbol-12-myristat-13-acetat (PMA) und 1 µg/ml Ionomyzin (beide von Sigma-Aldrich) in einem Brutschrank restimuliert. Eine Stunde nach polyklonaler Restimulation wurde der Sekretionshemmer Brefeldin A (10 µg/ml, Sigma-Aldrich) zugegeben. Anschließend wurden tote Zellen mit Hilfe des LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (life technologies) markiert und für 20 Minuten bei Raumtemperatur in 2% Paraformalaldehyd (PFA; Sigma -Aldrich) fixiert. Die Zytokinfärbung erfolgte in 0.5%-iger Saponinlösung (zur Permeabilisierung der Zellmembran) und folgenden Antikörpern: PacBlue-Anti-CD4 (Klon RM4-5; 1:400), PE-Cy7–anti–IFN-γ (Klon XMG 1.2; 1:400), (beide von BD Biosciences) FITC-anti-IL17A (Klon TC11-18H10.1; 1:200, BioLegend, USA), PE-anti-IL10 (Klon JESS-16E3; 1:100), APC-anti-IL22 (Klon IL22JOP; 1:100) (beide von eBioscience). Die Daten der Duchflusszytometrie wurden an einem MACSQuant Analyzer (Miltenyi Biotec, Deutschland) erhoben und mit FlowJo Software v10.1 (Tree star, USA) analysiert.

1.4.9 Real-Time PCR

Die Höhe der Expression pro- und anti-inflammatorischer Zytokine wie IL-22, IL-17A, IFN-γ und IL-10 mRNA wurde in schockgefrorenen Biopsien aus Ileum und Kolon mittels Light Cycler Data Analysis Software (Roche) bestimmt (67). Die mRNA des Hypoxanthin-Guanin-Phosphoribosyl-Transferase (HPRT) „Housekeeping“ Gens wurde als Referenz benutzt und die Daten in arbiträren Einheiten (arbitrary units, fold expression) angegeben.

1.4.10 Zytokinmessungen in Überständen von *ex vivo* Biopsien aus Kolon und extra-intestinalen Organen

Kolongewebe wurde längs geschnitten und in sterilem PBS ausgewaschen. MLN und Milz- sowie ca. 1 cm^2 große Kolonbiopsate wurden in 24-well-Platten (Nunc, Deutschland) in jeweils 500µl serumfreiem RPMI-1640 Medium mit Penicillin (100 U/ml) und Streptomycin (100 µg/ml, PAA Laboratories, Österreich) für 18 Stunden bei 37°C kultiviert. Im Anschluss wurden die Zytokine MCP-1, TNF, IL-6, IL-12p70 und IL-10 in den Überständen mittels „Inflammation Cytometric Bead Assays“ (CBA; BD Biosciences) und FACS Canto II (BD Biosciences) quantifiziert.

1.4.11 Statistische Analysen

Graphische Darstellungen wurden mit dem Programm GraphPad Prism v6.0 (GraphPad Software, USA) erstellt. Die Analysen der LightCycler- Ergebnisse wurden mit Hilfe des Programms Relquant 1.0 (Roche Molecular Biochemicals, Schweiz) durchgeführt. Mittelwerte, Mediane, Standartabweichungen (SD) und signifikante Unterschiede wurden mit dem Mann-Whitney-U-Test bzw. one-way Analyse der Varianz (ANNOVA) mit Turkey’s *post-hoc* Korrektur Test für multiple Vergleiche berechnet. Wahrscheinlichkeitswerte (p-Werte) $\leq 0{,}05$ wurden als statistisch signifikant erachtet.

1.5 Ergebnisse

In diesem Teil werden Auszüge der Ergebnisse der drei hier zusammengefassten Publikationen, in ihrer Reihenfolge (Publikation 1, 2, und 3) präsentiert. Keine der in unseren Untersuchungen gewählten Interventionen (antibiotische Behandlung, FMT, VSL#3 Rekolonisierung, oder *C. jejuni* Infektion) führte zur klinischen Beeinträchtigung der Mäuse (wie z.B. Durchfall, Gewichtsverlust, Blut im Stuhl). Im Folgenden werden die wichtigsten immunologischen Konsequenzen einer Breitband-Antibiose sowie der jeweiligen Rekolonisierungen aufgezeigt. Auf die jeweilige graphische Darstellung der Ergebnisse wird entsprechend auf die Abbildungsnummer (Abb.) der Originalpublikation hingewiesen.

1.5.1 Eradikation und Wiederherstellung der kommensalen Darmmikrobiota nach Breitband-Antibiose und fäkaler Mikrobiota-Transplantation

Um die Auswirkung der antibiotischen Behandlung auf die kommensalen Darmbakterien zu untersuchen und deren Eradikation zu bestätigen, führten wir kulturelle sowie kulturunabhängige

molekulare Analysen der Faecesproben behandelter Mäuse durch. Ab der dritten Woche der Behandlung konnten bei diesen Mäusen keine aeroben, anaeroben und mikroaerophilen Bakterien kultiviert werden. Darüber hinaus führten wir quantitative 16S rRNA Analysen der wichtigsten Bakteriengruppen des murinen Gastrointestinaltraktes (i.e. Enterobakterien, Enterokokken, Lactobacillen, Bifidobakterien, „*Bacteroides/Prevotella*"-Gruppe, „*Clostridium coccoides*" und „*Clostridium leptum*" Untergruppen) in konventionell besiedelten und sekundär abiotischen (ABx) Mäusen durch und verglichen diese zu autoklaviertem Futter (**Publikation 1, Abb. 1**). Wir konnten feststellen, dass bei ABx Mäusen eine signifikante Reduktion der Genkopienzahlen pro ng DNA für sämtliche untersuchten Bakteriengruppen vorlag. Bemerkenswerterweise waren die Genkopienzahlen der ABx Mäuse vergleichbar zu denen im autoklavierten Futter. Somit konnte von einer relevanten und ausreichenden Eradikation der intestinalen Mikrobiota ausgegangen werden. Hingegen konnte die vergleichbare Zusammensetzung der Floren von SPF Mäusen und Mäusen am Tag 7 (d7) und d28 nach FMT den Erfolg der bakteriellen Rekolonisierung bestätigen (**Publikation 1, Abb. S1**).

1.5.2 Auswirkungen der antibiotischen Therapie und fäkalen Mikrobiota-Transplantation auf das angeborene und adaptive Immunsystem im murinen Dünn- und Dickdarm

Um die immunologischen Konsequenzen der jeweiligen Interventionen zu analysieren führten wir zunächst quantitative immunhistologische Analysen definierter Zellpopulationen des angeborenen und erworbenen Immunsystems im Ileum und Kolon *in situ* durch (**Publikation 1, Abb. 3**). Wir konnten feststellen, dass eine antibiotisch erzielte Eradikation der kommensalen Darmbakterien zu einer deutlichen Reduktion der CD3+ T- und B220+ B-Lymphozyten, der FOXP3+ Treg sowie der F4/80+ Monozyten/Makrophagen in beiden intestinalen Kompartimenten führte ($p<0.01$-0.001; **Publikation 1, Abb. 3 A-H**). Diese Zellpopulationen konnten im Kolon bereits am d7 nach FMT wiederhergestellt werden, und am d28 nach Rekolonisierung waren die Zellzahlen der jeweiligen Populationen weitgehend vergleichbar zu denen konventioneller Mäuse sowohl im Dünn- als auch im Dickdarm.

1.5.3 Auszüge der durchflusszytometrischen Untersuchungsergebnisse zum Einfluss der antibiotischen Behandlung und fäkalen Mikrobiota-Transplantation auf intestinale, periphere und systemische Immunzellpopulationen

Um die Auswirkungen der Eradikation und anschließenden Rekonstitution der intestinalen Bakterien auf das murine Immunsystem detaillierter zu untersuchen, wurden Lymphozyten aus der LP des Dünn- und Dickdarms, der MLN und der Milz isoliert und durchflusszytometrisch

bezüglich ihrer Oberflächenmarker sowie pro- und anti-inflammatorischer Zytokine untersucht. Sowohl der Anteil als auch die absolute Zellzahl der CD4+ Zellen in der Schleimhaut des Dünn- und Dickdarms waren nach Fünffach-Antibiose signifikant reduziert. Bereits am d7 nach FMT konnte jedoch deren Wiederherstellung erreicht werden, wobei die CD4+ Zellzahl am d28 erneut abfiel ($p<0.01$-0.001; **Publikation 1, Abb. 4 A-D**). Ähnlich führte die Eradikation der Mikrobiota zu einer Reduktion der intestinalen CD8+ Zellen ($p<0.01$-0.001; **Publikation 1, Abb. 5 A-D**). Im Ileum konnten bereits am d7 nach FMT die naiven Ausgangsbedingungen erreicht werden, wohingegen im Kolon der Anteil der CD8+ Zellen sich erst am d28 normalisieren konnte und die absolute Zellzahl nach FMT erniedrigt blieb ($p<0.05$-0.001). Interessanterweise waren die Zahl der CD4+ Zellen sowie der Anteil und die Anzahl der CD8+ Zellen in der Milz von ABx Mäusen höher als die jeweiligen Parameter der konventionellen SPF Mäuse ($p<0.01$; **Publikation 1, Abb. 4H,** $p<0.001$; **Abb. 5 G-H**). Des Weiteren untersuchten wir die CD4+ Zellen der jeweiligen Kompartimente auf die Produktion pro- und anti-inflammatorischer Zytokine nach Antibiose und FMT hin. Die Eradikation der Mikrobiota führte zu einer starken Reduktion von IFN-γ produzierenden CD4+ Zellen im Dünn- und Dickdarm ($p<0.001$; **Publikation 1, Abb. 7A-B**), IL17+CD4+ und IL22+CD4+ Zellen ($p<0.01$-0.001; **Publikation 1, Abb. 8**), und IL10+ CD4+ Zellen ($p<0.001$; **Publikation 1, Abb. 9**) in allen untersuchten lymphatischen Kompartimenten. Eine Wiedereinführung der bakteriellen Flora mittels FMT regte die Zytokinexpression der CD4+ Zellen an. Am d7 nach FMT konnte teilweise eine „Überstimulation“ des Immunsystems beobachtet werden, wodurch die Zytokinexpression der CD4+ Zellen nach FMT noch höher als in konventionell besiedelten Tieren war. So wiesen z.B. Mäuse am d7 nach FMT einen höheren Anteil an IL-22 produzierenden CD4+ Zellen im Ileum und Kolon ($p<0.05$-0.001 vs. SPF; **Publikation 1, Abb. 8B, D**), IL-17 produzierenden CD4+ Zellen in MLN und Milz ($p<0.001$ vs. SPF; **Publikation 1, Abb. 8E, G**) und IL-10 produzierenden CD4+ Zellen in Dünndarm und MLN ($p<0.01$-0.001 vs. SPF; **Publikation 1, Abb. 9 A, C**). Am d28 nach FMT konnten, mit Ausnahme von der IFN-γ Produktion im Dünndarm, weitgehend die jeweiligen basalen Zytokinniveaus naiver Mäuse erreicht werden. Zusammenfassend konnten wir zeigen, dass eine antibiotisch erreichte Eradikation der intestinalen Darmbakterien weitreichende Folgen für das lokale und systemische murine Immunsystem hat und eine orale Applikation von Darmbakterien nach Beendigung der Therapie, eine effektive Therapiemöglichkeit für die Normalisierung der jeweiligen Immunzellpopulationen darstellt.

1.5.4 Auszüge der Untersuchungsergebnisse zu den immunomodulierenden Eigenschaften des Probiotikums VSL#3

Anschließend sind wir der Frage nachgegangen, ob die Gabe des Probiotikums VSL#3 genauso effektiv wie die FMT hinsichtlich der Wiederherstellung der Immunzellpopulationen der lymphatischen Organe nach antibiotischer Therapie sei. Kulturelle Analysen der Faecesproben nach peroraler Applikation von VSL#3 ergaben, dass sich die probiotischen Bakterienstämme ab d3 stabil im murinen Gastrointestinaltrakt ansiedeln konnten (**Publikation 2, Abbildung 1A**). Keine der in dieser Studie gewählten Interventionen (antibiotische Therapie, VSL#3 Rekolonisierung, FMT) führte zu Veränderungen der Casp3+ apoptotischen Darmepithelzellen (n.s. vs. naiven Kontrollen; **Publikation 2, Abb. 2A**), wohingegen die epitheliale Proliferationsrate in ABx Mäusen, beurteilt anhand der Expression des Proliferationsmarkers Ki67 (68), stark reduziert war. Darüber hinaus konnte eine komplette Wiederherstellung der proliferierenden Zellen sowohl nach Rekolonisierung mit VSL#3 Bakterien als auch mit komplexer muriner Mikrobiota erreicht werden ($p<0.001$; **Publikation 2, Abb. 2B**). Die durchflusszytometrischen Untersuchungen der lymphatischen Organe zeigten, dass mit VSL#3 besiedelte Mäuse höhere Zahlen von CD4+ Lymphozyten in der LP des Dünn- und Dickdarms als ABx Mäuse aufwiesen ($p<0.05$-0.001 vs ABx; **Publikation 2, Abb. 4 B, D**). Außerdem stieg nach antibiotischer Therapie die Anzahl der CD4+ Lymphozyten in der Milz, unabhängig von der anschließenden Rekolonisierung ($p<0.05$; **Publikation 2, Abb 4H**) an. Weiterhin konnte mittels VSL#3 Rekolonisierung eine verstärkte Expression des Treg-Oberflächenmarkers CD25 in allen untersuchten Organen erreicht werden ($p<0.01$-0.001 vs ABx; **Publikation 2, Abb. 7 A, C, E, G**). Diese beobachtete Steigerung nach VSL#3 Rekolonisierung war genauso stark ausgeprägt wie der Anstieg dieser Zellpopulation in Kolon, MLN und Milz nach FMT.

Anschließend untersuchten wir die Auswirkung der VSL#3 Rekolonisierung im Vergleich zur FMT auf die Zytokinproduktion von CD4+ Lymphozyten in der LP des Dünn- und Dickdarms, MLN und Milz nach antibiotischer Therapie. Mäuse, die mit VSL#3 behandelt wurden, zeigten ähnlich niedrige Anteile an IFN-γ produzierenden CD4+ Zellen im Dünn- und Dickdarm (n.s. vs. ABx; **Publikation 2, Abb. 9 A, C**), sowie IL17- bzw. IL22 produzierenden CD4+ Zellen in allen untersuchten immunologischen Kompartimenten, im Vergleich zu sekundär abiotischen Mäusen ohne anschließende Rekolonisierung (n.s. vs. ABx; **Publikation 2, Abb. 10 A-H**). Hingegen stellte sich VSL#3 als ein starker Stimulator des anti-inflammatorischen Zytokins IL-10 dar. In der LP des Dünndarms war die IL-10 Expression der CD4+ Lymphozyten nach VSL#3-Gabe sogar noch höher als in Mäusen, die mit komplexer muriner Flora rekolonisiert wurden ($p<0.001$; **Publikation 2, Abb. 9B**). In allen anderen Kompartimenten waren VSL#3 und FMT

gleichermaßen effektiv bezüglich der Wiederherstellung der CD4+IL10+ Subpopulation, die bei Abwesenheit von intestinaler antigener Stimulation in sekundär abiotischen Mäusen stark supprimiert war ($p<0.05$-0.001; **Publikation 2, Abb. 9 D, F, H**). Diese Ergebnisse wurden außerdem mittels mRNA Analysen von aus Dünn- und Dickdarm entnommenen Biopsien unterstützt (**Publikation 2, Abb. 11**). Die Genexpressionsraten für IL-17, IL-22 und IFN-γ blieben nach VSL#3 Rekolonisierung ähnlich niedrig wie in ABx Mäusen, wohingegen eine starke Erhöhung der IL-10 mRNA im Kolon der mit VSL#3 besiedelten Mäuse beobachtet werden konnte. Eine Tendenz zur Erhöhung von IL-10 mRNA durch VSL#3 konnte auch im Ileum beobachtet werden (nicht signifikant). Zusammenfassend konnte in dieser Publikation gezeigt werden, dass das Probiotikum VSL#3 die Folgen der Eradikation von Darmbakterien nicht nur lokal, sondern auch systemisch antagonisieren kann, wobei durch dieses Probiotikum v.a. anti-inflammatorische Immunantworten angeregt werden.

1.5.5 Auszüge der Untersuchungsergebnisse zur Rolle des Probiotikums VSL#3 im murinen *Campylobacter jejuni* Infektionsmodell

Als Nächstes untersuchten wir, ob die beschriebenen anti-inflammatorischen Eigenschaften von VSL#3 auch im murinen *C. jejuni* Infektionsmodell zur Geltung kommen. Es stellte sich hierbei zunächst die Frage, ob eine prophylaktische und/oder therapeutische Gabe von VSL#3 nach antibiotischer Eradikation der intestinalen Mikrobiota und Infektion mit *C. jejuni* zu einer Reduktion der Pathogenlasten im Darm führt. Darüber hinaus untersuchten wir, ob durch die Gabe von VSL#3 die infektionsbedingten pro-inflammatorischen Immunantworten herunter- und/oder die anti-inflammtorischen Immunantworten hochreguliert werden. Kulturelle Florenanalysen von Faecesproben zu verschiedenen Zeitpunkten zeigten, dass sowohl VSL#3 als auch *C. jejuni* sich stabil im murinen GIT ansiedeln konnten. Die bakteriellen Lasten der jeweiligen Bakterienpopulationen waren stabil und nicht durch die Ko-Kolonisierung beeinflussbar. Weder die prophylaktische noch die therapeutische Gabe von VSL#3 konnte zu einer Reduktion des Pathogens im Darm führen (**Publikation 3, Abb. 1 und 2**). Weil die Campylobakteriose mit einer Erhöhung apoptotischer Darmepithezellen vergesellschaftet ist (15), untersuchten wir die Anzahl Caspase 3 positiver Zellen mittels *in situ* Immunhistochemie im Kolon. Eine *C. jejuni* Monoinfektion führte zu einer deutlichen Erhöhung apoptotischer Zellen im Vergleich zu naiven und mit VSL#3 besiedelten Mäusen ($p<0.001$; **Publikation 3, Abb. 3A**). Sowohl eine prophylaktische als auch therapeutische VSL#3 Ko-Kolonisierung der mit *C. jejuni* infizierten Tiere konnte jedoch, diese *C. jejuni* induzierte Apoptose abmildern, und führte zu einem Abfall apoptotischer Zellen um ca. 50% ($p<0.005$-0.001; **Publikation 3, Abb. 3A**). Weil die

Zellproliferation des Epithels einen unabdingbaren Prozess für die Regeneration desselben darstellt, untersuchten wir auch die Anzahl der Ki67 positiven Zellen des Dickdarmepithels. Alle behandelten Mäuse (VSL#3 und *C. jejuni* Mono- sowie Ko-Kolonisierung) wiesen hierbei mehr Ki67+ proliferierende Zellen als naive Kontrollmäuse ($p<0.001$; **Publikation 3, Abb. 3B**).
Ein weiteres typisches Merkmal *C. jejuni* bedingter intestinaler Entzündung ist die Rekrutierung pro-inflammatorischer Immunzellen in die entzündete Mukosa und LP des Darms (15). Durch *in situ* immunhistochemische Untersuchungen von Dickdarm-Biopsaten stellten wir fest, dass eine Monobesiedlung mit VSL#3 Bakterien, zu keiner Erhöhung von T- und B-Lymphozyten, Treg oder Monozyten/Makrophagen im Vergleich zu naiven, konventionellen Mäusen führte (n.s. vs. Naive; **Publikation 3, Abb. 4**). Hingegen verursachte eine *C. jejuni*-Monoinfektion einen signifikanten Anstieg respektiver Immunzellpopulationen ($p<0.001$; **Publikation 3, Abb. 4**). Die prophylaktische und therapeutische Gabe von VSL#3 konnte hingegen die Anzahl der rekrutierten Immunzellen im Darm verringern ($p<0.05$-0.001; **Publikation 3, Abb. 4**). Die Infektion mit *C. jejuni* war ferner mit einer Erhöhung pro-inflammatorischer Zytokine wie TNF, MCP-1 und IL-6 im Kolon assoziiert ($p<0.05$-0.001; **Publikation 3, Abb. 5A-C**). Die prophylaktische Gabe von VSL#3 konnte allerdings die *C. jejuni* induzierte Erhöhung der MCP-1- und IL-6-Konzentrationen im Dickdarm abschwächen. Darüber hinaus führten sowohl die prophylaktische als auch die therapeutische Gabe von VSL#3 zu einer höheren Produktion des anti-inflammatorischen Zytokins IL-10 im Vergleich zu mit *C. jejuni* monoinfizierten Mäusen ($p<0.05$; **Publikation 3, Abb. 5D**). Die prophylaktische Gabe von VSL#3 konnte außerdem den *C. jejuni* induzierten systemischen TNF-Konzentrationsasnstieg (in der Milz) leicht abmildern ($p<0.05$; **Publikation 3, Abb. 7A**). Zusammenfassend lässt sich somit feststellen, dass eine Behandlung mit VSL#3 zu einer abgemilderten entzündlichen Immunantwort während einer *C. jejuni*-Infektion *in vivo* führt.

1.6 Diskussion

Die sich mehrende Evidenz bezüglich der Bedeutsamkeit der Darmmikrobiota bei physiologischen und pathologischen Prozessen im humanen Wirt erfordert die Entwicklung passender Mausmodelle für die Untersuchung der komplexen Mikrobiota-Wirts-Interaktionen. Durch das keimfreie Mausmodell wurden erste Hinweise auf den Einfluss der Mikrobiota auf das Immunsystem geliefert. Dennoch weisen diese Tiere zahlreiche Veränderungen und Entwicklungsstörungen des Immunsystems auf, die v.a. das darmassoziierte lymphatische Gewebe (GALT) betreffen, und sich beispielsweise durch geringere und kleinere PP und MLN, veränderte Kryptenmorphologie und verringerte Dicke der Mukosa auszeichnen (69-71). Die Assoziierung

von Dysbiose und Antibiotikagebrauch mit zahlreichen Pathologien (26-31) betont nicht nur die wichtige Rolle der Mikrobiota für die Gesundheit des Wirtes, sondern auch die Notwendigkeit der weiteren Aufklärung des Wechselspiels zwischen Mikrobiota, Antibiotika und Wirtsimmunität in normal entwickelten Tieren. Die von uns generierten sekundär abiotischen Mäuse stellen hierfür ein geeignetes Modell dar, u.a. weil die Entwicklungsstörungen keimfreier Mäuse umgegangen werden, und nach einer antibiotischen Therapie die Rekolonisation mit komplexer wirtseigener und -fremder Mikrobiota, einer Kombination aus mehreren sowie einzelnen kommensalen Baktierienspezies sowie Pathogenen möglich ist (72). Nach einer 8-10 wöchigen Fünffach-Antibiose, war die intestinale Mikrobiota der Mäuse nahezu vollständig eradiziert, wie durch kulturelle und molekulare Methoden bestätigt werden konnte (15, 61, 72). Bemerkenswerterweise waren in den Faecesproben von ABx Mäusen und im autoklavierten Futter vergleichbare Genkopienzahlen der wichtigsten Bakteriengruppen nachweisbar werden. Es muss zudem bedacht werden, dass die Unterscheidung vom genetischen Material aus toten oder lebendigen Bakterien hierbei nicht möglich ist.

Weder die antibiotische Behandlung noch die anschließende FMT führte zu einer Beeinträchtigung des klinischen Zustandes der Tiere. Darüber hinaus konnten wir hier feststellen, dass nach Pausieren der Antibiotikagabe und anschließender FMT die Florenzusammensetzung stabil und der konventionell besiedelter, unbehandelter Mäuse ähnlich war. Unsere immunhistochemischen Analysen ergaben sowohl im Dünn- als auch im Dickdarm eine starke Reduktion von T- und B-Lymphozyten, Treg und Monozyten/Makrophagen nach antibiotischer Behandlung und eine komplette Wiederherstellung dieser Zellpopulationen am d28 nach FMT. Diese Mikrobiota-abhängige Dynamik von Immunzellpopulationen in der Darmmukosa und LP wird sowohl für Zellen des angeborenen (73) als auch des erworbenen Immunsystems (74, 75), durch frühere Studien unterstützt. Mittels durchflusszytometrischer Analysen konnten wir außerdem feststellen, dass das Fehlen oder Wiedereinführen intestinaler Antigene unterschiedliche Antworten auf verschiedenen Ebenen des Immunsystems (lokal/peripher vs. zentral/systemisch) auslösten. So war eine Reduktion der CD4+ und CD8+ Zellzahlen in der LP des Dünn- und Dickdarms mit einer Erhöhung derselben in der Milz assoziiert. Dies deutet auf eine mögliche „Zentralisierung“ dieser Zellpopulationen bei Abwesenheit intestinaler antigener Stimulation hin. Außerdem zeigt dies, dass bei der Entwicklung von therapeutischen Modulationsmöglichkeit der Mikrobiota nicht nur die mukosalen Auswirkungen, sondern auch die potentiellen systemischen Folgen solcher Interventionen bedacht und grundlegend untersucht werden müssen.

Antibiotisch behandelte Mäuse zeigten außerdem eine geringere Produktion von IFN-γ im Ileum und Kolon sowie von IL-17, IL-22 und IL-10 im Dünn- und Dickdarm, in den MLN und in der

Milz. Diese Effekte konnten jedoch vollständig durch FMT normalisiert werden. Die Bedeutung von Mikrobiota-vermittelten Signalen für die Expansion der zytokin-produzierenden CD4+ Zellen im Darm ist bereits in früheren Studien dargelegt worden (76). Darüber hinaus konnte gezeigt werden, dass eine Rekolonisierung keimfreier Mäuse mit komplexer muriner Mikrobiota gleichzeitig pro- und anti-inflammatorische Immunantworten induzierte (77). Die Reduktion der IL-17 Produktion, dem Schlüssel-Zytokin von Th17 Zellen, die für den mukosalen Schutz gegen pathogene Bakterien und Pilze bedeutsam sind (78), kann außerdem eine mögliche Erklärung für die bekannte Anfälligkeit von antibiotisch behandelten Tieren gegenüber Pathogenen (14, 24) liefern. Zusammenfassend zeigte sich in diesen Experimenten, dass durch die FMT zahlreichen immunologischen „Kollateralschäden", die mit einer Breitband-Antibiose einhergehen, entgegengewirkt werden konnte.

Oral zu überreichende probiotische Substanzen sind praktikabler als eine fäkale Transplantation und genießen außerdem eine bessere Akzeptanz bei den Patienten. Aus diesem Grund verglichen wir den immunomodulierenden Einfluss von VSL#3 und FMT nach einer antibiotika-vermittelten Depletierung der intestinalen Mikrobiota. Wir konnten feststellen, dass VSL#3 Bakterien sich stabil im Darm ansiedeln konnten, und zu keinen makroskopischen (Durchfall, Gewichtsverlust, Blut im Stuhl) oder mikroskopischen (Apoptoserate) Anzeichen einer Entzündung des GIT führten. Weiterhin waren VSL#3 und FMT gleichermaßen in der Lage, die durch die Antibiotika-Behandlung verminderten Proliferationsraten im Dünn- und Dickdarmepithel zu normalisieren. Reikvam *et al.* haben schon die Unerlässlichkeit der Darmbakterien für die Regenerationsfähigkeit des Kolonepithels gezeigt (79). Die Proliferation der Enterozyten ist außerdem ein wichtiger Gewebereparaturmechanismus, dessen Verlust zu einer Störung der epithelialen Struktur und Barriere führen kann (80).
Die durchflusszytometrischen Analysen der aus verschiedenen lymphatischen Organen isolierten Lymphozyten ergaben, dass die Erniedrigung der CD4+ Lymphozyten im Dünndarm nach einer Antibiotika-Behandlung, durch eine VSL#3 Rekolonisierung wieder rückgängig zu machen war. Hingegen blieben die CD4+ Lymphozyten in der Milz antibiotisch behandelter Mäuse, ungeachtet der anschließenden Gabe von VSL#3 oder komplexer Mikrobiota, erhöht. Dies deutet auf die Möglichkeit mikrobiota-unabhängiger, immunmodulierender Eigenschaften antibiotischer Substanzen hin, wie sie bereits für die Chinolone (81) und Makrolide (82) beschrieben worden sind. VSL#3 Bakterien führten außerdem zu einer Wiederherstellung der Treg Zellpopulation und ihres Schlüsselzytokins IL-10, ohne Th-1- oder Th-17-Immunantworten zu induzieren. So blieben pro-inflammatorische Zytokine wie IFN-γ, IL-17 und IL-22 nach VSL#3 Rekolonisierung in

sämtlichen untersuchten immunologischen Kompartimenten erniedrigt, wobei ihre Expression nur mittels FMT angeregt werden konnte. Das Konzept, dass VSL#3 anti-inflammatorische Immunantworten in Th-1- oder Th-17 -vermittelten entzündlichen Erkrankungen induziert, wurde bereits vorgeschlagen (41) und ist im klinischen Sinne überaus attraktiv. Auch ein anderes probiotisches Gemisch, bestehend aus *L. acidophilus, L. casei, L. reuteri, B. bifidium* und *Streptococcus thermophilus* konnte durch die Induktion regulatorischer dendritischer und T-Zellen das Krankheitsbild mehrerer immunvermittelter Pathologien, wie etwa TNBS Kolitis, atopische Dermatitis und rheumatoide Arthritis verbessern (83). Die Reduktion der IFN-γ- und IL-17-Konzentration in mit VSL#3 besiedelten Mäusen, werfen jedoch die Frage auf, ob diese Mäuse durch den fehlenden Schutz durch Th-1 und Th-17 Zellen, möglicherweise anfälliger für intestinale Pathogene sind. In Anbetracht des aktuellen Kenntnisstandes erscheint dies jedoch eher unwahrscheinlich. Bei Patienten mit *C. difficile* assoziiertem Durchfall wurden z.B. anti-inflammatorische, krankheitsabmildernde Effekte von Probiotika festgestellt (57). Darüber hinaus wirken Probiotika über viele verschiedene Mechanismen wie etwa die Hemmung von Wachstum, Stoffwechsel und Adhäsion enteropathogener Bakterien (53-55), die Verstärkung der intestinalen Barrierefunktion (37), die Verstärkung der Kolonisationsresistenz gegenüber pathogenen Keimen durch Konkurrenz um Nährstoffe und Nischen (45), sowie die Modulation des angeborenen und adaptiven Immunsystem (46). Pagnini *et al*. konnten außerdem zeigen, dass VSL#3 in der Lage ist, die intestinale Produktion von TNF zu erhöhen und die epitheliale Barrierefunktion zu verbessern (84). Es ist also denkbar, dass bei Eindringen enteropathogener Bakterien auch durch VSL#3 eine ausreichende, jedoch nicht überschießende, pro-inflammatorische Immunantwort ausgelöst werden kann, die in unserem (klinisch gesunden) Mausmodell fehlt. Zusammenfassend weisen unsere Daten auf die Fähigkeit von VSL#3 hin, im Anschluss einer antibiotischen Therapie, immunomodulatorisch auf angeborene und erworbene Immunzellpopulationen nicht nur auf mukosaler Ebene (LP des Dünn- und Dickdarms) sondern auch peripherer (MLN) und systemischer Ebene (Milz) zu wirken. Dabei scheint es hauptsächlich anti-inflammatorische Immunantworten anzuregen, ohne die Th-1 und Th-17-Achse zu beeinflussen.

C. jejuni stellt einen der häufigsten Verursacher infektiöser bakterieller Durchfallerkrankungen dar. Das Potenzial neuartiger probiotischer Therapiemaßnahmen bei dieser Infektionskrankheit ist nicht vollständig erforscht. Wir gingen deshalb der Frage nach, ob die beobachteten anti-inflammatorischen Eigenschaften von VSL#3 auch eine Rolle bei der *C. jejuni*-Infektion sekundär abiotischer Mäuse spielen könnte. Hierfür führten wir Ko-Kolonisierungsexperimente mit VSL#3 und *C. jejuni* durch, wobei VSL#3 entweder 5 Tage vor (prophylaktische Gabe) oder nach

(therapeutische Gabe) Infektion mit *C. jejuni* peroral appliziert wurde. Kinetikanalysen von Bakterienlasten in Faecesproben über einen Zeitraum von 3 Wochen ergaben, dass sowohl VSL#3 als auch *C. jejuni* sich stabil im murinen GIT ansiedeln konnten, die Ko-Kolonisierung mit VSL#3 die Bakterienlasten des Pathogens allerding nicht reduzieren konnte. Diese Ergebnisse stehen im Widerspruch zu einer anderen Versuchsreihe von Wagner *et al* (45). Die Autoren untersuchten keimfreie BALB/c Mäuse, die mit komplexer humaner Flora rekonstituiert und mit einem probiotischen Gemisch aus drei *Lactobacillus*- und zwei *Bifidobacterium*-Stämmen behandelt wurden, was zu einer kompletten Beseitigung des Pathogens aus dem murinen GIT führte (45). Die im Vergleich zu den von uns erhobenen Daten unterschiedlichen Ergebnisse sind möglicherweise entweder auf die Unterschiede der Zusammensetzung der verwendeten Probiotika oder auf die verschiedenen verwendeten Mausmodelle zurückzuführen. Während C57BL/6j Mäuse zu Th-1- und BALB/c Mäuse zu Th-2 dominierten Immunantworten (85) neigen, ist ihre unterschiedliche Reaktion auf verschiedene Pathogene dokumentiert (86, 87). Außerdem kann möglicherweise die von Wagner *et al*. zur Rekoloniserung benutzte humane Flora durch ihre Diversität und Komplexität viel eher eine physiologische Kolonisationsresistenz wiederherstellen, als das aus 8 Stämmen bestehende probiotische Gemisch VSL#3.

Wie bereits früher von unserer Arbeitsgruppe berichtet, wiesen *C. jejuni* infizierte Mäuse vermehrt Apoptosen von Darmepithelzellen auf (15, 88), ein Effekt, der allerdings durch VSL#3 Gabe abgemildert werden konnte. Dies steht im Einklang mit Ergebnissen aus einer anderen Studie, bei der die Fähigkeit von VSL#3 die epitheliale Apoptose bei der Dextransodiumsulphat (DSS) Kolitis zu verringern, beschrieben wurde (89). Darüber hinaus konnte durch die prophylaktische und therapeutische Gabe von VSL#3 die *C. jejuni* induzierte Akkumulation angeborener (Monozyten/Makrophagen) und erworbener (T- und B-Lymphozyten, Treg) Immunzellen in die Mukosa und LP des Kolons abgeschwächt werden. Ein ähnlicher VSL#3 Effekt wurde bereits auch im TNBS Kolitismodell beschrieben (90). Eine VSL#3 Ko-Kolonisierung führte außerdem zu einer erhöhten IL-10-Sekretion im Kolon im Vergleich zu einer *C. jejuni* Monoinfektion. Sowohl durch die therapeutische als auch prophylaktische Gabe von VSL#3 konnte das im Kolon sezernierte pro-inflammatorische IL-6 reduziert werden. Die prophylaktische VSL#3 Gabe konnte zusätzlich auch die MCP-1 Produktion im Kolon verringern. Die anti-inflammatorischen Eigenschaften von VSL#3 waren nicht auf den GIT begrenzt, so konnte ein diskreter Abfall der TNF Produktion in der Milz durch die prophylaktische Gabe von VSL#3 beobachtet werden. Die immunomodulierenden Eigenschaften von VSL#3 können möglicherweise auf dessen Einfluss auf Toll-like Rezeptoren (TLR) zurückzuführen sein. Wir haben bereits früher berichtet, dass die *C. jejuni* induzierte Immunopathologie von TLR-4- und TLR-9-Signalwegen abhängt (15), während

in einer anderen Studie gezeigt wurde, dass VSL#3 die *in vitro* Expression von TLR-2, TLR-3, TLR-4 und TLR-9 herunterregulierte (91). Es bleibt außerdem ungeklärt, ob die beobachteten Effekte durch einzelne Bakterienstämme verursacht werden, oder ob die synergistischen Wechselwirkungen mehrerer Stämme eine wichtigere Rolle spielen könnte. Abschließend konnten wir feststellen, dass VSL#3 lokale und systemische Folgen einer *C. jejuni*-induzierten Entzündungsreaktion abmildern kann, und somit zukünftig eine versprechende Therapieoption dieser bakteriellen Durchfallerkrankung darstellen könnte.

Der Zusammenschau der Daten ergibt, dass die intestinale Mikrobiota eine außerordentliche Rolle für die Aufrechterhaltung der immunologischen Homöostase spielt, und zwar nicht nur auf mukosaler, peripherer sondern auch auf systemischer, zentraler Ebene. Des Weiteren haben auch Probiotika das Potenzial, das angeborente und erworbene Immunsystem, v.a. in Richtung anti-inflammatorischer Immunantworten, zu modulieren. Deshalb bieten sich probiotische Interventionen als mögliche Strategien für die Prophylaxe und Therapie enteropathogener Infektionen, etwa durch *C. jejuni,* an.

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„Ich, Ira Ekmekçiu, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Die Mikrobiota-vermittelte Modulation intestinaler und extra-intestinaler Immunzellpopulationen in sekundär abiotischen Mäusen und im murinen *Campylobacter jejuni* Infektionsmodell“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Publikation 1:

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Beitrag im Einzelnen: Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Rekolonisierung mit VSL#3, tägliche Tiervisite, Sektion; Lymphozytenisolierung, Durchführung durchflusszytometrischer Färbungen, mikrobiologische Auswertungen, Analysen der entnommenen Proben, Anfertigung und Beurteilung histologischer Schnitte, Auswertung der Durchflusszytometriedaten, statistische Aufarbeitung der Daten, Literaturrecherche, Verfassung des Manuskripts.

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The probiotic compound VSL#3 modulates mucosal, peripheral and systemic immunity following murine broad-spectrum antibiotic treatment. Front Cell Infect Microbiol 2017

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<u>Publikation 3</u>:

Ekmekciu I, Fiebiger U, Stingl K, Bereswill S, Heimesaat MM

Amelioration of intestinal and systemic sequelae of murine Campylobacter jejuni infection by probiotic VSL#3 treatment. Gut Pathog 2017

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Immune Responses to Broad-Spectrum Antibiotic Treatment and Fecal Microbiota Transplantation in Mice

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Compelling evidence demonstrates the pivotal role of the commensal intestinal microbiota in host physiology and the detrimental effects of its perturbations following antibiotic treatment. Aim of this study was to investigate the impact of antibiotics induced depletion and subsequent restoration of the intestinal microbiota composition on the murine mucosal and systemic immunity. To address this, conventional C57BL/6j mice were subjected to broad-spectrum antibiotic treatment for 8 weeks. Restoration of the intestinal microbiota by peroral fecal microbiota transplantation (FMT) led to reestablishment of small intestinal $CD4^+$, $CD8^+$, and $B220^+$ as well as of colonic $CD4^+$ cell numbers as early as 7 days post-FMT. However, at d28 following FMT, colonic $CD4^+$ and $B220^+$ cell numbers were comparable to those in secondary abiotic (ABx) mice. Remarkably, $CD8^+$ cell numbers were reduced in the colon upon antibiotic treatment, and FMT was not sufficient to restore this immune cell subset. Furthermore, absence of gut microbial stimuli resulted in decreased percentages of memory/effector T cells, regulatory T cells, and activated dendritic cells in the small intestine, colon, mesenteric lymph nodes (MLN), and spleen. Concurrent antibiotic treatment caused decreased cytokine production (IFN-γ, IL-17, IL-22, and IL-10) of $CD4^+$ cells in respective compartments. These effects were, however, completely restored upon FMT. In summary, broad-spectrum antibiotic treatment resulted in profound local (i.e., small and large intestinal), peripheral (i.e., MLN), and systemic (i.e., splenic) changes in the immune cell repertoire that could, at least in part, be restored upon FMT. Further studies need to unravel the distinct molecular mechanisms underlying microbiota-driven changes

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Abbreviations: ABx, secondary abiotic; BSA, bovine serum albumin; CFU, colony-forming units; DC, dendritic cells; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FMT, fecal microbiota transplantation; GF, germfree; HBSS, Hanks' balanced salt solution; HPF, high power field; IBD, inflammatory bowel disease; LPL, lamina propria lymphocytes; LPS, lipopolysaccharide; MLN, mesenteric lymph nodes; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PP, Peyer's patches; PMA, phorbol myristate acetate; SPF, specific-pathogen-free; Th, T helper; TNBS, trinitrobenzenesulphonic acid; Treg, regulatory T cells.

in immune homeostasis subsequently providing novel therapeutic or even preventive approaches in human immunopathologies.

Keywords: microbiota, antibiotics, secondary abiotic (gnotobiotic) mice, fecal microbiota transplantation, innate and adaptive immunity, bacterial recolonization, mucosal and systemic immune responses

INTRODUCTION

The human gastrointestinal tract harbors a plethora of microorganisms, including bacteria, viruses, fungi, helminths, and protozoa that are referred to as commensal microbiota. Bacteria, however, constitute the vast majority of the intestinal microbiota (1). A recent study revealed that the ratio of human cells and bacteria is close to 1:1 with absolute numbers approximating 10^{13} each (2). Hence, a more detailed examination of distinct host-microbiota interactions remains of utmost interest. Under steady-state conditions, this interaction is largely defined by mutual benefits. The host provides the microbiota with a physiological niche in a nutrient rich environment, while the microbiota exerts various beneficial functions for the host such as vitamin production (3), digestion of dietary compounds (4), and protection from pathogens (5).

One important aspect underlining the indispensability of gut microorganisms is their contribution to the development, maturation, and regulation of the immune system (6). Studies in isolator-raised germfree (GF) mice revealed fundamental impairments regarding the development and differentiation of immune compartments including Peyer's patches (PP) and mesenteric lymph nodes (MLN) as indicated by decreased IgA levels, and cellular defects of intestinal epithelial and lamina propria (LP) lymphocytes (7). Given that the alteration of the intestinal microbiota composition, termed dysbiosis, is associated with defined immunopathological conditions including inflammatory bowel diseases (8, 9), allergies (10), type 2 diabetes mellitus (11), obesity (12), anxiety, depression (13), and autism (14), the orchestrated interplay between the commensal microbiota and host cells plays a pivotal role in maintaining immune homeostasis and host cell physiology.

This rationale is further supported by compelling evidence derived from studies with defined bacterial strains that induce the development and expansion of distinct immune cell subsets. For instance, segmented filamentous bacteria have been identified as potent inducers of the IL-17-producing T helper (Th) 17 cells (15), while *Clostridium* species of clusters IV and XIVa promoted accumulation of regulatory T cells (Treg) in the colonic LP of mice (16).

Antibiotic treatment, besides being one of the greatest achievements in the history of medicine, results in disruption of intestinal microbial communities as collateral damage with long-term consequences after cessation of therapy (17). Many antibiotic compounds have been shown to render the host susceptible to infection by several pathogens including *Salmonella* species (18), vancomycin-resistant *Enterococcus* spp. (19), and *Clostridium difficile* (20). *C. difficile* toxin-induced enterocolitis, for instance, represents one of the biggest antibiotics-related health-care problems with potentially fatal outcome (20). Remarkably, even short-term application of antimicrobial compounds such as clindamycin induces long-lasting decreases in enteric microbial diversity and renders mice susceptible to *C. difficile* colonization and infection (17).

Furthermore, there is profound evidence regarding the impact of antibiotic treatment on immune cell homeostasis. For instance, mice treated with vancomycin or colistin from birth on displayed decreased numbers of isolated lymphoid follicles, a tertiary lymphoid tissue, in the small and large intestines (21). Moreover, treatment of mice with an antibiotic cocktail consisting of neomycin, vancomycin, and metronidazole resulted in lower intestinal expression of regenerating islet-derived protein 3 gamma, an antimicrobial peptide directed against Gram-positive bacteria (22), whereas treatment with vancomycin resulted in reduced Treg numbers in the colon (16). Reduction of the Treg population could also be observed in the murine MLN and PP upon microbiota depletion by broad-spectrum antibiotic treatment (23). Whether the observed effects on the immune system following antimicrobial treatment were rather primarily due to the alterations of microbial communities and/or distinct compound-related mechanisms, however, remains unanswered.

In this study, we therefore aimed to further elucidate the interplay of the triangle relationship between intestinal microbiota, antibiotics and the immune system in more detail. To address this, we performed a comprehensive survey of distinct immune cell subsets, including $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, memory T cells, activated dendritic cells (DC), and Treg in intestinal and systemic compartments of mice that were virtually depleted of microbiota through broad-spectrum antibiotic treatment as compared to secondary abiotic (ABx) mice following fecal microbiota transplantation (FMT) and to conventionally colonized mice. Moreover, we analyzed both pro- and anti-inflammatory cytokines including IFN-γ, IL-17, IL-22, and IL-10 expressed by $CD4^+$ lymphocytes following broad-spectrum antibiotic treatment and FMT.

MATERIALS AND METHODS

Mice

All animals were bred, raised, and housed in the facilities of the "Forschungseinrichtungen für Experimentelle Medizin" (Charité – University Medicine Berlin, Germany) under specific-pathogen-free (SPF) conditions. Female age-matched C57BL/6j wild-type mice were used.

Generation of Secondary Abiotic (Gnotobiotic) Mice and Reconstitution of the Intestinal Commensal Microbiota by Fecal Transplantation

In order to virtually deplete the intestinal microbiota, 8–10 weeks old mice were transferred to sterile cages and subjected to quintuple antibiotic treatment for 8 weeks as previously described

(24). Three days prior to peroral FMT, the antibiotic cocktail was withdrawn and replaced by sterile drinking water. Successful eradication of the cultivable intestinal microbiota was confirmed as described previously (24). Fresh murine fecal samples were collected from 10 age- and sex-matched SPF control mice, pooled, dissolved in 10 ml sterile phosphate-buffered saline (PBS; Gibco, Life Technologies, Paisley, UK) and the supernatant perorally applied by gavage (in 0.3 ml PBS) in order to reconstitute secondary abiotic (i.e., gnotobiotic) mice with a complex intestinal microbiota.

Sampling Procedures

Mice were sacrificed by isoflurane treatment (Abbott, Greifswald, Germany) at day 7 or day 28 post-FMT. Luminal large intestinal samples as well as *ex vivo* biopsies from spleen, MLN, ileum, and colon were taken under sterile conditions. Ileal and colonic *ex vivo* biopsies were collected from each mouse in parallel for immunological, microbiological, and immunohistochemical analysis. For immunohistochemical stainings, ileum and colon samples were immediately fixed in 5% formalin and embedded in paraffin, and sections (5 µm) were stained with distinct antibodies as described below.

Immunohistochemistry

In situ immunohistochemical analysis of ileal and colonic paraffin sections was performed as previously described (25–28). Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Glostrup, Denmark, 1:100), CD3 (#N1580, Dako, 1:10), Foxp3 (FJK-16s, eBioscience, San Diego, CA, USA, 1:100), B220 (eBioscience, 1:200), and F4/80 (# 14-4801, clone BM8, eBioscience, 1:50) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 400× magnification) was determined microscopically by an independent blinded investigator.

Lymphocyte Isolation from Spleen and MLN

Single cell suspensions were generated from spleens and MLN, and erythrocytes were removed from splenic samples by 1.66% ammonium chloride. All samples were resuspended in defined volumes of PBS/0.5% bovine serum albumin (BSA) and subjected to further processing (23).

LP Lymphocyte Isolation

Segments of the murine gut were removed and freed from fat, connective tissue, and PP, cut longitudinally, and cleared from luminal content and mucus with ice-cold PBS. The isolation of lamina propria lymphocytes (LPL) followed a standard protocol with minor modifications (29). Briefly, the intestines were cut into 0.5 cm pieces and incubated twice with 25 ml Hanks' balanced salt solution (HBSS; Gibco) containing 1 mM dithioerythritol (Carl Roth) for 20 min at 37°C and 220 rpm. Afterward, the intestines were introduced to HBSS containing 1.3 mM ethylenediaminetetraacetic acid (Life Technologies, Eugene, OR, USA). Subsequently the cells were placed in digestion solution, containing 0.5 mg/ml collagenase A (Roche, Mannheim, Germany), 0.5 mg/ml DNAse I (Roche), 10% fetal calf serum (FCS), and 1 mM of each $CaCl_2$ and $MgCl_2$ (both Carl Roth). Digestion was performed through incubation for 45 min at 37°C and 220 rpm. After the incubation, the digested tissues were washed with RPMI containing 5% FCS, resuspended in 5 ml 44% Percoll (GE Healthcare, Uppsala Sweden), and overlaid on 5 ml 67% Percoll in a 15 ml Falcon tube. Percoll gradient separation was performed by centrifugation at 600 *g* for 20 min at room temperature. LPL were collected from the interphase, washed once, and suspended in PBS/0.5% BSA.

Surface and Intracellular Stainings and Flow Cytometry

Surface staining was performed using following antibodies: FITC-anti-CD4 (Clone RM4-5; 1:200), PerCP-anti-CD8 (Clone 53-6.7; 1:100), PacBlue-anti-B220 (Clone RA3-6B2, 1:200), APC-Cy7-anti-CD25 (Clone PC61, 1:200), PE-anti-CD44 (Clone IM7, 1:200), and APC-anti-CD86 (Clone B7-2, 1:200) (all from BD Biosciences, San Jose, CA, USA).

For intracellular staining, cells were restimulated for 5 h with 10 ng/ml phorbol myristate acetate and 1 µg/ml ionomycin, in a tissue culture incubator at 37°C (both Sigma-Aldrich). Ten micrograms per milliliter brefeldin A (Sigma-Aldrich) were added to the cell suspensions after 1 h of polyclonal restimulation. Then cells were treated with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Life Technologies) and hereafter fixed with 2% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. Cells were stained in 0.5% saponin (Sigma-Aldrich) using following antibodies: PacBlue-anti-CD4 (Clone RM4-5; 1:400), PE-Cy7-anti-IFN-γ (Clone XMG 1.2; 1:400) (both from BD Biosciences), FITC-anti-IL17A (Clone TC11-18H10.1; 1:200, BioLegend, San Diego, CA, USA), PE-anti-IL10 (Clone JESS-16E3; 1:100), and APC-anti-IL22 (Clone IL22JOP; 1:100) (both from eBioscience). All data were acquired on a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and were analyzed with FlowJo Software v10.1 (Tree star, Ashland, OR, USA).

Real-time PCR

Expression levels of pro- and anti-inflammatory cytokines including IFN-γ, IL-22, IL-17A, and IL-10 mRNA were determined in snap frozen ileal and colonic *ex vivo* biopsies using Light Cycler Data Analysis Software (Roche) as stated elsewhere (30). The mRNA of the housekeeping gene for hypoxanthine-phosphoribosyltransferase was used as reference; the mRNA expression levels of the individual genes were normalized to the lowest measured value and expressed as fold expression (arbitrary units) (31).

For molecular analysis of the intestinal microbiota, DNA was extracted from fecal samples as described previously (24). Briefly, DNA extracts and plasmids were quantified using Quant-iT PicoGreen reagent (Invitrogen, Paisley, UK) and adjusted to 1 ng/µl. Then, abundance of the main bacterial groups of murine intestinal microbiota was assessed by quantitative real time-PCR with group-specific 16S rRNA gene primers (Tib MolBiol, Berlin,

Germany) as described previously (5, 32, 33). The number of 16S rRNA gene copies per microgram DNA of each sample was determined, and frequencies of respective bacterial groups calculated proportionally to the eubacterial (V3) amplicon.

Statistical Analysis

Medians, means, SDs, and significance levels were determined using Mann–Whitney *U* test or one-way analysis of variance with Tukey's post hoc test for multiple comparisons (GraphPad Prism Software v6, La Jolla, CA, USA) as indicated. Two-sided p values ≤ 0.05 were considered significant. Data shown were pooled from two independent experiments ($n = 10$–15 per group).

RESULTS

Depletion and Reconstitution of the Murine Intestinal Microbiota following Broad-Spectrum Antibiotic Treatment and FMT

To confirm successful depletion of the intestinal microbiota, we applied cultural analyses of fecal samples derived from ABx mice. In fact, all fecal samples were culture negative for aerobic, microaerobic, and obligate anaerobic species as assessed by direct plating and enrichment procedures (not shown). To additionally

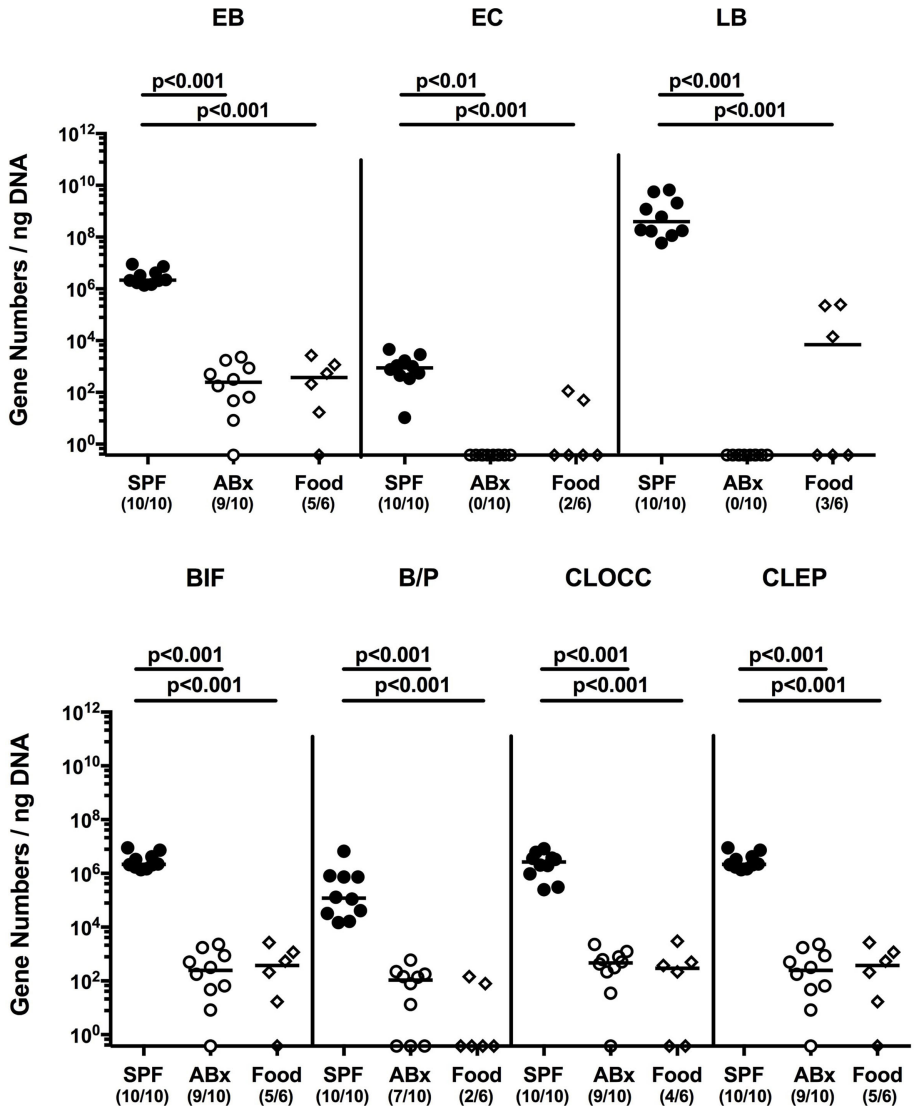


FIGURE 1 | Intestinal microbiota composition of conventional and secondary abiotic mice as compared to autoclaved food pellets. The intestinal microbiota composition was analyzed in fecal samples derived from conventionally colonized (SPF) mice and mice subjected to an 8 weeks course of broad-spectrum antibiotic treatment [thereby generating secondary abiotic (ABx) mice] by quantitative real-time PCR amplifying variable regions of the bacterial 16S rRNA gene and compared to the bacterial composition detected in sterilized (autoclaved) food pellets. The following main intestinal bacterial groups were determined (expressed as 16S rRNA gene numbers per nanogram DNA): enterobacteria (EB), enterococci (EC), lactic acid bacteria (LB), bifidobacteria (BIF), *Bacteroides/Prevotella* spp. (BP), *Clostridium coccoides* group (CLOCC), and *Clostridium leptum* group (CLEP). Numbers of samples harboring the respective bacterial group out of the total number of analyzed samples are given in parentheses.

assess abundance of fastidious and uncultivable intestinal bacteria, we next determined the main bacterial groups abundant in the murine intestinal tract by quantitative 16S rRNA-based PCR analysis of fecal samples derived from conventionally colonized and ABx mice as compared to respective bacterial groups abundant in autoclaved food pellets. In ABx mice, bacterial 16S rRNA gene numbers were decreased by up to 10 orders of magnitude as compared to conventional SPF controls ($p < 0.001$; **Figure 1**). Remarkably, mean 16S rRNA gene numbers in fecal samples derived from ABx mice and autoclaved food pellets were comparable, indicating a successful and biologically relevant depletion of the intestinal microbiota following broad-spectrum antibiotic treatment. Notably, one cannot differentiate whether detected 16S rRNA gene numbers in ABx mice were derived from avital ("dead") or viable fastidious/uncultivable bacterial cells. We next determined the efficiency of intestinal microbiota reconstitution upon FMT of ABx mice. As assessed by molecular methods, 16S rRNA gene numbers of the main bacterial intestinal microbiota groups were comparable in conventional mice and ABx mice at days 7 and 28 post-FMT (Figure S1 in Supplementary Material) indicating a successful reconstitution of the intestinal microbiota by FMT.

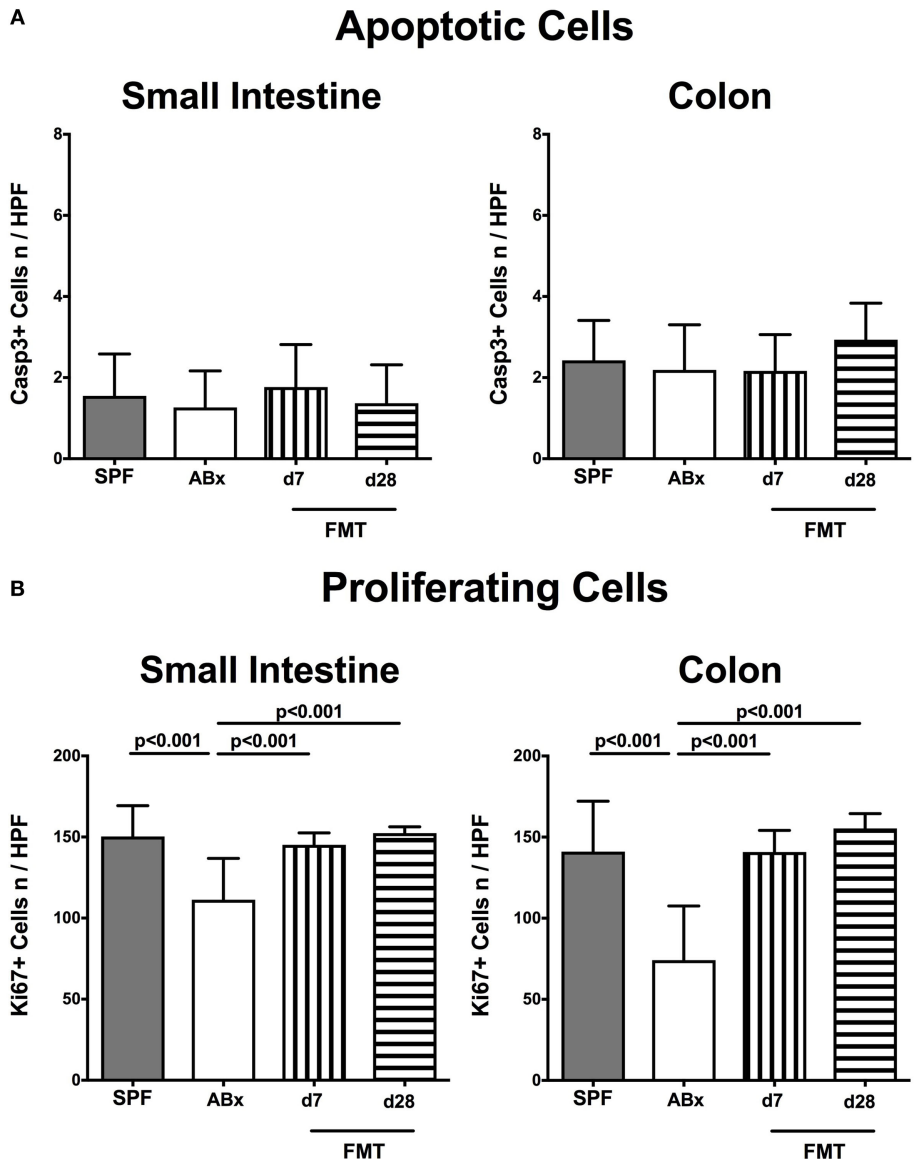


FIGURE 2 | **Apoptotic and proliferating cells in small and large intestinal epithelia of secondary abiotic and microbiota-reconstituted mice**. The average numbers of **(A)** apoptotic (positive for caspase-3, Casp3) and **(B)** proliferating cells (positive for Ki67) in at least six representative high power fields (HPFs, 400× magnification) per animal were determined in immunohistochemically stained small intestinal and colonic *ex vivo* biopsies derived from naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and abiotic mice reconstituted with murine microbiota at day (d) 7 (bars with vertical lines) and d28 (bars with horizontal lines) following fecal microbiota transplantation (FMT).

Macroscopic and Microscopic Sequelae of Broad-Spectrum Antibiotic Treatment and FMT

Given that neither antibiotic treatment nor FMT affected mice clinically and resulted in macroscopic sequelae such as wasting, diarrhea, or occurrence of blood in fecal samples (not shown), we assessed potential microscopic changes in intestinal *ex vivo* biopsies derived from ABx and FMT mice. To address this, we determined numbers of apoptotic cells in small and large intestinal paraffin sections following staining against caspase-3, given that apoptosis is an established parameter used for histopathological evaluation and grading of intestinal inflammation (5). Neither small nor large intestinal epithelial apoptotic cell numbers differed in conventionally colonized, ABx, and reconstituted mice at days 7 and 28 post-FMT (**Figure 2A**). Interestingly, quantification of Ki67 expression cells as a sensitive measure for cell proliferation and regeneration (34) revealed reduced numbers of proliferating cells in both ileal and colonic epithelia of ABx mice ($p < 0.001$; **Figure 2B**). As early as 7 days post-FMT, however, Ki67$^+$ cell numbers reached basal counts again ($p < 0.001$ vs ABx; **Figure 2B**). Hence, neither broad-spectrum antibiotic treatment nor FMT results in increased intestinal apoptosis, whereas reconstitution with complex intestinal microbiota is essential for restoring cell proliferative and regenerative measures during physiological tissue turnover within the intestinal tract.

Impact of Broad-Spectrum Antibiotic Therapy and Subsequent FMT on Innate and Adaptive Immune Cell Subsets in Murine Small and Large Intestines *In Situ*

To examine the impact of the intestinal microbiota on abundances of distinct immune cell populations in the small and large intestines, we microscopically quantitated respective immune cell subsets in small intestinal and colonic paraffin sections applying *in situ* immunohistochemistry. In microbiota-depleted mice, significantly reduced numbers of CD3$^+$ T lymphocytes ($p < 0.001$; **Figures 3A,E**), B220$^+$ B lymphocytes ($p < 0.001$; **Figures 3B,F**), Foxp3$^+$ regulatory T cells (Treg, $p < 0.001$; **Figures 3C,G**) as well as of F4/80$^+$ monocytes and macrophages ($p < 0.01$–0.001; **Figures 3D,H**) in both ileum and colon as compared to conventional mice could be observed. Following FMT colonic, but not ileal numbers of respective immune cell populations increased

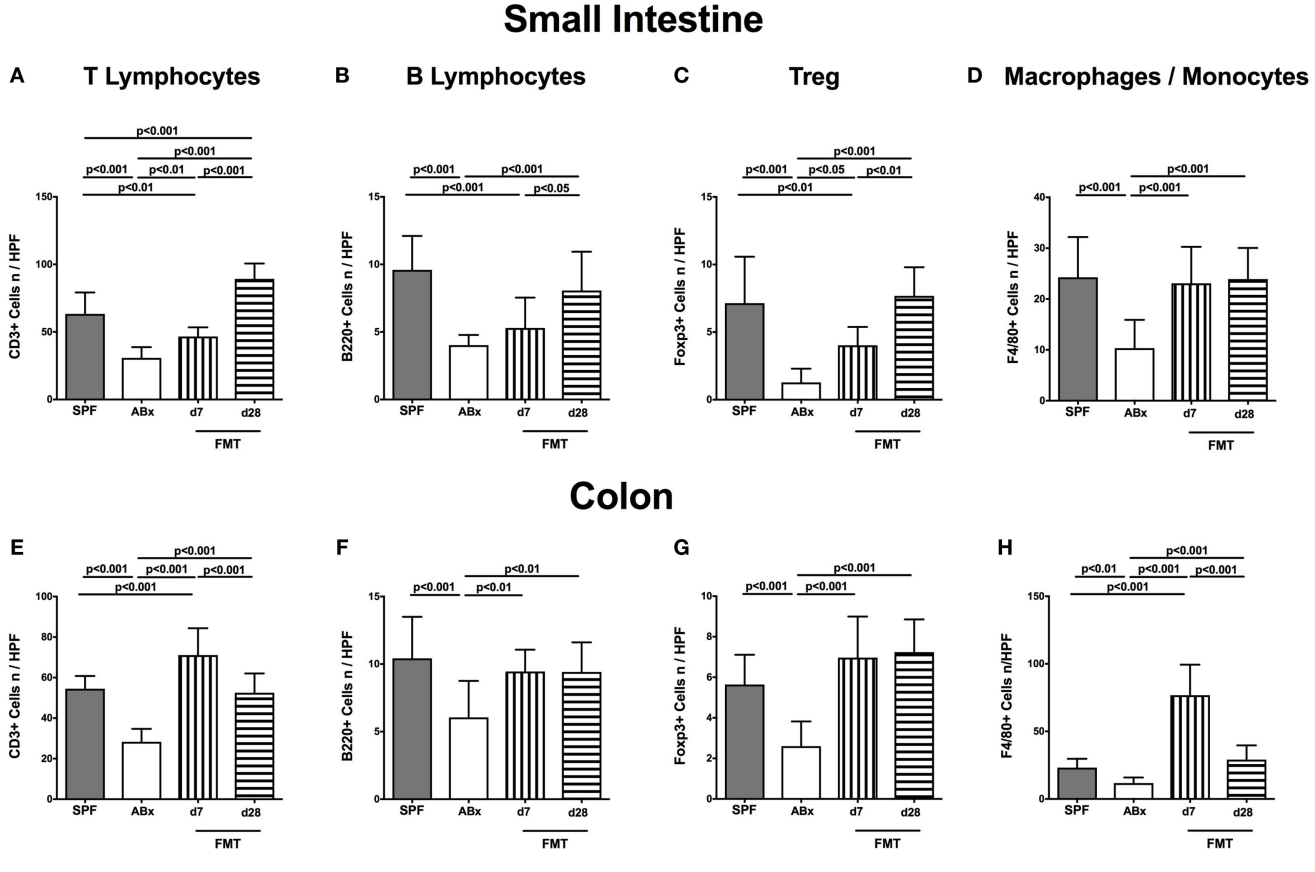
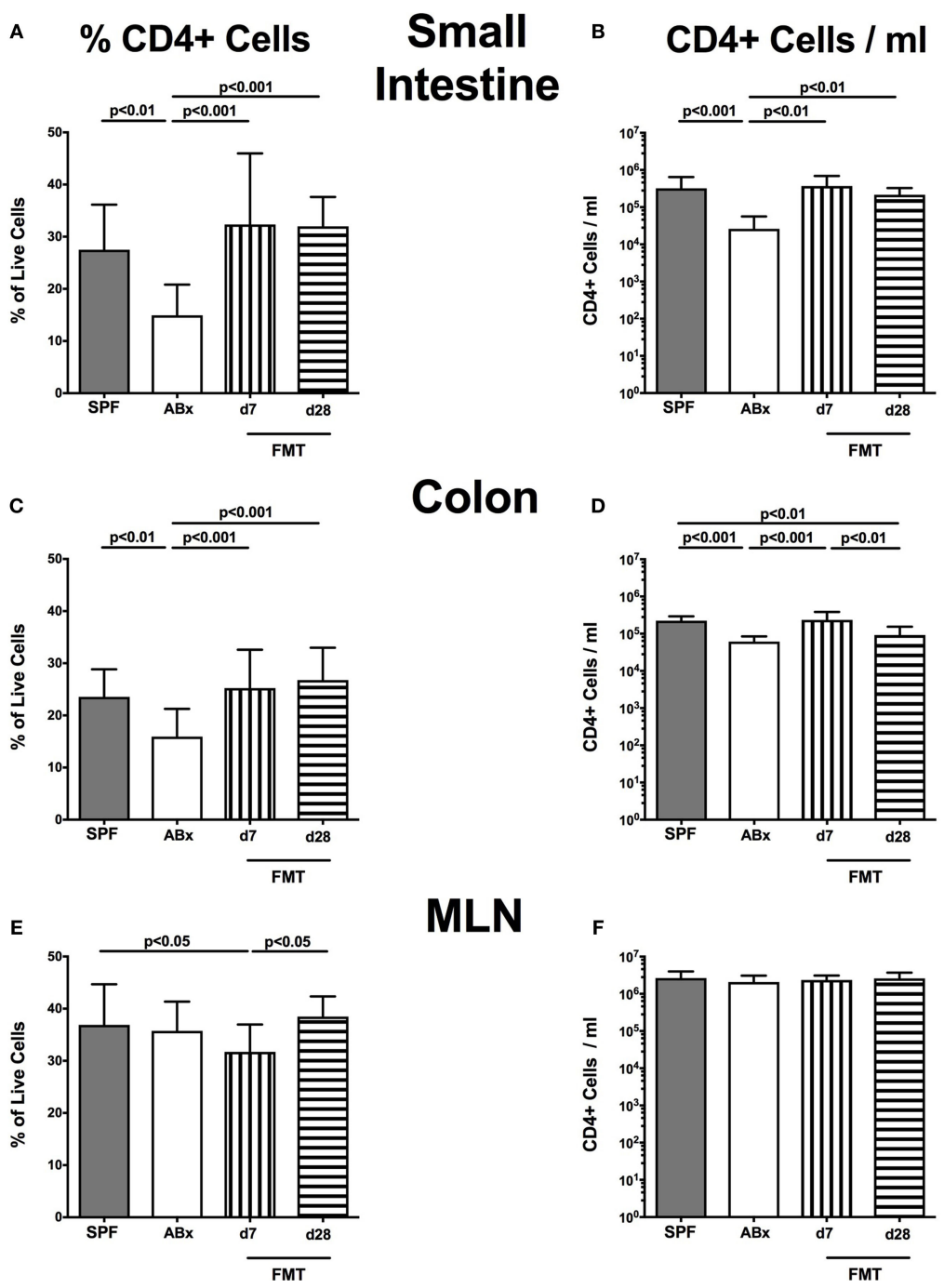


FIGURE 3 | Ileal and colonic immune cell populations in secondary abiotic and microbiota-reconstituted mice. The average numbers of T lymphocytes [positive for CD3 **(A,E)**], B lymphocytes [positive for B220 **(B,F)**], regulatory T cells [Treg, positive for Foxp3 **(C,G)**], and macrophages/monocytes [positive for F4/80 **(D,H)**] from at least six representative high power fields (HPFs, 400× magnification) per animal were determined in *ex vivo* biopsies taken from the small intestine [upper panel **(A–D)**] and colon [lower panel **(E–H)**] of conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and with microbiota-reconstituted abiotic mice at day (d) 7 (bars with vertical lines) or d28 (bars with horizontal lines) following fecal microbiota transplantation (FMT).

back to counts observed in control mice as early as 7 days post-FMT ($p < 0.01$–0.001 vs ABx, **Figures 3E,H**). In small intestines, however, T and B lymphocytes as well as Treg numbers were lower at day 7 post-FMT as compared to SPF mice but reached comparable or even higher counts thereafter (**Figures 3A,C**). Hence, our data underline the essential association of the complex commensal microbiota and the repertoire of innate and adaptive immune cell populations in both the small and large intestines.

Impact of Broad-Spectrum Antibiotic Treatment and Subsequent FMT on Distinct Lymphocyte Populations in Murine Intestinal and Systemic Compartments

To further elaborate the role of the intestinal microbiota on adaptive immunity in mucosal, peripheral, and systemic



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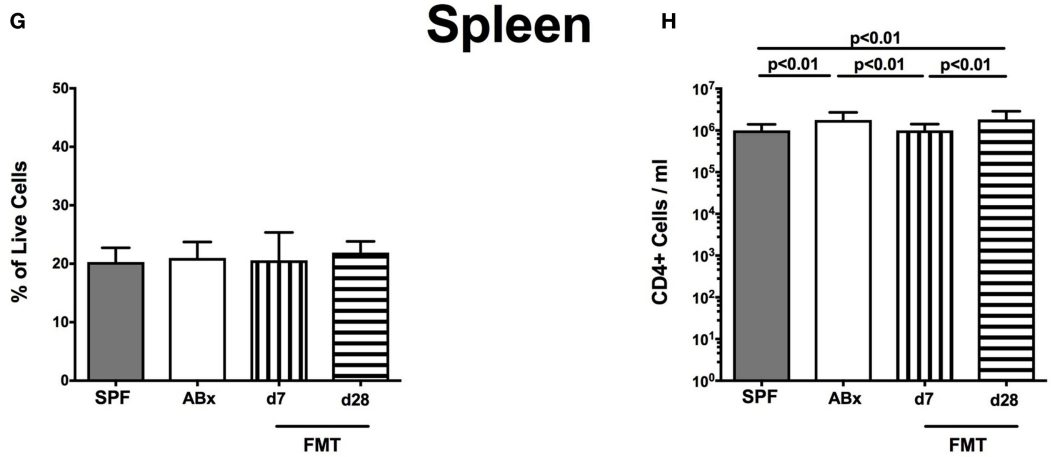


FIGURE 4 | CD4⁺ cells in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice. The percentages [left panel **(A,C,E,G)**] and cell numbers [right panel **(B,D,F,H)**] of the CD4⁺ lymphocyte population within the small intestine **(A,B)**, colon **(C,D)**, mesenteric lymph nodes (MLN) **(E,F)**, and spleen **(G,H)** of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice on day (d) 7 (bars with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

compartments, we isolated lymphocytes of the small and large intestinal LP, MLN, and spleen and analyzed defined immune cell populations by flow-cytometric analysis. Gating strategies are depicted in Figures S2A–F in Supplementary Material.

Broad-spectrum antibiotic treatment induced a significant reduction of both relative abundances and absolute numbers of CD4⁺ helper T lymphocytes in the small and large intestinal LP that could be restored at day 7 post-FMT, whereas colonic CD4⁺ cell concentrations further declined thereafter ($p < 0.01$–0.001; **Figures 4A–D**). Interestingly, within the MLN, but not other compartments, decreased percentages of CD4⁺ cells could be observed at day 7 post-FMT ($p < 0.05$; **Figures 4E,F**). In the spleen, frequencies of CD4⁺ cells were not affected by microbiota depletion (n.s.; **Figure 4G**). Notably, increased splenic CD4⁺ cell numbers were determined in ABx mice that slightly declined until day 7 post-FMT but increased to even supra-basal levels thereafter ($p < 0.01$; **Figure 4H**). These results point toward a potential "systemic accumulation" of these cells in the splenic compartment in absence of the intestinal microbiota.

In the small intestinal, LP decreased frequencies and cell numbers of CD8⁺ cytotoxic T cells were observed following microbiota depletion ($p < 0.01$–0.001; **Figures 5A,B**) that could be completely restored upon FMT. In the colonic LP, CD8⁺ cell frequencies were reduced upon antibiotic treatment and reestablished rather late following microbiota reconstitution (i.e., until day 28 post-FMT) ($p < 0.001$, d28 vs ABx; **Figure 5C**). Notably, colonic CD8⁺ cell numbers were profoundly affected by antibiotic treatment of mice, irrespective whether recolonized or not as indicated by lower counts as compared to SPF controls ($p < 0.05$–0.001; **Figure 5D**). In line with CD4⁺ cell numbers, CD8⁺ cells within MLN were not affected by antibiotic treatment and/or subsequent bacterial recolonization (n.s.; **Figures 5E,F**).

Relative abundances as well as absolute numbers of splenic CD8⁺ cells increased following antibiotic treatment ($p < 0.05$–0.001; **Figures 5G,H**), decreased as early as 7 days post-FMT but increased again 28 days after FMT to higher levels than in SPF mice.

We next addressed whether also B lymphocytes were affected following antibiotic treatment and restoration of the intestinal microbiota. In fact, also absolute numbers of B220⁺ cells decreased in small intestines, colon, and MLN upon microbiota depletion, but conversely increased in the spleen. FMT could sufficiently restore small intestinal, but not colonic B220⁺ cell counts as early as 7 days thereafter (Figures S3B,D in Supplementary Material). Again, microbiota depletion-induced elevation of splenic B220⁺ cells was reversed until day 7 post-FMT ($p < 0.05$; Figure S3H in Supplementary Material). Interestingly, proportions of B220⁺ B lymphocytes within small intestine, colon, and spleen were rather unaffected by the colonization status of mice (Figures S3A,C,G in Supplementary Material), whereas an increased proportion of B cells could be observed in MLN at day 7 post-FMT as compared to the other groups ($p < 0.001$; Figure S3E in Supplementary Material).

Taken together, these data point out that a virtual eradication of the intestinal microbiota by broad-spectrum antibiotic treatment affects both absolute numbers and relative abundances of distinct adaptive immune cell population not only locally (i.e., in the intestinal tract) but also has far-reaching consequences on systemic immune functions.

Impact of Broad-Spectrum Antibiotic Treatment and Subsequent FMT on Memory/Effector T Cells, Treg, and Activated DC in Murine Intestinal and Systemic Compartments

We next surveyed the impact of broad-spectrum antibiotic therapy and FMT on defined T cell subsets and on the activation

status of distinct cell populations. To address this, the surface marker CD44 that is expressed upon antigen contact (35) on $CD4^+$ and $CD8^+$ cells was analyzed. Upon broad-spectrum antibiotic treatment, both CD44 positive $CD4^+$ and $CD8^+$ memory/effector cells were less abundant in all surveyed lymphoid compartments ($p < 0.01$–0.001; **Figure 6**). This effect could, however, be reversed upon FMT as indicated by higher abundances of CD44 positive $CD4^+$ and $CD8^+$ cells at days 7 and 28 post-FMT as compared to ABx mice ($p < 0.05$–0.001; **Figure 6**). At day 7 post-FMT, $CD8^+CD44^+$ in the MLN were even more

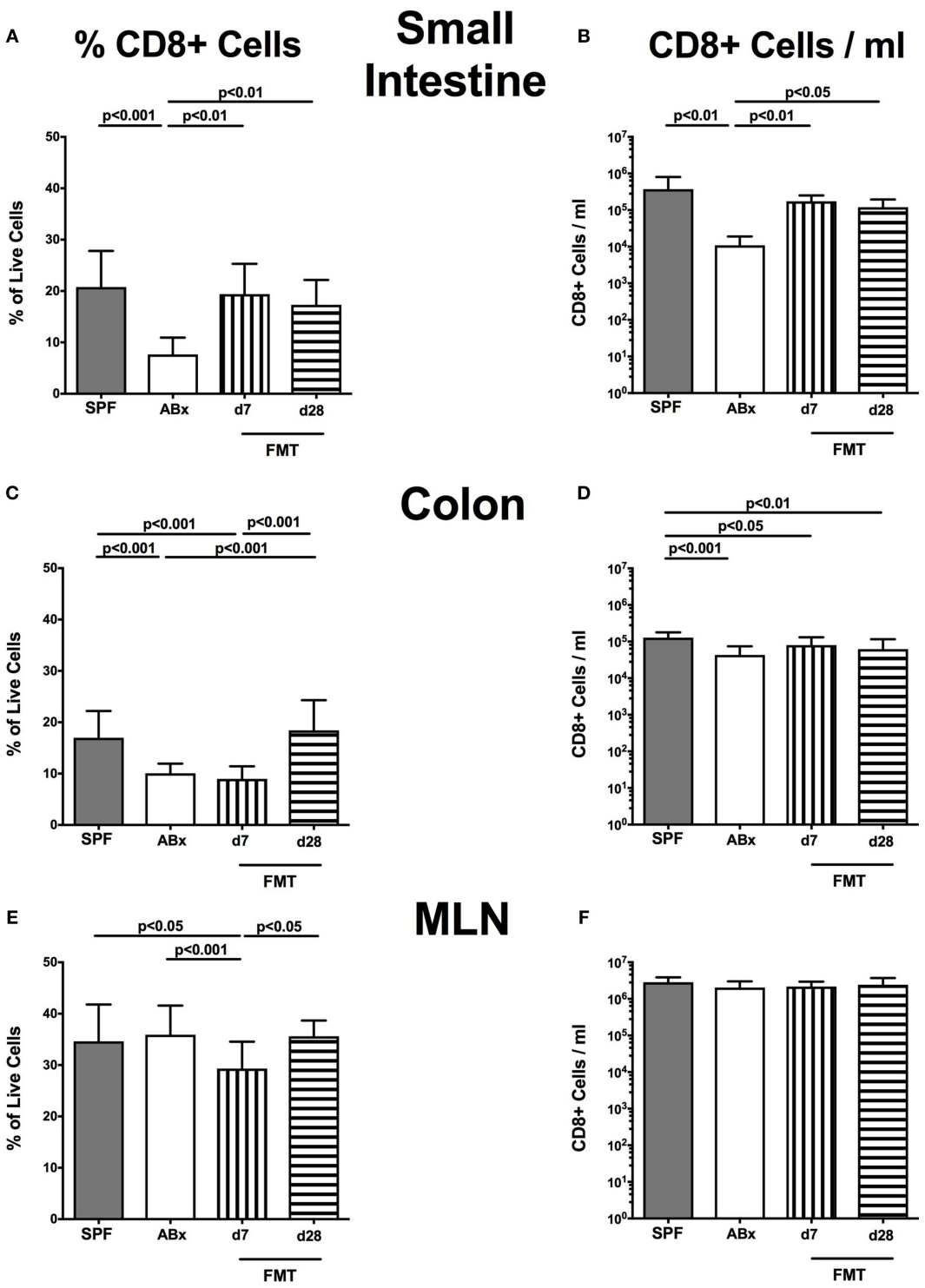


FIGURE 5 | **Continued**

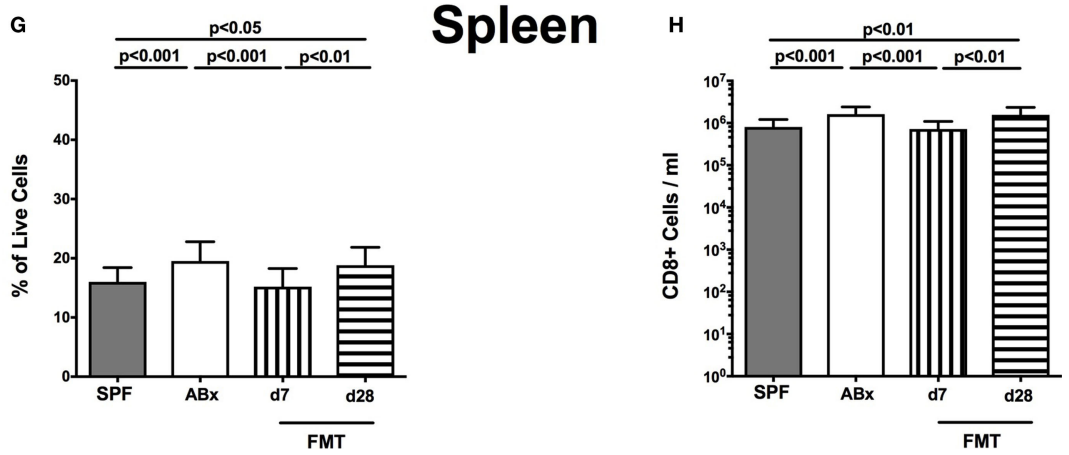


FIGURE 5 | CD8⁺ cells in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice. The percentages [left panel **(A,C,E,G)**] and cell numbers [right panel **(B,D,F,H)**] of the CD8⁺ lymphocyte population within the small intestine **(A,B)**, colon **(C,D)**, mesenteric lymph nodes (MLN) **(E,F)**, and spleen **(G,H)** of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

abundant than in SPF mice but reached basal levels thereafter ($p < 0.05$; **Figure 6F**).

As already observed for the other immune cell subsets, also $CD4^+CD25^+$ Treg were strongly diminished in small and large intestines, MLN, and spleen of ABx mice ($p < 0.01$–0.001; Figures S4A,C,E,G in Supplementary Material), but this reduction could be reversed as early as 7 days following FMT ($p < 0.001$; Figures S4A,C,E,G in Supplementary Material). Interestingly, in all analyzed immunological compartments, Treg numbers were even higher at day 7 post-FMT as compared to naive mice but declined back to basal levels thereafter ($p < 0.01$–0.001; Figures S4A,C,E,G in Supplementary Material).

As for Treg, a strong downregulation of CD86 expression, a costimulatory molecule marking activated DC (36), could be observed in the small intestine, colon, MLN, and spleen upon broad-spectrum antibiotic treatment ($p < 0.001$; Figures S4B,D,F,H in Supplementary Material). Within 7 days following FMT, however, $CD86^+$ cells reached basal levels again (Figures S4B,D,F,H in Supplementary Material). As already described for Treg, activated DC were even more abundant in spleens at day 7 post-FMT as compared to naive mice ($p < 0.001$; Figures S4H in Supplementary Material) but declined to basal levels thereafter.

Hence, memory/effector T cells, Treg, and activated DC are highly microbiota dependent, are disturbed following broad-spectrum antibiotic treatment, but can be restored upon FMT.

Impact of Broad-Spectrum Antibiotic Treatment and Subsequent FMT on Cytokine Production in Murine Intestinal and Systemic Compartments

We further addressed whether microbiota depletion and subsequent reconstitution by FMT affected pro- and anti-inflammatory cytokine expression of $CD4^+$ cells in intestinal and systemic lymphoid compartments by performing intracellular cytokine stainings. Gating strategies are depicted in Figures S2G–I in Supplementary Material. Decreased IFN-γ-expressing $CD4^+$ cells were detected in small and large intestines of ABx mice but increased back to naive levels until day 7 following microbiota reconstitution ($p < 0.001$; **Figures 7A,B**). Of note, at day 7, but not day 28 following FMT, IFN-γ-expressing $CD4^+$ cells were more prominent in MLN as compared to naive controls ($p < 0.05$; **Figure 7C**). Splenic $CD4^+$IFN-γ^+ cells, however, were virtually unaffected by antibiotic treatment and bacterial reconstitution (n.s.; **Figure 7D**). Notably, $CD4^+$ cells expressing IL-17 and IL-22 were downregulated in small and large intestines, in MLN as well as in the spleen upon broad-spectrum antibiotic treatment but could be reversed as early as 7 days following FMT ($p < 0.01$–0.001; **Figure 8**). In addition, $CD4^+IL17^+$ cells in the MLN and spleen as well as small and large intestinal $CD4^+$ $IL\text{-}22^+$ cells were even higher at day 7 following FMT as compared to naive animals ($p < 0.05$–0.001; **Figures 8B,D,E,G**) but declined back to basal levels thereafter ($p < 0.01$–0.001). Furthermore, a strong reduction of $CD4^+$ lymphocytes producing the anti-inflammatory cytokine IL-10 could be determined in all immunological sites following antibiotic therapy ($p < 0.001$; **Figure 9**). FMT could completely restore this cell population in all examined lymphoid compartments. Importantly, higher levels of IL-10 productions were observed in the small intestinal LP and MLN of mice at day 7 post-FMT than in their naive counterparts ($p < 0.01$–0.001; **Figures 9A,C**).

These findings were further supported by results obtained from mRNA analysis of respective cytokines measured in ileal and colonic *ex vivo* biopsies (Figure S5 in Supplementary Material). IFN-γ expression was downregulated upon antibiotic treatment in the small intestine only, an effect that was fully reversed until day 28 following FMT (Figure S5A in Supplementary Material). Moreover, antibiotic treatment resulted in strong suppression of both IL-17 and IL-22 mRNA expression. In the colon, expression

of respective cytokines fully recovered as early as 7 days post-microbiota reconstitution, whereas small intestinal IL-17 mRNA levels measured at day 28 following FMT were approximately 300 times higher than in ABx mice, but still significantly lower as compared to naive SPF control mice (Figures S5B,C in Supplementary Material). In recolonized mice, a trend toward higher small intestinal expression of IL-22 mRNA could be observed (n.s.; Figures S5B,C in Supplementary Material). Furthermore ABx mice also displayed lower levels of IL-10 mRNA expression in both small and large intestines than their conventional counterparts (Figure S5D in Supplementary Material). At day 7 post-FMT, a strong IL-10 response could be only observed in the colon, whereas 28 days after microbiota reassociation, IL-10 expression reached naive levels in both compartments (Figure S5D in Supplementary

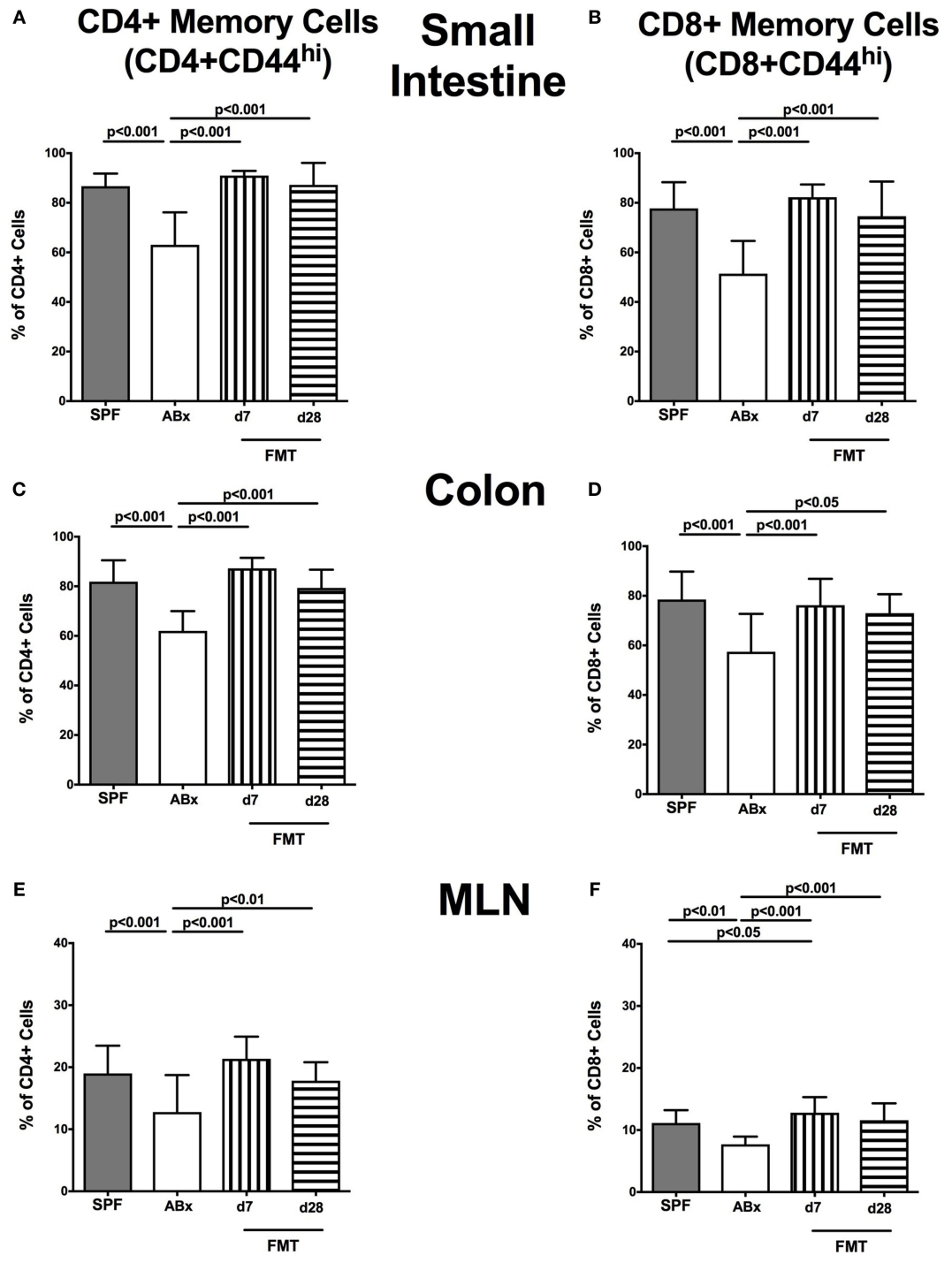


FIGURE 6 | **Continued**

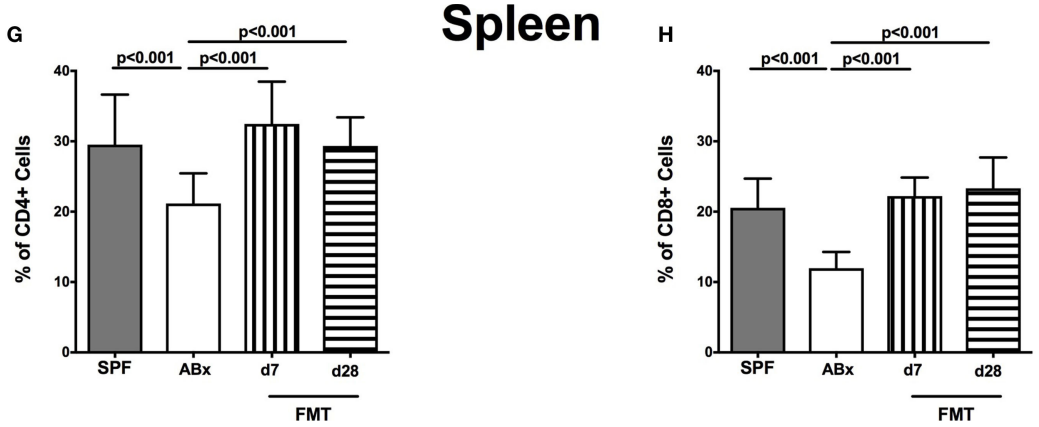


FIGURE 6 | Memory/effector T cell compartment in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice. The proportions of $CD4^+$ memory/effector cells [$CD4^+CD44^{hi}$, gated on $CD4^+$ cells, left panel **(A,C,E,G)**] and $CD8^+$ memory/effector cells [$CD8^+CD44^{hi}$, gated on $CD8^+$ cells, right panel **(B,D,F,H)**] within the small intestine **(A,B)**, colon **(C,D)**, mesenteric lymph nodes (MLN) **(E,F)**, and spleen **(G,H)** of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

Material). Hence, long-term broad-spectrum antibiotic treatment leads to suppression of both pro- and anti-inflammatory cytokines. These effects can, however, almost fully be reversed by recolonization with complex murine intestinal microbiota.

DISCUSSION

The complex mutualistic microbiota–host relationships, particularly the microbial stimuli-induced changes of immune cell homeostasis have elicited increased interest in recent years. Although isolator-raised GF mice have greatly advanced our understanding of the microbiota impact on the immune system, they do not address the role of microbiota-induced changes in host immunity later in life, given that profound immunological changes such as hypoplastic PP, reduced numbers of IgA-producing plasma cells (37), and LP $CD4^+$ T cells (38), smaller germinal centers in MLN and poorly formed B and T cell zones in the spleens and lymph nodes could be shown in these mice (6, 7). Furthermore, increased evidence regarding pathologies related to antibiotic therapy [reviewed in Ref. (39)] including antibiotic-associated diarrhea (40), allergic inflammation (41), and asthma (42) has underlined the impact of antibiotics induced perturbations of the intestinal microbiota on host physiology.

In the present study, we applied a mouse model suitable to dissect the interplay between microbiota, antibiotics, and host immune system in conventionally raised and developed mice. Following broad-spectrum antibiotic treatment secondary abiotic (i.e., gnotobiotic), mice were virtually lacking the intestinal microbiota as shown by cultural as well as by highly sensitive culture-independent molecular methods (5, 24, 43). Most strikingly, quantitative 16S rRNA based real-time PCR analysis presented here revealed that gene copy numbers of the main bacterial intestinal groups did not differ between fecal samples taken from secondary abiotic mice and autoclaved food pellets. Notably, it is rather impossible to differentiate whether detected 16S rRNA gene copies in ABx mice were derived from avital ("dead") or viable (fastidious/uncultivable) bacterial cells. Hence, secondary abiotic mice provide the following advantages [as reviewed in Ref. (43)]: first, they circumvent the developmental anomalies of isolator-raided GF mice and offer second, the opportunity to analyze the antibiotics induced disruption of the commensal intestinal microbiota composition and subsequent adverse consequences ("collateral damages") for host immunity. Third, upon cessation of antibiotic treatment, secondary abiotic mice can be stably reassociated with single bacterial species, with a combination of defined commensals or pathogens or with a complex commensal microbiota derived from mice or even a different host including humans (5, 24, 43). Finally, neither secondary abiotic nor with complex microbiota reassociated mice display any adverse clinical sequelae such as wasting or diarrhea or microscopic signs of intestinal inflammation including epithelial apoptosis. We could further confirm that the intestinal microbiota composition was stable for at least 4 weeks following FMT and similar to conventionally colonized naive mice.

The reintroduction of complex microbiota into the host *via* FMT is a well-known therapy dating back to the Chinese Dong-jin dynasty in the fourth century (44) and has undergone a renaissance recently as a therapeutic option for the treatment of recurrent and refractory *C. difficile* toxin induced acute necrotizing pseudo-membranous enterocolitis (45–49).

Initial small and large intestinal *in situ* analysis of distinct innate as well as adaptive immune cell populations revealed that macrophages and monocytes as well as T lymphocytes, Treg, and B lymphocytes, respectively, were drastically reduced following antibiotic therapy but could be restored by FMT in a time-dependent fashion. This reinforced the microbiota dependent dynamics of mucosal immune cell homeostasis as prior evidence has revealed for both innate (22, 50) and adaptive immune responses (51, 52).

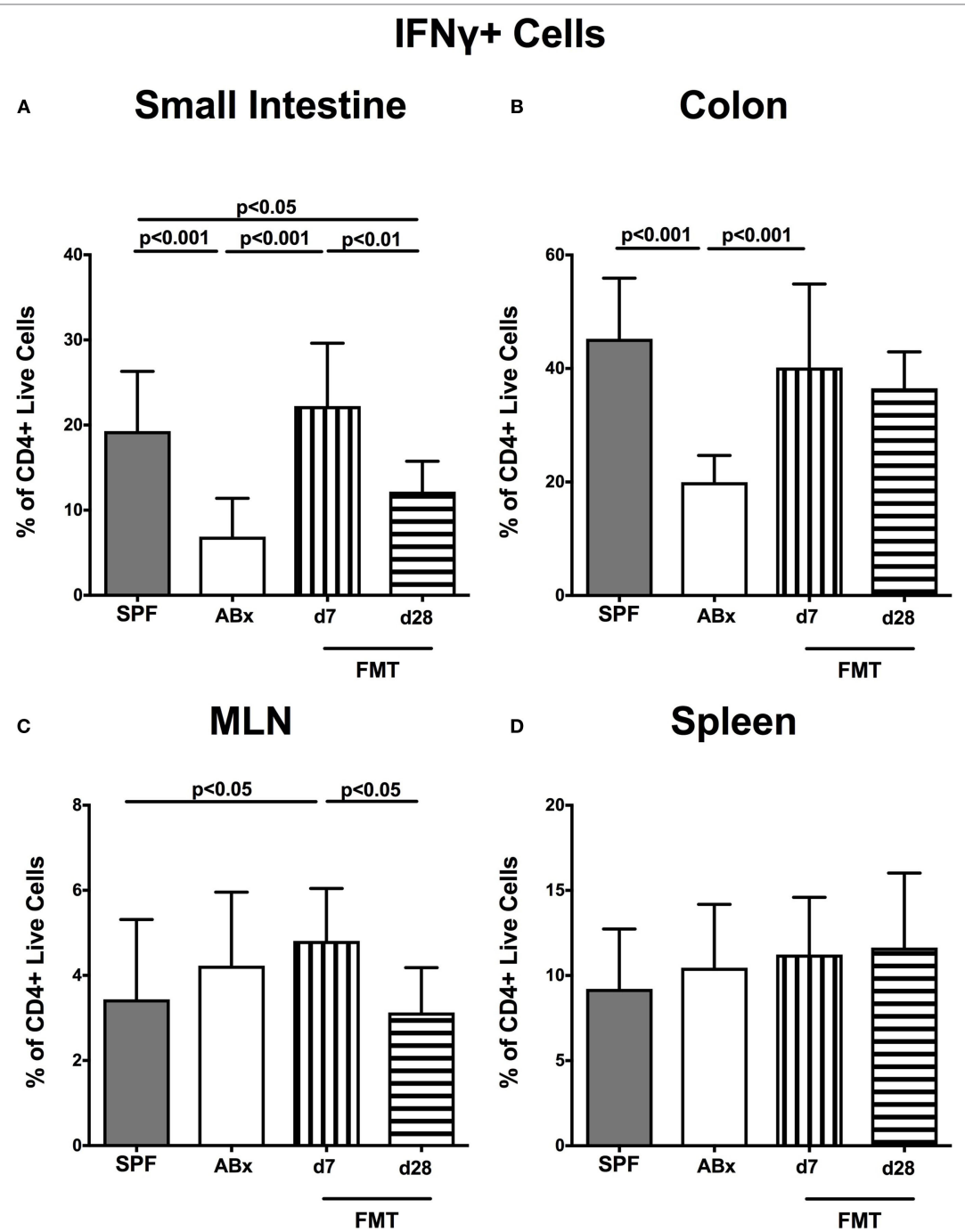
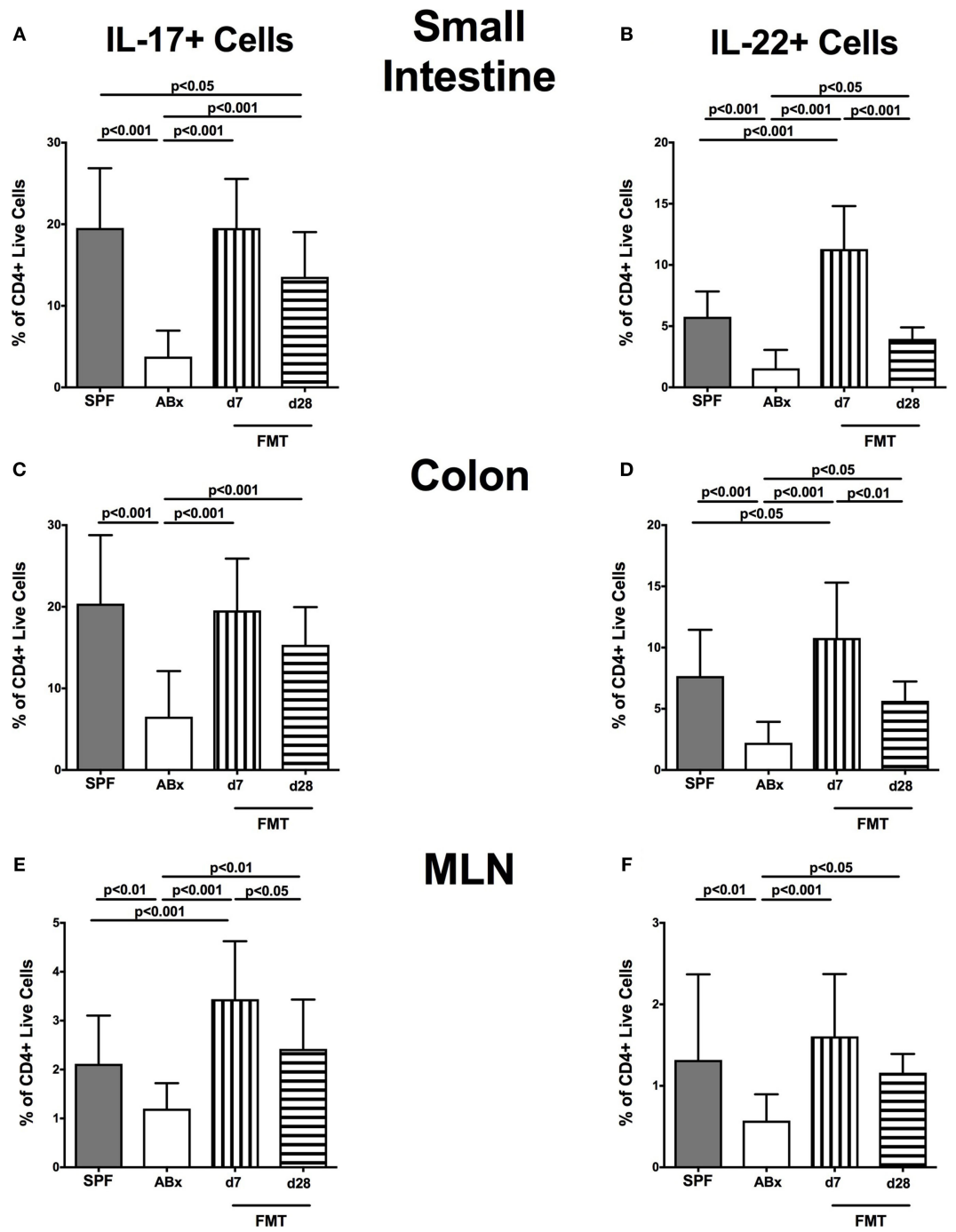


FIGURE 7 | IFN-γ-producing CD4⁺ cells in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice. The percentages of IFN-γ-producing CD4⁺ cells in the **(A)** small intestine, **(B)** colon, **(C)** mesenteric lymph nodes (MLN), and **(D)** spleen of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

These data were supported by more detailed flow-cytometric analyses of lymphocytes isolated from different intestinal compartments including the small intestine, colon, and MLN. Overall, broad-spectrum antibiotics decreased distinct immune cell subsets such as Th cells, cytotoxic T cells, memory and effector T cells, B lymphocytes, Treg as well as activated DC, whereas reintroduction of the complex microbiota could sufficiently reverse the immune-depressive effects exerted by the antibiotic compounds. We could further observe, however, that immunological sites of the different levels (i.e., local/intestinal, extra-intestinal/systemic) do not always respond in the same manner and to a comparable extent to the absence of or reassociation

with intestinal microbiota. For instance, a decline of $CD4^+$, $CD8^+$, and $B220^+$ cell numbers in the small and large intestinal LP following antibiotic therapy was conversely associated with an increase of the respective immune cell populations in the spleen, pointing toward a possible centralization of lymphocytes due to missing interactions with bacterial antigens in the intestinal tract. Moreover, our data suggest an inverse relationship of the mentioned lymphocytic cell subsets between colon and spleen at different time points post-FMT, given that a colonic decrease of $CD4^+$ and $B220^+$ cells was, conversely, paralleled by an increase of the respective cell types at day 28 post-FMT in the spleen. One therefore needs to take into consideration that, while tempting to develop novel approaches to conveniently manipulate gut microbiota, changes in immune cell populations are not restricted to local, i.e., intestinal sites but might also lead to global/systemic consequences. This is also supported by previous studies with



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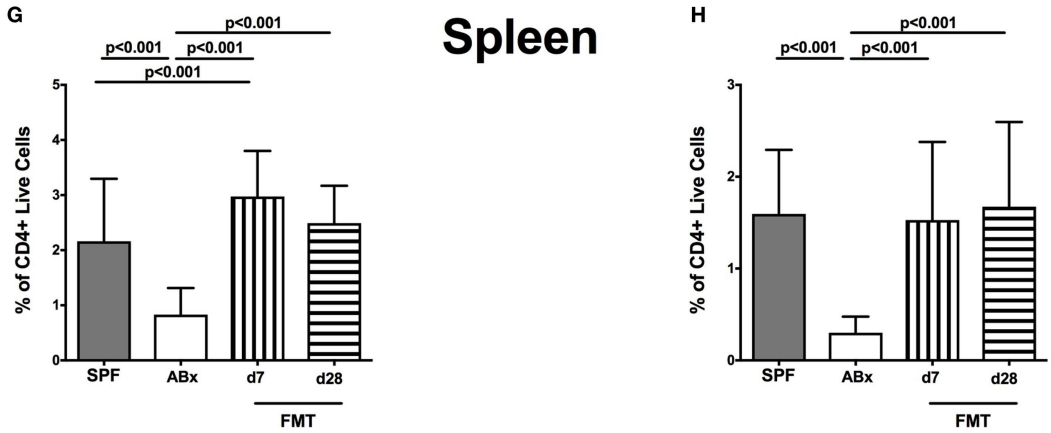

FIGURE 8 | IL-17- and IL-22-producing CD4⁺ cells in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice. The percentages of IL-17- [left panel **(A,C,E,G)**] and IL-22- [right panel **(B,D,F,H)**] producing CD4⁺ cells in the small intestine **(A,B)**, colon **(C,D)**, mesenteric lymph nodes (MLN) **(E,F)**, and spleen **(G,H)** of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

probiotic strains such as *Lactobacillus reuteri* 100-23 inducing systemic anti-inflammatory IL-10 production (53) or *Lactobacillus casei* (DN-114 001) alleviating skin inflammation (54).

One explanation for kinetic differences in reconstituting cell types following FMT of ABx mice could be that a minimum of time is required to fully compensate for the prominent collateral damages to the intestinal ecosystem and immune system that were caused by long-term antibiotic treatment. To accomplish this following FMT, the bacteria need to allocate niches, redevelop an intraluminal equilibrium for both bacteria–bacteria and microbiota–immune cell interactions. Together with data showing long-lasting consequences of antibiotic therapy on the human gut ecosystem (55, 56), these findings emphasize the need for considering long-term effects on immunity in patients undergoing antibiotic treatment.

Strikingly, recolonization with complex intestinal microbiota could not sufficiently recover CD8⁺ cell numbers in the colonic LP, suggesting that antibiotic treatment affects this cell population through commensal-independent mechanisms. While underlying mechanisms still need to be unraveled, this would fit with prior data already describing microbiota-independent immunomodulatory effects of antibiotic compounds such as macrolides (57) and fluoroquinolones (58). Immunomodulatory properties of macrolides were especially recognized due to their effectiveness in treating diffuse panbronchitis, a complex pulmonary disorder afflicting mainly East Asians (59), and have been confirmed by numerous *in vitro* and *in vivo* experiments. In mammalian host cells, for instance, macrolides impact the mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 (ERK 1/2) and nuclear factor-kappa B (NF-κB) pathways subsequently leading to inhibition of mucus secretion, suppression of the production and secretion of pro-inflammatory cytokines, inhibition of cell proliferation, suppression of iNOS-mediated NO production, and inhibition of chemotaxis [as reviewed in Ref. (57, 59)]. Moreover, *in vitro* experiments revealed that the fluoroquinolone moxifloxacin decreased the TNF and IL-1 production by lipopolysaccharide (LPS)-stimulated human monocytes (60). Fluoroquinolones have also been shown to protect mice from both lethal and sublethal LPS challenges by significantly reducing serum levels of pro-inflammatory cytokines such as IL-6, IL-12, and TNF (61, 62). Evidence suggests that fluoroquinolones affect the intracellular cyclic adenosine-3,5-monophosphate and phosphodiesterases as well as transcription factors such as NF-κB, activator protein 1, and NF of activated T cells [as reviewed in Ref. (58)].

Remarkably, one study revealed that two-thirds of intestinal gene expression alterations in antibiotic-treated mice occur microbiota independently, particularly affecting mitochondrial genes coding for electron transport chains, oxidation-reduction, ATP biosynthesis, and cellular and mitochondrial ribosomes (63).

In terms of activation status and cytokine profiling of immune cell populations, we could observe a rather different situation. Antibiotic treatment resulted in a strong reduction of Treg, activated DC, and of CD4⁺ and CD8⁺ memory/effector cells in all examined immunological sites, whereas a virtually complete recovery of these cell populations could be observed upon recolonization with complex microbiota, given that at day 28 post-FMT, the overall situation resembled that of naive untreated mice. Similarly, antibiotic-treated mice revealed declined production of IFN-γ in colon and ileum, and of IL-17, IL-22, and IL-10 in small and large intestines, MLN, and spleen. These effects could, however, be completely restored following FMT. Our data are well in line with previous findings stating the importance of microbiota-driven signaling for the expansion of cytokine-producing CD4⁺ cells in the gut (64). Moreover, it has been shown that fecal reassociation of GF mice simultaneously drives pro-inflammatory and regulatory immune responses (65). Taken together, these results emphasize the indispensable importance of the intestinal microbiota for differentiation of immune cells and maintenance of immune system homeostasis and confirm their capacity of restoring several impairments following antibiotic treatment. Furthermore, the antibiotics induced reduction of the

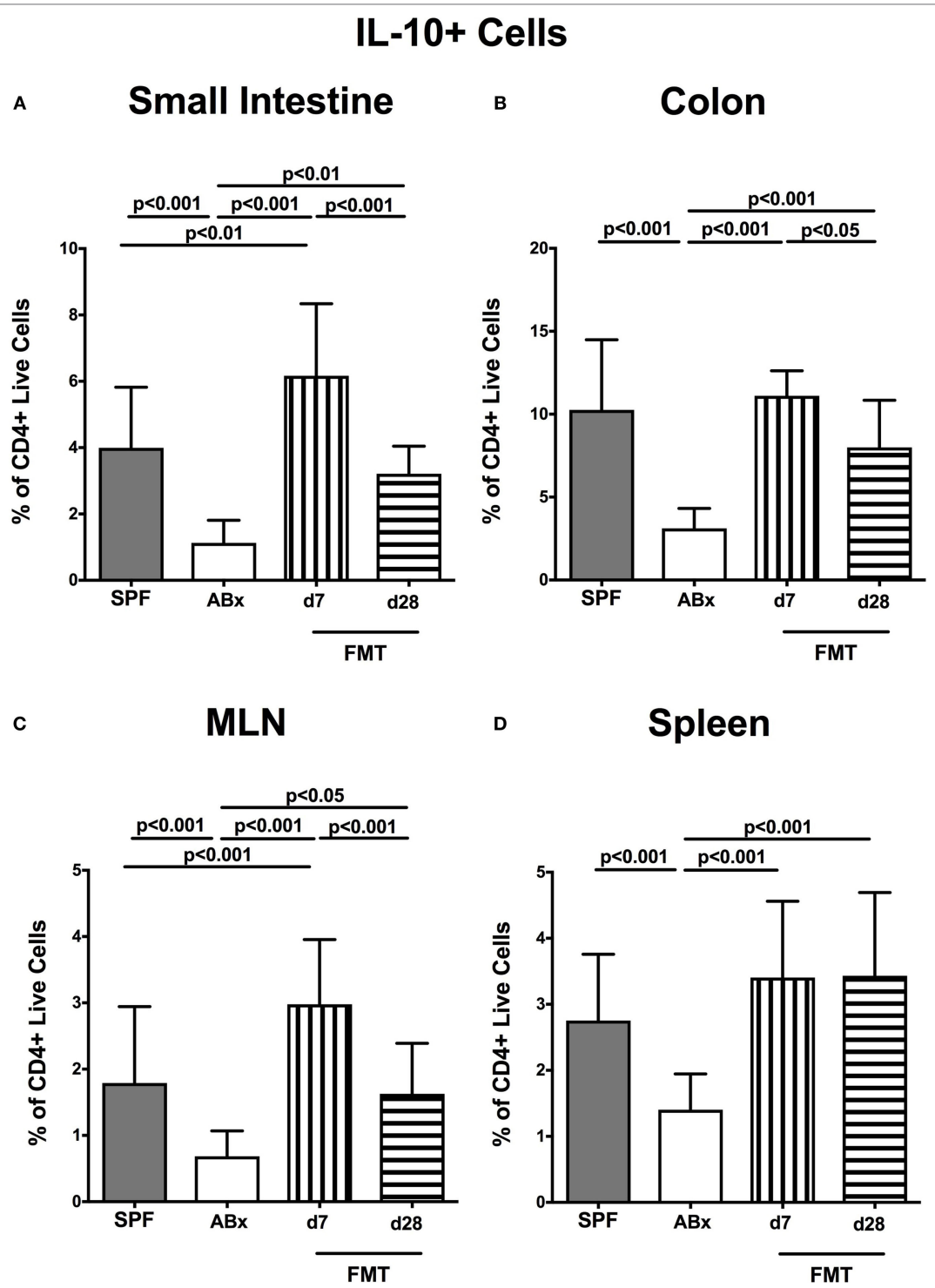

FIGURE 9 | IL-10-producing CD4⁺ cells in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice. The percentages of IL-10-producing CD4⁺ cells in the **(A)** small intestine, **(B)** colon, **(C)** mesenteric lymph nodes (MLN), and **(D)** spleen of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

Th17 cell compartment, which is important in protection against bacterial and fungal pathogens, particularly those encountered at mucosal surfaces (66), is well in line with the increased susceptibility of microbiota-depleted mice to pathogens (67, 68). This increased susceptibility to infection and inflammation may also be further amplified by the observed lower IL-10 levels in

the lymphoid compartments with subsequent consequences for anti-inflammatory Treg-mediated responses.

Summary and Conclusion

In the present study, we have focused on the effects of a complex murine microbiota on the immune system following antibiotics induced impairments not only of the intestinal ecosystem but also of peripheral as well as systemic immune functions. Whether the here displayed beneficial restoring effects exerted by reintroduced microbial antigens are due to the large bacterial loads, complexity and/or diversity of the introduced complex microbiota, or whether distinct species in concert with each other play a more important role in an orchestrated fashion, with the host immune system as the conductor, should be unraveled in more detail, but appears literally rather as a search for the needle in the hay stack. Nevertheless, it remains an outstanding and challenging issue to characterize the effects of single species and their products on the balance between pro-inflammatory and regulatory immune responses.

We are all aware of the fact that a rational and responsible antibiotic treatment is unavoidable under specific clinical conditions, but it is crucial to keep the effects of this therapy on the immune system in mind. These effects might be due to potential immune-modulating properties of the antimicrobial compound itself and/or due to microbiota-modulating (-depleting) sequelae of therapy or prophylaxis. Further knowledge of the orchestrated microbiota–host interplay could offer valuable contributions to the development of novel therapeutic approaches including strategies to enhance immunity and manipulating microbiota composition toward more beneficial (i.e., probiotic) species.

ETHICS STATEMENT

All animal experiments were carried out according to the European Guideline for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, Germany, registration numbers G0097/12 and G0184/12). Animal welfare was examined twice daily by assessment of clinical conditions.

AUTHOR CONTRIBUTIONS

IE performed experiments, analyzed data, and wrote paper. EK, UF, and UE performed experiments, analyzed data, and co-edited paper. CN and PB suggested critical parameters in design of experiments and supplied antibodies. AS provided advice in design and performance of experiments. AK analyzed data and co-edited paper. SB provided advice in design and performance of experiments and co-edited paper. MH designed and performed experiments, analyzed data, and co-wrote paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00397/full#supplementary-material.

FIGURE S1 | **Intestinal microbiota of conventional and reconstituted secondary abiotic mice**. At day (d) 7 (open squares; $n = 16$) and d28 (black squares; $n = 18$) following fecal microbiota transplantation, the intestinal microbiota composition was assessed in fecal samples 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 nanogramg DNA): total eubacterial load (TL), enterobacteria (EB), enterococci (EC), lactic acid bacteria (LB), bifidobacteria (BIF), *Bacteroides/Prevotella* spp. (BP), *Clostridium coccoides* group (CLOCC), *Clostridium leptum* group (CLEP), and *Mouse Intestinal Bacteroides* (MIB). Conventionally colonized mice served as naive controls (N, black circles; $n = 13$).

FIGURE S2 | **Gating strategies of extracellular stainings on one representative splenic sample after gating for lymphocytes and including only single cells**. **(A)** Exclusion of dead cells *via* LIVE/DEAD Fixable Aqua Dead Cell Stain kit (L/D) in the forward scatter, **(B)** $CD4^+$ and $CD8^+$ cells gated on living cells, **(C)** B cells and activated DC gated on $CD4^-CD8^-$ cells, **(D)** regulatory T cells, and **(E)** $CD4^+$ memory/effector cells gated on $CD4^+$ cells, **(F)** $CD8^+$ memory/effector cells gated on $CD8^+$ cells. Gating strategies of intracellular stainings on one representative ileal sample after gating for lymphocytes and including only single cells. **(G)** Identification of $CD4^+$ living cells by exclusion of dead cells *via* LIVE/DEAD Fixable Aqua Dead Cell Stain kit (L/D), **(H)** IFN-γ and IL-10 gating, and **(I)** IL-17 and IL-22 gating.

FIGURE S3 | **$B220^+$ cells in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice**. The percentages [left panels **(A,C,E,G)**] and cell numbers [right panels **(B,D,F,H)**] of the $B220^+$ lymphocyte population within the small intestine **(A,B)**, colon **(C,D)**, mesenteric lymph nodes (MLN) **(E,F)**, and spleen **(G,H)** of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

FIGURE S4 | **Regulatory T cells (Treg) and activated DC in intestinal and systemic compartments of secondary abiotic and microbiota-reconstituted mice**. The frequencies of Treg ($CD4^+$ $CD25^+$, gated on $CD4^+$ cells) [left panels **(A,C,E,G)**] and activated DC [$CD86^+$, gated on $CD4^-CD8^-$ live cells, right panels **(B,D,F,H)**] in the small intestine **(A,B)**, colon **(C,D)**, mesenteric lymph nodes (MLN) **(E,F)**, and spleen **(G,H)** of naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) post-fecal microbiota transplantation (FMT) are depicted.

FIGURE S5 | **mRNA analysis of pro- and anti-inflammatory cytokines in small intestinal and colonic tissue**. RT-PCR results of **(A)** IFN-γ, **(B)** IL-17, **(C)** IL-22, and **(D)** IL-10 expression in small intestinal (upper panel) and colonic (lower panel) tissues derived from naive conventional mice (SPF, gray bars), secondary abiotic mice (ABx, white bars), and recolonized mice at day (d) 7 (boxes with vertical lines) and d28 (bars with horizontal lines) following fecal microbiota transplantation (FMT) are depicted.

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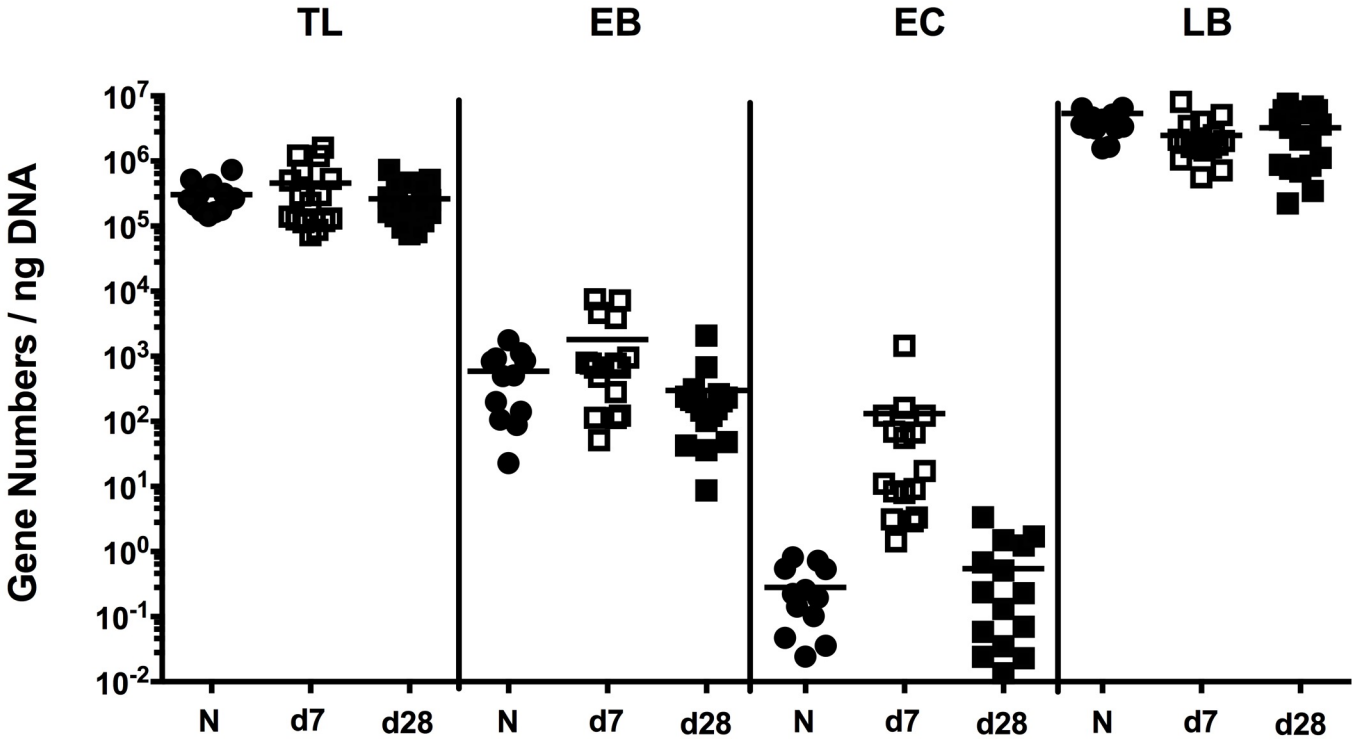
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figure S1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EC
LB
Gene Numbers / ng DNA
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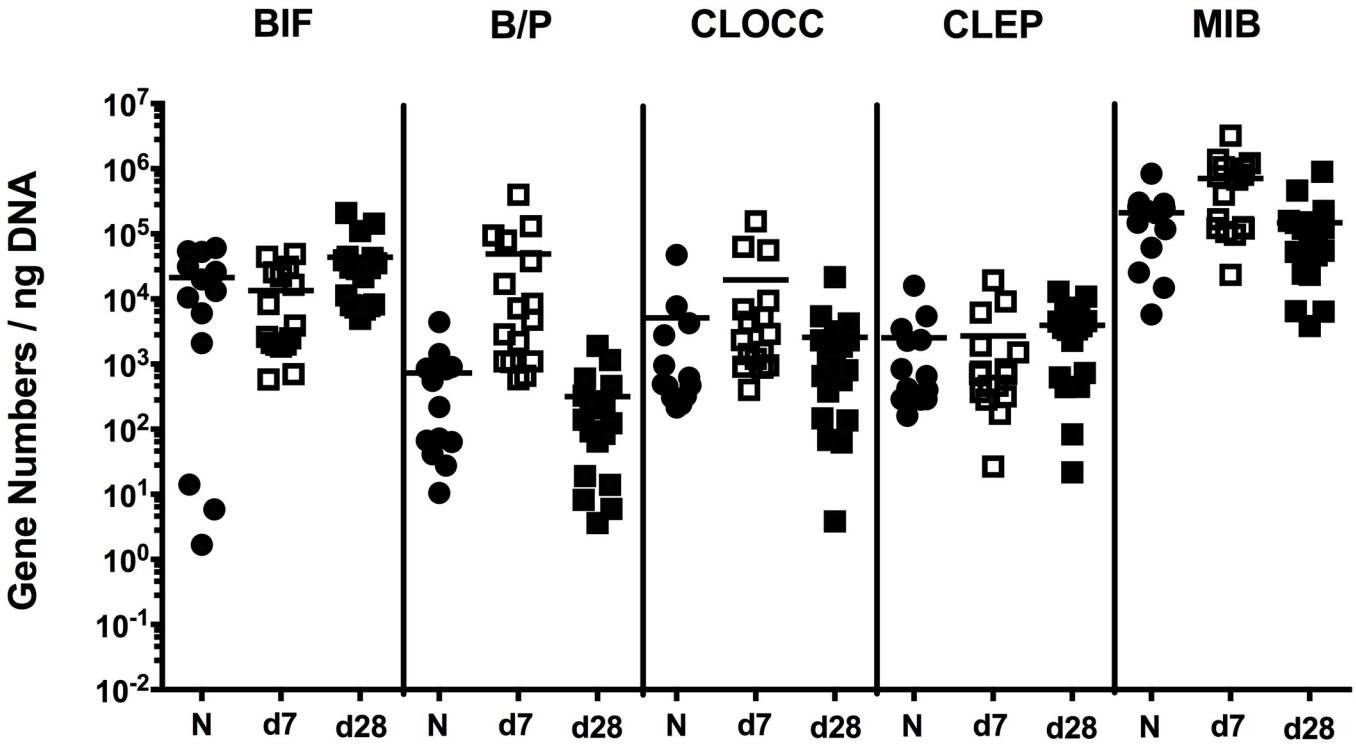
N
d7
d28


BIF
B/P
CLOCC
CLEP
MIB
Gene Numbers / ng DNA
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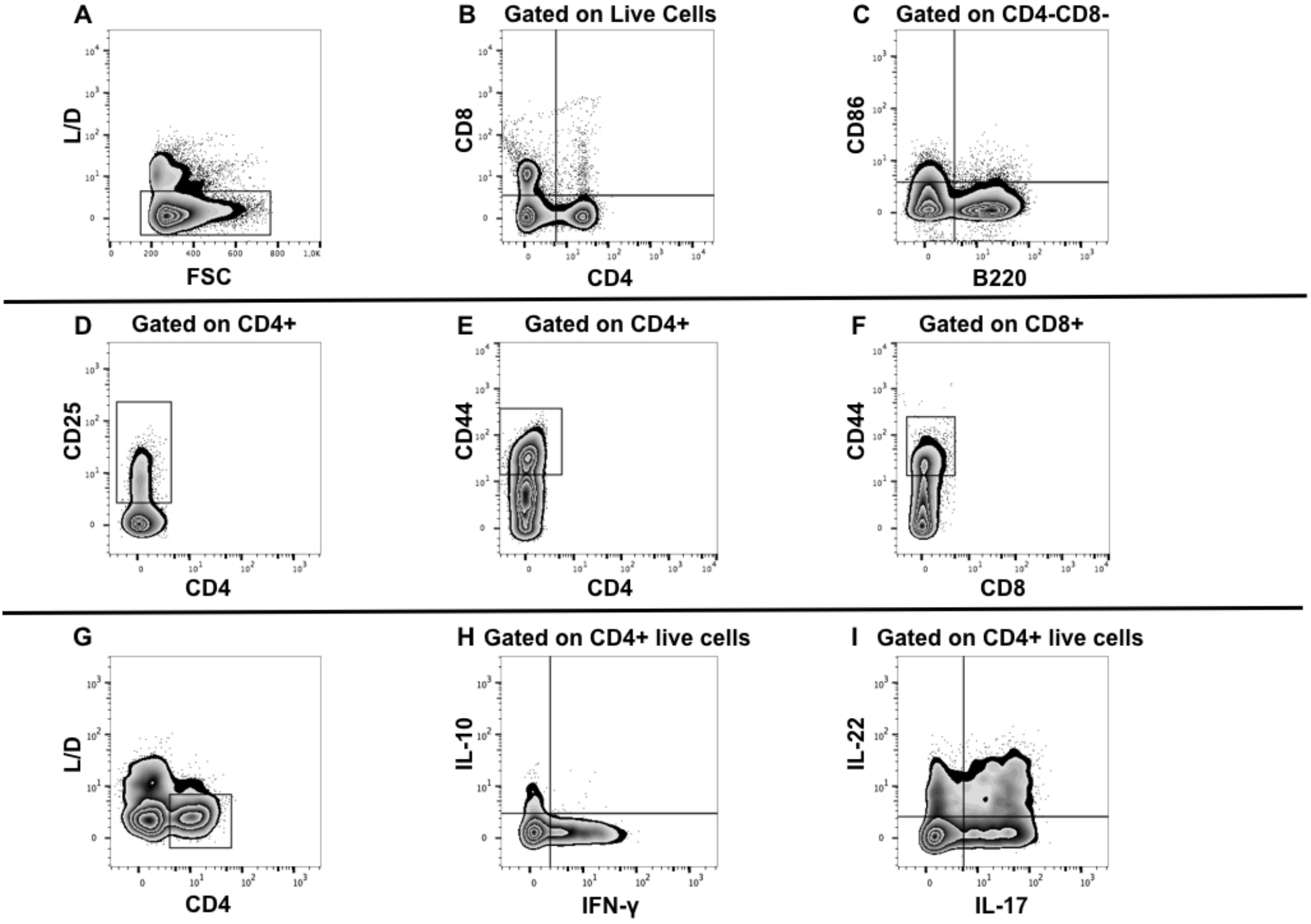
10^{-2}
N
d7
d28

Figure S2


A
L/D
FSC
B Gated on Live Cells
CD8
CD4
C Gated on CD4-CD8-
CD86
B220
D Gated on CD4+
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CD4
E Gated on CD4+
CD44
CD4
F Gated on CD8+
CD44
CD8
G
L/D
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IFN-γ
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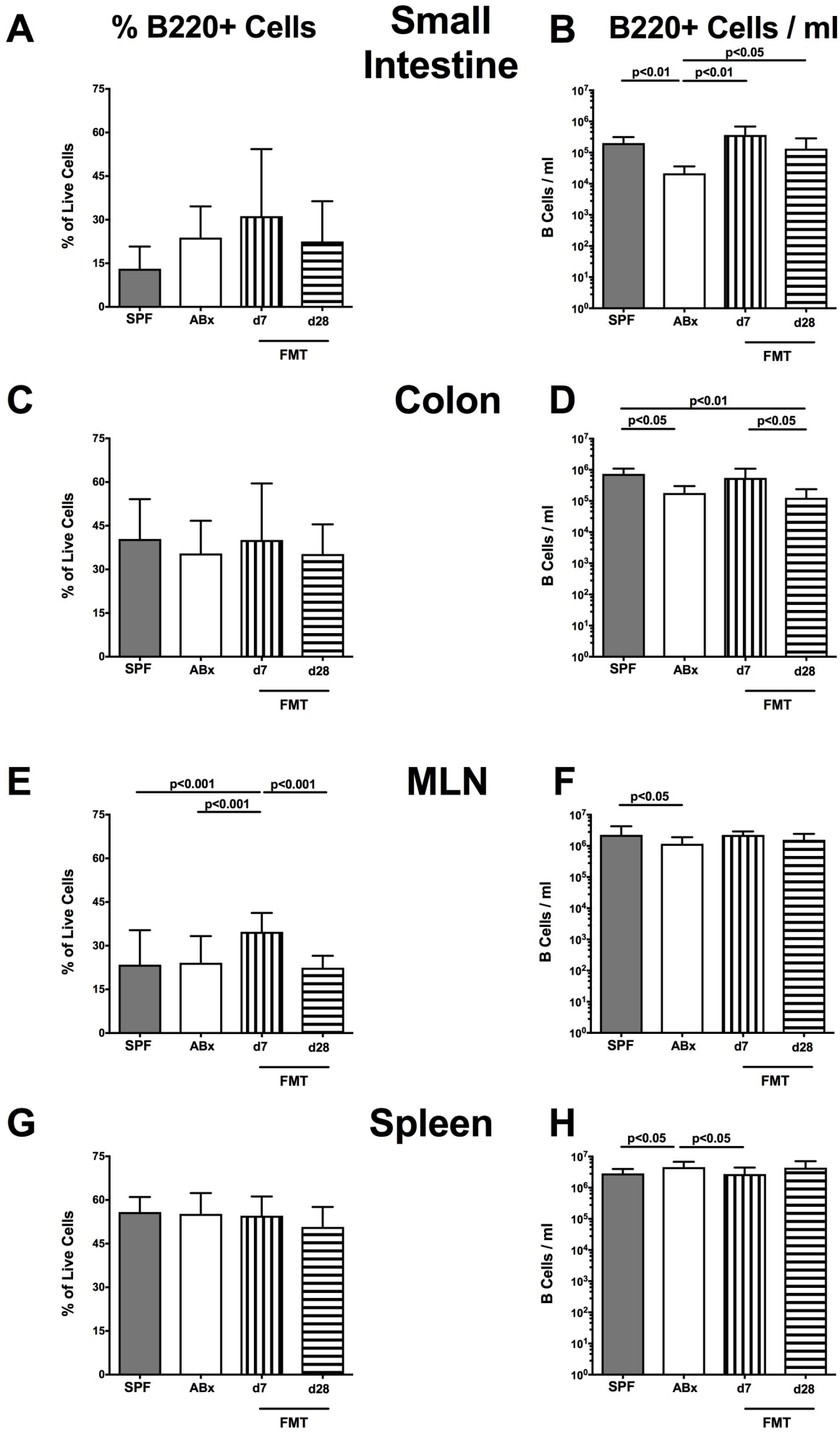
IL-22
IL-17

Figure S3


A
% B220+ Cells
Small Intestine
B
B220+ Cells / ml
p<0.01
p<0.01
p<0.05
% of Live Cells
B Cells / ml
SPF
ABx
d7
d28
FMT
C
Colon
D
p<0.01
p<0.05
p<0.05
E
MLN
F
p<0.001
p<0.001
p<0.001
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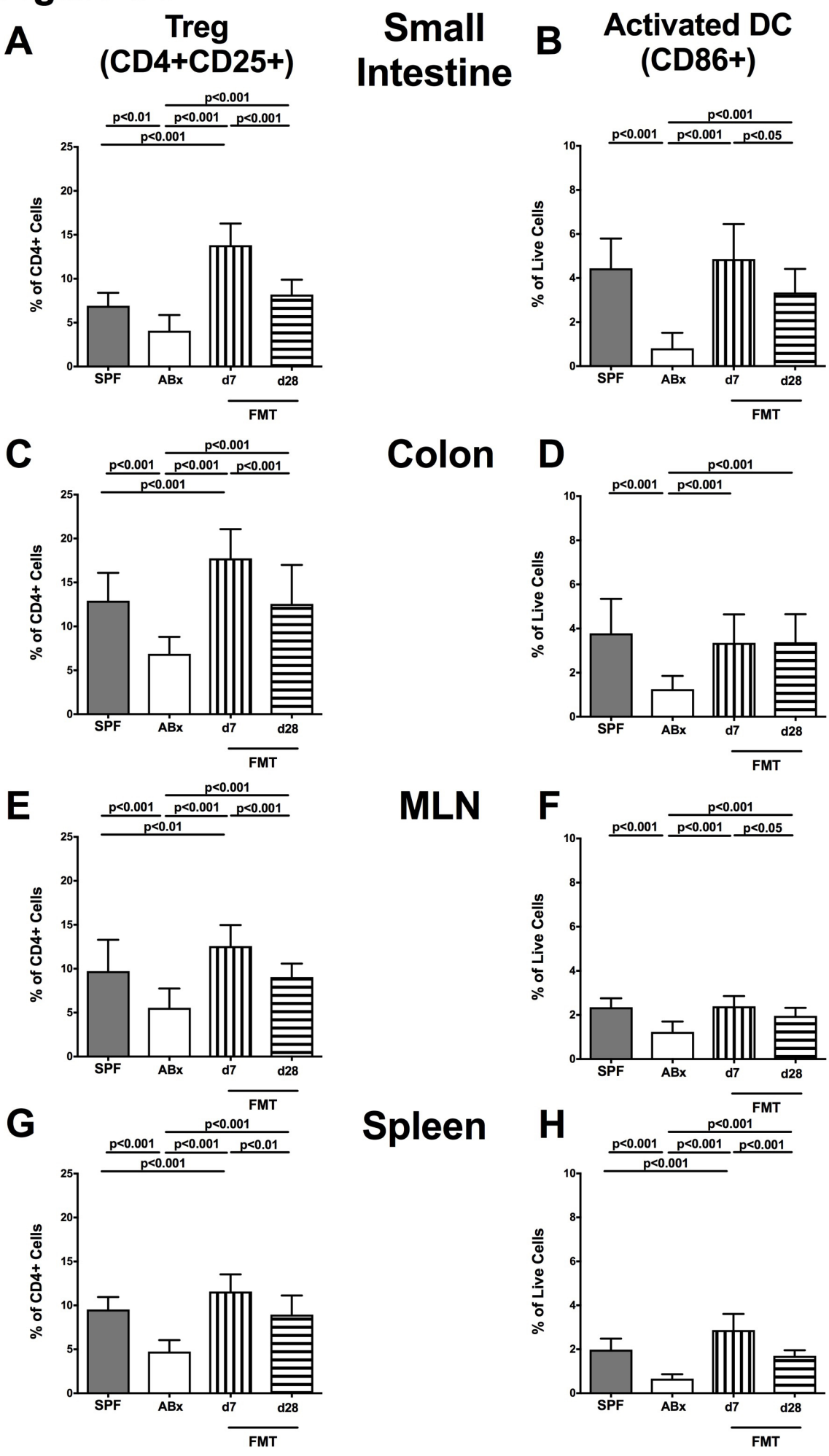
G
Spleen
H
p<0.05
p<0.05

Figure S4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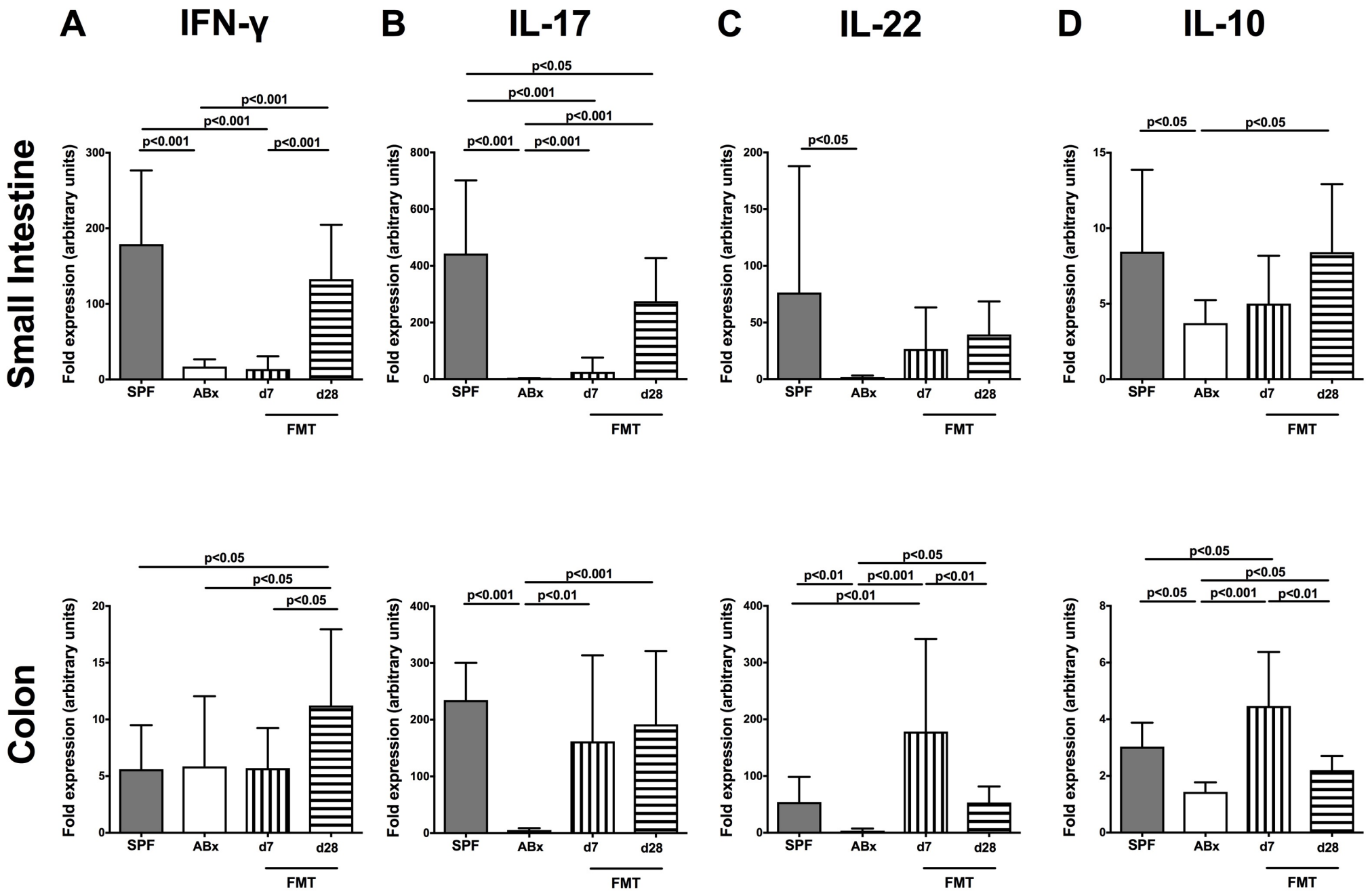
Treg (CD4+CD25+)
Small Intestine
B
Activated DC (CD86+)
% of CD4+ Cells
% of Live Cells
SPF
ABx
d7
d28
FMT
p<0.001
p<0.01
p<0.05
C
Colon
D
E
MLN
F
G
Spleen
H

Figure S5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IFN-γ
B
IL-17
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IL-22
D
IL-10
Small Intestine
Colon
Fold expression (arbitrary units)
SPF
ABx
d7
d28
FMT
p<0.001
p<0.05
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The Probiotic Compound VSL#3 Modulates Mucosal, Peripheral, and Systemic Immunity Following Murine Broad-Spectrum Antibiotic Treatment

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There is compelling evidence linking the commensal intestinal microbiota with host health and, in turn, antibiotic induced perturbations of microbiota composition with distinct pathologies. Despite the attractiveness of probiotic therapy as a tool to beneficially alter the intestinal microbiota, its immunological effects are still incompletely understood. The aim of the present study was to assess the efficacy of the probiotic formulation VSL#3 consisting of eight distinct bacterial species (including *Streptococcus thermophilus, Bifidobacterium breve, B. longum, B. infantis, Lactobacillus acidophilus, L. plantarum, L. paracasei, and L. delbrueckii* subsp. *Bulgaricus*) in reversing immunological effects of microbiota depletion as compared to reassociation with a complex murine microbiota. To address this, conventional mice were subjected to broad-spectrum antibiotic therapy for 8 weeks and perorally reassociated with either VSL#3 bacteria or a complex murine microbiota. VSL#3 recolonization resulted in restored CD4+ and CD8+ cell numbers in the small and large intestinal lamina propria as well as in B220+ cell numbers in the former, whereas probiotic intervention was not sufficient to reverse the antibiotic induced changes of respective cell populations in the spleen. However, VSL#3 application was as efficient as complex microbiota reassociation to attenuate the frequencies of regulatory T cells, activated dendritic cells and memory/effector T cells in the small intestine, colon, mesenteric lymph nodes, and spleen. Whereas broad-spectrum antibiotic treatment resulted in decreased production of cytokines such as IFN-γ, IL-17, IL-22, and IL-10 by CD4+ cells in respective immunological compartments, VSL#3 recolonization was sufficient to completely recover the expression of the anti-inflammatory cytokine IL-10 without affecting pro-inflammatory mediators. In summary, the probiotic compound VSL#3 has an extensive impact on mucosal, peripheral, and systemic innate as well as adaptive immunity, exerting beneficial anti-inflammatory effects in intestinal as well as systemic compartments. Hence, VSL#3 might be considered a therapeutic immunomodulatory tool following antibiotic therapy.

Keywords: probiotics, antibiotics, innate and adaptive immunity, microbiota, fecal microbiota transplantation, secondary abiotic (gnotobiotic) mice, mucosal and peripheral and central immunity

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INTRODUCTION

In the past decades the commensal gut microbiota has been established as an indispensable major key factor in host physiology. The microbiota has been shown to be involved in numerous physiological processes, including vitamin synthesis (LeBlanc et al., 2013), food digestion (Hooper et al., 2002), fat metabolism (Backhed et al., 2004), intestinal angiogenesis (Stappenbeck et al., 2002), enteric nerve function (Husebye et al., 1994), protection from pathogens (Sekirov et al., 2008; Bereswill et al., 2011), and immune system development (Cebra, 1999). Moreover, perturbations of the complex host resident intestinal ecologic system, termed dysbiosis, have been linked to a wide range of pathological conditions including intestinal disorders such as inflammatory bowel diseases (IBD; Baumgart and Carding, 2007), irritable bowel syndrome (IBS; Carroll et al., 2010), and coeliac disease (De Palma et al., 2010), as well as extra-intestinal pathologies such as allergy and asthma (Noverr and Huffnagle, 2004), arthritis (Taurog et al., 1994), type 1 diabetes mellitus (Wen et al., 2008), obesity (Backhed et al., 2007), multiple sclerosis (Ochoa-Reparaz et al., 2009), and distinct cardiovascular diseases (Serino et al., 2014).

With this growing body of evidence concerning the pivotal role of the microbiota in health and disease, the potential of altering and modulating the microbiota composition in beneficial ways has become an increasing focus of attention. Microbiota-modulating intervention strategies include administration of probiotics, defined as live microorganisms conferring a health benefit on the host when administered in adequate amounts (FAO/WHO, 2002). So far, the most emphasis has been laid on investigating the role of probiotic compounds in intestinal inflammation. For instance, *Escherichia coli* strain Nissle 1917 was shown to prevent acute and chronic colitis (Kamada et al., 2005) and to enhance mucosal barrier functions in mice (Ukena et al., 2007; Wassenaar, 2016). Moreover, treating IL-10 deficient mice with *Lactobacillus plantarum* attenuated the severity of colonic inflammation by reducing mucosal IL-12p40 and IFN-γ levels (Schultz et al., 2002). Similarly, the application of VSL#3, a probiotic mixture of eight different bacterial strains (namely *Streptococcus thermophilus*, *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii* subsp. *Bulgaricus*) was demonstrated as an effective therapy in both murine IL-$10^{-/-}$ colitis (Jijon et al., 2004) and trinitrobenzenesulphonic acid (TNBS) induced colitis through enhancement of IL-10 and TGF-β expressing T cells (Di Giacinto et al., 2005). Additionally, the induction of TGF-β following oral VSL#3 administration was shown to be effective in ameliorating inflammation in a murine model of T-helper (Th-) 2 cells mediated food allergy (Barletta et al., 2013). The efficacy of probiotic compounds has also been examined in clinical trials with IBD patients. For instance, *E. coli* Nissle 1917 exerted similar efficacy as compared to the established standard medication (i.e., mesalazine) in maintenance therapy of ulcerative colitis (UC) (Kruis et al., 2004). Moreover, a meta-analysis including three controlled trials demonstrated the capability of VSL#3 to induce remission in UC patients (Jonkers et al., 2012). Several studies have also confirmed the protective role of VSL#3 in preventing relapses of pouchitis (Gionchetti et al., 2003), a condition developed by ∼50% of UC patients following ileo-anal pouch anastomosis (Shen and Lashner, 2008). In contrast, studies in patients suffering from Crohn's disease (CD) did not unravel a beneficial role of probiotics, neither in induction nor maintenance of remission of this inflammatory disease (Shen et al., 2014).

Several mechanisms to explain the beneficial role of probiotics have been proposed including enhancement of intestinal barrier functions (Ukena et al., 2007), amendment of microbiota diversity, and modulation of the innate and adaptive immune system (Grabig et al., 2006). However, these mechanisms remain in need of further investigation.

In the present study, we focussed on the impact of the commercial probiotic compound VSL#3 on restoring distinct immune cell functions that were affected in mice upon broad-spectrum antibiotic treatment. We therefore performed a comprehensive analysis of the mucosal (i.e., ileal and colonic lamina propria lymphocytes, LPL), peripheral (i.e., mesenteric lymph nodes, MLN) and systemic (i.e., splenic lymphocytes) immune responses in conventional mice with a depleted microbiota following 8 weeks of broad-spectrum antibiotic treatment and upon reassociation with either VSL#3 or fecal microbiota transplantation (FMT) as compared to mice without antibiotic challenge.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted according to the European Guideline for animal welfare (2010/63/EU) with approval from the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, Germany, registration number G0184/12 and G0097/12).

Mice

Animals were bred and maintained in the facilities of the "Forschungseinrichtungen für Experimentelle Medizin" (FEM, Charité – Universitätsmedizin, Berlin, Germany) under specific pathogen-free (SPF) conditions. Female age matched C57BL/6j wildtype mice were used.

Generation of Microbiota Depleted Mice and Bacterial Recolonization

To eradicate the murine intestinal microbiota 8–10 week old mice were transferred to sterile cages and treated with a quintuple broad-spectrum antibiotic cocktail as previously described (Heimesaat et al., 2006). Three days prior recolonization

Abbreviations: ABx, secondary abiotic; BSA, bovine serum albumin; CD, Crohn's disease; CFU, colony forming units; DC, dendritic cells; DTE, dithioerythritol; EDTA, ethylene diamine tetraacetic acid; FCS, fetal calf serum; FMT, fecal microbiota transplantation; HBSS, Hanks balanced salt solution; HPF, high power field; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; LPL, lamina propria lymphocytes; MLN, mesenteric lymph nodes; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; SPF, special pathogen free; Th, T helper cell; TNBS, trinitrobenzenesulphonic acid; Treg, regulatory T cells; UC, ulcerative colitis.

experiments the antibiotic cocktail was withdrawn and replaced by autoclaved drinking water. For FMT, fresh murine fecal samples were collected from 10 individual female 3 months old naive mice (harboring a conventional SPF microbiota), pooled, dissolved in 10 ml sterile phosphate buffered saline (PBS; Gibco, life technologies, Paisley, UK), and bacterial loads were evaluated by cultural and molecular methods before peroral challenge of mice with 0.3 ml of the suspension by gavage. Another group of mice received an oral suspension of VSL#3 bacteria. VSL#3 is a commercially available probiotic mixture (Manufacturer: SIIT S.r.l, Trezzano sul Naviglio, Italy; distributed by Actial Farmaceutica, Funchal, Madeira, Portugal) consisting of the following eight bacterial species: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus delbrueckii* subsp. *Bulgaricus*. A total of 4.5×10^{11} probiotic bacteria were dissolved in 50 ml PBS. By gavaging 0.3 ml, each mouse received $\sim 10^9$ viable probiotic bacteria as confirmed by cultural analyses of the suspensions. Mice were continuously kept in a sterile environment (autoclaved food and drinking water or sterile antibiotic cocktail) and were handled under strict aseptic conditions to prevent contaminations.

Clinical Score

To survey clinical signs of inflammation, a standardized cumulative clinical score (maximum 12 points), addressing the occurrence of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Krefeld, Germany; 4: overt blood visible), diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces), and the clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromized locomotion, pre-final aspect) was applied daily as described earlier (Haag et al., 2012).

Sampling Procedures

Mice were sacrificed by isofluran treatment (Abbott, Greifswald, Germany) at day (d) 28 post recolonization. Tissue samples from spleen, MLN, ileum and colon, and luminal samples from colon were removed under sterile conditions. Ileal and colonic *ex vivo* biopsies were collected from each mouse in parallel for immunological, microbiological, and immunohistochemical analysis. For immunohistochemical stainings, ileum and colon samples were immediately fixed in 5% formalin and embedded in paraffin, and sections (5 μm) were stained with the respective antibodies as described below.

Bacterial Colonization Densities Following Recolonization of Secondary Abiotic Mice with VSL#3 or Complex Murine Microbiota

Total intestinal loads of VSL#3 bacteria were quantified in serial dilutions of fecal and large intestinal luminal samples streaked onto Columbia-Agar supplemented with 5% sheep blood and Columbia-CNA Agar supplemented with colistin and nalidixic acid (both Oxoid) in parallel and incubated under aerobic (with 5% CO_2), microaerophilic (in jars using CampGen gas packs; Oxoid) and obligate anaerobic (in jars using Anaerogen gas packs; Oxoid) conditions for at least 2 days. Bacterial species were identified according to their typical morphological appearances. The total VSL#3 bacterial loads of intestinal samples were approximated as the sum of identified colony forming units (CFU) derived from the respective culture conditions. The detection limit of viable bacteria was ≈100 CFU per g.

The complex intestinal microbiota composition in conventionally colonized SPF mice and mice subjected to FMT was assessed by quantitative 16S rRNA based real time PCR as described previously (Heimesaat et al., 2010, 2014; Rausch et al., 2013; Thoene-Reineke et al., 2014).

Immunohistochemistry

In situ immunohistochemical analysis of ileal and colonic paraffin sections was performed as previously described (Heimesaat et al., 2007, 2010; Alutis et al., 2015). Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Glostrup, Denmark, 1:100), CD3 (#N1580, Dako, 1:10), FOXP3 (FJK-16s, eBioscience, San Diego, CA, USA, 1:100), and B220 (eBioscience, 1:200) were used. For detection, the LSAB method was applied with FastRed (Dako) as chromogen. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 400× magnification) was determined microscopically by a blinded investigator.

Lymphocytes Isolation from Spleens and Mesenteric Lymph Nodes

Single cell suspensions were generated from spleens and MLN, and erythrocytes were removed from splenic samples by 1.66% ammonium chloride. All samples were resuspended in defined volumes of PBS/0.5% BSA and subjected to further processing (Cording et al., 2013).

LPL Isolation

Segments of the murine gut were removed and freed from fat, connective tissue and PP, cut longitudinally and cleared from luminal content and mucus with ice-cold PBS. The isolation of LPL followed a standard protocol with minor modifications (Sheridan and Lefrancois, 2012). Briefly, the intestines were cut into 0.5 cm pieces and incubated twice with 25 ml Hanks balanced salt solution (HBSS; Gibco) containing 1 mM dithioerythritol (DTE; Carl Roth) for 20 min at 37°C and 220 rpm. Afterwards the intestines were introduced to HBSS containing 1.3 mM ethylenediaminetetraacetic acid (EDTA; Life Technologies, Eugene, Oregon, USA). Subsequently the cells were placed in digestion solution, containing 0.5 mg/ml collagenase A (Roche, Mannheim, Germany), 0.5 mg/ml DNAse I (Roche), 10% FCS, and 1 mM of each CaCl2 and $MgCl_2$ (both Carl Roth). Digestion was performed through incubation for 45 min at 37° and 220 rpm. After the incubation the digested tissues were washed with RPMI containing 5% FCS, resuspended in 5 ml 44% Percoll (GE Healthcare, Uppsala, Sweden), and overlaid on 5 ml 67% Percoll in a 15 ml Falcon tube. Percoll gradient separation was performed by centrifugation at 600 g for 20 min at room temperature. LPL were collected from the interphase, washed once and suspended in PBS/0.5% BSA.

Surface and Intracellular Stainings and Flow Cytometry

Surface staining was performed using the following antibodies: FITC-anti-CD4 (Clone RM4-5; 1:200), PerCP-anti-CD8 (Clone 53-6.7; 1:100), PacBlue-anti-B220 (Clone RA3-6B2, 1:200), APC-Cy7-anti-CD25 (Clone PC61, 1:200), PE-anti-CD44 (Clone IM7, 1:200), APC-anti-CD86 (Clone B7-2, 1:200; all from BD Biosciences, San Jose, CA, USA).

For intracellular staining cells from spleen, MLN and intestinal LP were restimulated for 5 h with 10 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin, in a tissue culture incubator at 37°C (both Sigma-Aldrich). Ten micrograms per microliter of brefeldin A (Sigma-Aldrich) was added to the cell suspensions after 1 h of polyclonal restimulation. Then cells were treated with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (life technologies) and hereafter fixed with 2% paraformaldehyde (PFA; Sigma-Aldrich) for 20 min at room temperature. Cells were stained in 0.5% saponin (Sigma-Aldrich) using the following antibodies: PacBlue-Anti-CD4 (Clone RM4-5; 1:400), PE-Cy7-anti-IFN-γ (Clone XMG 1.2; 1:400), (both from BD Biosciences) FITC-anti-IL17A (Clone TC11-18H10.1; 1:200, BioLegend, San Diego, CA, USA), PE-anti-IL10 (Clone JESS-16E3; 1:100), APC-anti-IL22 (Clone IL22JOP; 1:100) (both from eBioscience). All data were acquired on a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed with FlowJo Software v10.1 (Tree star, Ashland, OR, USA).

Real-Time PCR

RNA was isolated from snap frozen ileal and colonic *ex vivo* biopsies, reverse transcribed, and analyzed for cytokine specific mRNA as described earlier (Munoz et al., 2009). Murine IL-22, IL-17A, IL-10, and IFN-γ mRNA expressions were analyzed using Light Cycler Data Analysis Software (Roche). Expression levels were calculated relative to the housekeeping gene for hypoxanthine-phosphoribosyltransferase expression and indicated as "Arbitrary Units" (fold expression).

Statistical Analysis

Medians and significance levels using appropriate tests as indicated (Mann Whitney *U*-test and one-way ANOVA with Tukey's *post-hoc* test for multiple comparisons) were determined using GraphPad Prism Software v6 (La Jolla, CA, USA). Two-sided probability (p) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

RESULTS

Macroscopic and Microscopic Intestinal Changes in Secondary Abiotic Mice upon Recolonization with VSL#3 or Fecal Microbiota Transplantation

Given a better acceptance by the patients and more practical mode of peroral application of a probiotic formulation as compared to FMT in both ambulant and hospital settings, we compared the immunopathological impact of the probiotic compound VSL#3 and complex microbiota in the with broad-spectrum antibiotics treated host. Therefore, we virtually depleted the intestinal microbiota of conventional mice by quintuple antibiotic treatment. These secondary abiotic (ABx) mice then received either 10^9 viable VSL#3 bacteria via the oral route or were subjected to FMT by gavage, whereas naive, conventionally colonized and ABx mice served as positive and negative controls, respectively. From day 3 until day 28 following VSL#3 peroral challenge, mice could be stably colonized as indicated by 10^9 CFU of VSL#3 bacteria per g feces (**Figure 1A**). In order to exclude that upon cessation of antibiotic treatment and peroral reconstitution with VSL#3 remnant commensal bacteria might grow back, we surveyed the intestinal microbiota composition applying highly sensitive and culture-independent 16S rRNA based molecular quantitative RT-PCR. In fact, only bifidobacteria and lactobacilli as main bacterial constituents of the applied probiotic compound increased in fecal samples until day 28 following probiotic challenge ($p < 0.001$; **Figure 1B**), whereas the other intestinal bacterial commensals remained virtually unchanged (n.s.; **Figure 1B**). Moreover, mice subjected to FMT showed at day 28 after recolonization a complex large intestinal microbiota composition that was comparable to the microbiota in conventional control animals (**Figure 1C**).

Given that mice were clinically/macroscopically uncompromised upon antibiotic treatment as well as following respective recolonization regimens as assessed by a clinical scoring system on a daily basis (not shown), we next assessed potential microscopic changes in the intestinal tract. To address this, we quantitatively surveyed apoptotic cell numbers in small and large intestinal epithelia applying *in situ* immunohistochemistry. In line with the uncompromised clinical conditions, apoptosis was neither induced by broad-spectrum antibiotic treatment nor by respective peroral reassociation (n.s.; **Figure 2A**; **Figure S1**). Numbers of epithelial cells positive for Ki67, a sensitive marker for cell proliferation and regeneration (Scholzen and Gerdes, 2000), however, were significantly reduced in both, ileum and colon following broad-spectrum antibiotic treatment. Notably, administration of either VSL#3 or complex SPF microbiota was sufficient to restore regenerative epithelial properties as indicated by higher small as well as large intestinal Ki67+ cell numbers as compared to ABx mice ($p < 0.001$), that were comparable to those observed in naive SPF mice (**Figure 2B**; **Figure S1**).

Adaptive Immune Cell Subsets in Small and Large Intestines *In situ* Following Broad-Spectrum Antibiotic Treatment and Recolonization with VSL#3 or Fecal Microbiota Transplantation

To further dissect the role of VSL#3 in modulating adaptive immune responses following microbial depletion, we quantified cell numbers of distinct immune cell populations in both small and large intestines of mice at day 28 post recolonization with either VSL#3 or FMT by *in situ* immunohistochemical staining of paraffin sections (**Figure 3**; **Figure S1**). Broad-spectrum antibiotic treatment was associated with reduced numbers of CD3+ T lymphocytes ($p < 0.001$; **Figures 3A,D**; **Figure S1**), B220+ B lymphocytes ($p < 0.001$; **Figures 3B,E**; **Figure S1**),

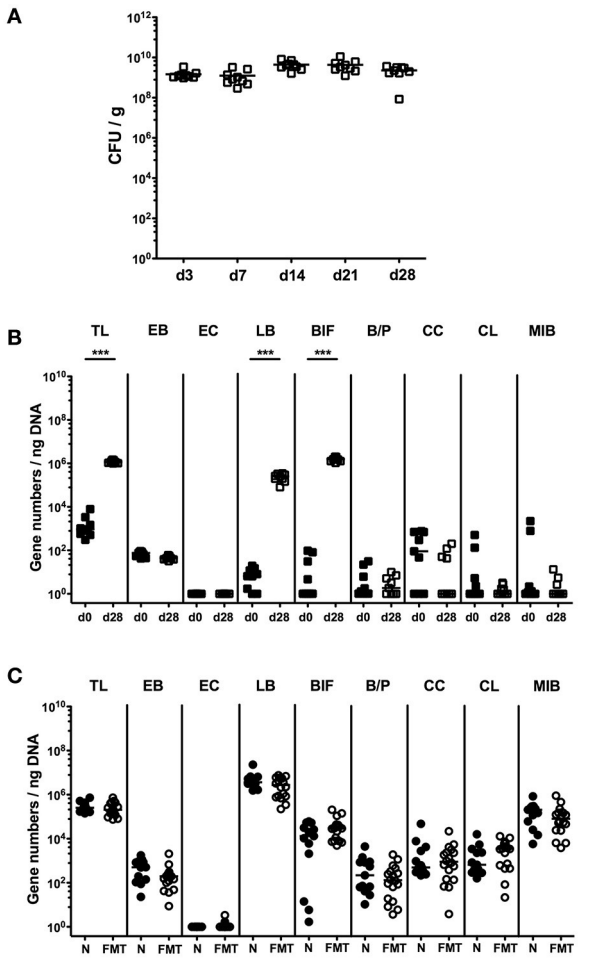

FIGURE 1 | Bacterial colonization densities following bacterial recolonization of secondary abiotic mice with VSL#3 or complex murine microbiota. Secondary abiotic mice were generated by broad-spectrum antibiotic treatment and perorally reassociated with the probiotic compound VSL#3 or subjected to fecal microbiota transplantation. **(A)** Bacterial colonization densities were assessed in fecal samples (CFU/g, colony forming units per gram) over time upon reassociation by culture on day (d) 0, 3, 7, 14, 21, 28 following peroral challenge with the probiotic compound VSL#3 (white squares). **(B)** Changes in intestinal microbiota composition of secondary abiotic mice (d0, closed squares) within 28 days following peroral VSL#3 reassociation (d28, open squares) or **(C)** fecal microbiota transplantation (FMT, white circles) were assessed in fecal samples by quantitative Real-Time PCR amplifying bacterial 16S rRNA variable regions of the main intestinal bacterial groups and expressed as 16S rRNA gene numbers per ng DNA: TL, Total eubacterial load; EB, *Enterobacteriaceae*; EC, *Enterococcus* spp.; LB, Lactic acid bacteria; BIF, Bifidobacteria; BP, *Bacteroides/Prevotella* spp.; CC, *Clostridium coccoides* group; CL, *Clostridium leptum* group; and MIB, *Mouse Intestinal Bacteroides*. Conventional, naive (N, black circles) mice served as controls. Medians (black bars) and significance levels determined by Mann Whitney *U*-test (***$p <$ 0.001) are indicated. Data were pooled from two independent experiments.

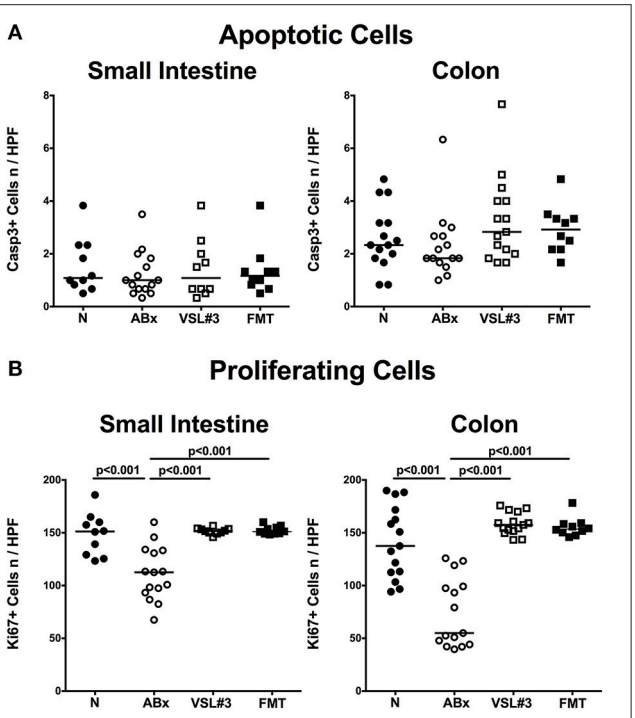

FIGURE 2 | Small intestinal and colonic epithelial apoptotic and proliferating cells following recolonization of secondary abiotic mice with VSL#3 or complex murine microbiota. The average numbers of **(A)** apoptotic (positive for Casp3) and **(B)** proliferating cells (positive for Ki67) in at least six representative high power fields (HPF, 400× magnification) per animal were determined in immunohistochemically stained small intestinal and colonic tissue of naive conventional mice (N), through antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 or fecal microbiota transplantation (FMT) on day 28 following peroral reassociation. Medians (black bars) and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments ($n = 10$–15/group).

and Foxp3+ Treg ($p < 0.001$; **Figures 3C,F**; **Figure S1**) in mucosa and lamina propria of both ileum and colon. Application of either VSL#3 or complex SPF microbiota, however, was sufficient to restore adaptive immune cell populations in the colon as indicated by T and B cell as well as Treg numbers that were comparable to naive mice at day 28 following respective recolonization (**Figures 3D–F**; **Figure S1**). Numbers of T and B lymphocyte numbers increased in small intestines of secondary abiotic mice following FMT, but not VSL#3 recolonization ($p < 0.001$; **Figures 3A,B**; **Figure S1**). Small intestinal Treg numbers were elevated upon either reassociation regimen and to the highest extent by FMT (**Figure 3C**; **Figure S1**).

To exclude that the observed effects were microbiota driven and not due to antibiotic withdrawal *per se*, we quantitatively assessed respective intestinal immune cell populations in secondary abiotic mice 4 weeks following cessation of broad-spectrum antibiotic treatment (**Figure S2**). However, withdrawal of antibiotic treatment (ABx%) did not restore any of the analyzed small or large intestinal immune cell populations as indicated by comparable cell numbers in ABx and ABx% mice (n.s.; **Figure S2**).

Hence, depending on the intestinal compartment, peroral application of VSL#3 or complex murine microbiota could sufficiently reverse antibiotics-induced decreases in intestinal

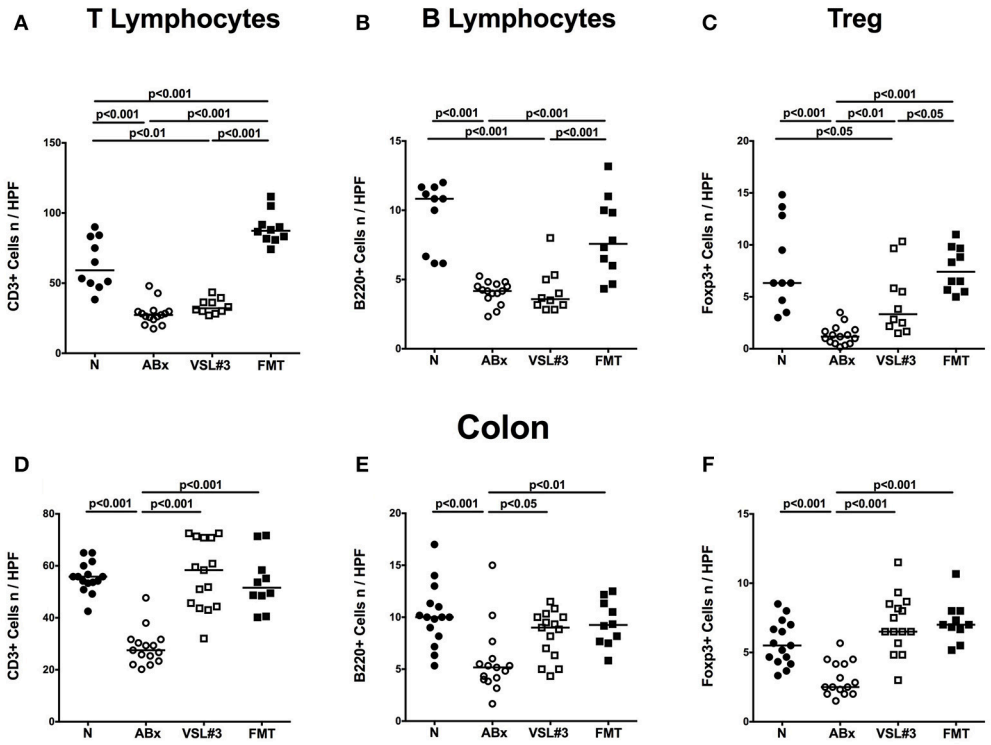

FIGURE 3 | Adaptive immune cell subsets in small and large intestines *in situ* following recolonization of secondary abiotic mice with VSL#3 or complex murine microbiota. The average numbers of T lymphocytes (positive for CD3, **A,D**), B lymphocytes (positive for B220, **B,E**), and regulatory T cells (positive for FOXP3, **C,F**) in the small intestinal (upper panel, **A–C**) and colonic (lower panel, **D–F**) tissue in at least six representative high power fields (HPF, 400× magnification) per animal were determined in immunohistochemically stained tissues of naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) on day 28 following peroral reassociation. Medians and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments (n = 10–15/group).

immune cell populations with the most prominent effect in the colon.

Distinct T Cell Populations in Intestinal and Systemic Compartments of Secondary Abiotic Mice Following Recolonization with VSL#3 or Fecal Microbiota Transplantation

We next elaborated the capacities of peroral VSL#3 application or FMT to induce, maintain and modulate distinct immune cell populations in mucosal, peripheral, and systemic immunological sites of mice that had been treated with broad-spectrum antibiotics. To address this, we performed flow-cytometric analyses of lymphocytes derived from the lamina propria of small and large intestine, MLN and spleen of mice at day 28 post-recolonization. The gating strategies are depicted in **Figures S3A–F**. We firstly focused on relative abundances and absolute numbers of the main lymphocytic groups, namely CD4+ (**Figures 4A–H**) and CD8+ (**Figures 5A–H**) T lymphocytes as well as B220+ B lymphocytes (**Figures 6A–H**). Antibiotic treatment resulted in decreased relative abundances and absolute numbers of CD4+ T helper cells in both the small and large intestines ($p < 0.05$–0.001; **Figures 4A–D**), whereas VSL#3 administration could sufficiently restore respective cell numbers at either mucosal site. Furthermore, abundances of CD4+ cells increased in MLN upon VSL#3 treatment ($p < 0.05$; **Figure 4E**). Following FMT, CD4+ cell frequencies were higher in small and large intestines as compared to ABx mice ($p < 0.001$; **Figures 4A,C**). Whereas frequencies of splenic CD4+ cells were rather unchanged upon antibiotic treatment and peroral reassociation (n.s.; **Figure 4G**), increased CD4+ cell numbers could be observed in the spleens of mice that had undergone antibiotic therapy, regardless whether subsequently recolonized or not ($p < 0.05$; **Figure 4H**).

We further analyzed CD8+ cytotoxic T cell responses in intestinal and systemic compartments upon antibiotic treatment and subsequent bacterial reassociation. Both VSL#3 treatment and FMT could sufficiently restore the antibiotics induced CD8+ cell frequency reduction in the small and large intestines as indicated by higher small and large intestinal CD8+ cell abundances as compared to ABx mice

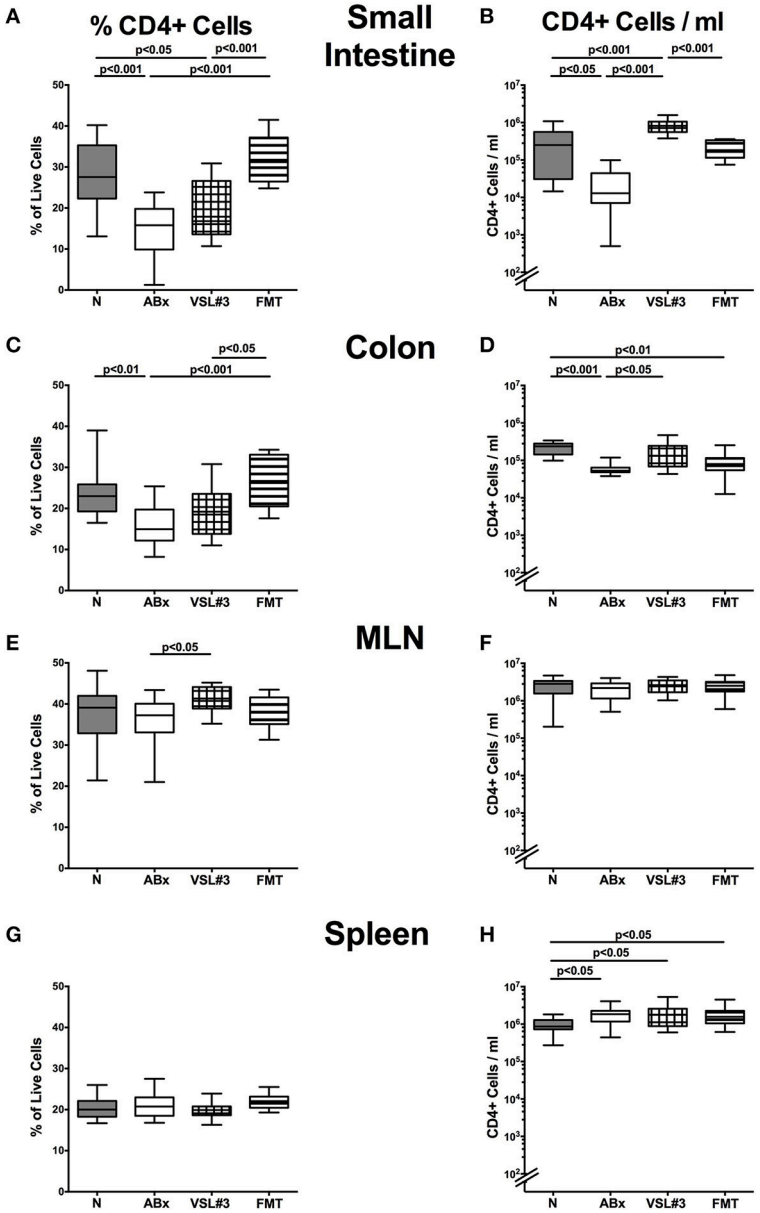

FIGURE 4 | CD4+ cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. Conventionally colonized adult mice were treated with broad-spectrum antibiotics for 8 weeks and differentially recolonized by gavage thereafter. Subsequently, lymphocytes from small intestinal and colonic lamina propria, MLN and spleen were isolated, and analyzed by flow cytometry. The percentages (left panel **A,C,E,G**) and absolute cell numbers (right panel **B,D,F,H**) of the CD4+ lymphocyte population in small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments (n = 10–15/group).

($p < 0.01$; **Figures 5A,C**), which also held true for absolute CD8+ cell numbers upon VSL#3 treatment ($p < 0.05$–0.001; **Figures 5B,D**). Whereas CD8+ cells were rather unchanged in MLN of ABx mice with and without bacterial recolonization, both frequencies and absolute numbers of splenic CD8+ cells increased upon broad-spectrum antibiotic treatment ($p < 0.001$; **Figures 5G,H**). Interestingly, VSL#3, but not FMT could reverse this effect on CD8+ cell abundances ($p < 0.001$ vs. ABx mice; **Figure 5G**). Hence, again peroral VSL#3 application or FMT were able to reverse antibiotics induced decreases in T cells, depending on the respective immunological compartment.

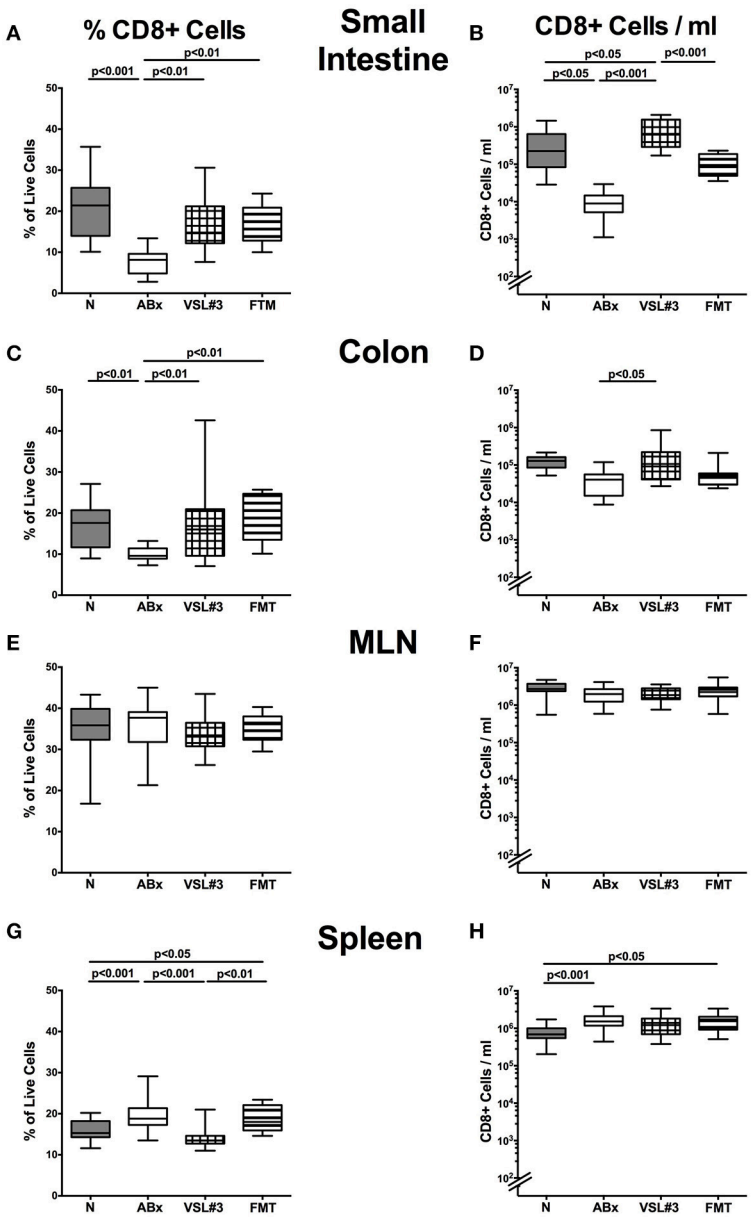

FIGURE 5 | CD8+ cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. The percentages (left panel **A,C,E,G**) and absolute cell numbers (right panel **B,D,F,H**) of the CD8+ lymphocyte population of small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (p-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments (n = 10–15/group).

B Lymphocytes in Intestinal and Systemic Compartments of Secondary Abiotic Mice Following Recolonization with VSL#3 or Fecal Microbiota Transplantation

We next expanded our comprehensive survey on lymphocyte populations during antibiotic treatment and bacterial recolonization to B220+ B cells. Whereas decreased B220+ cell counts were detected in small and large intestines as well as in MLN following broad-spectrum antibiotic treatment ($p < 0.05$–0.01; **Figures 6B,D,F**), VSL#3, but not FMT resulted in elevated small intestinal B lymphocytes ($p < 0.001$ vs. ABx; **Figure 6B**). In the splenic compartment, B220+ cell numbers increased following antibiotic treatment, but also upon additional VSL#3 challenge ($p < 0.05$ and $p < 0.01$, respectively; **Figure 6H**). In addition, B220+ cells were

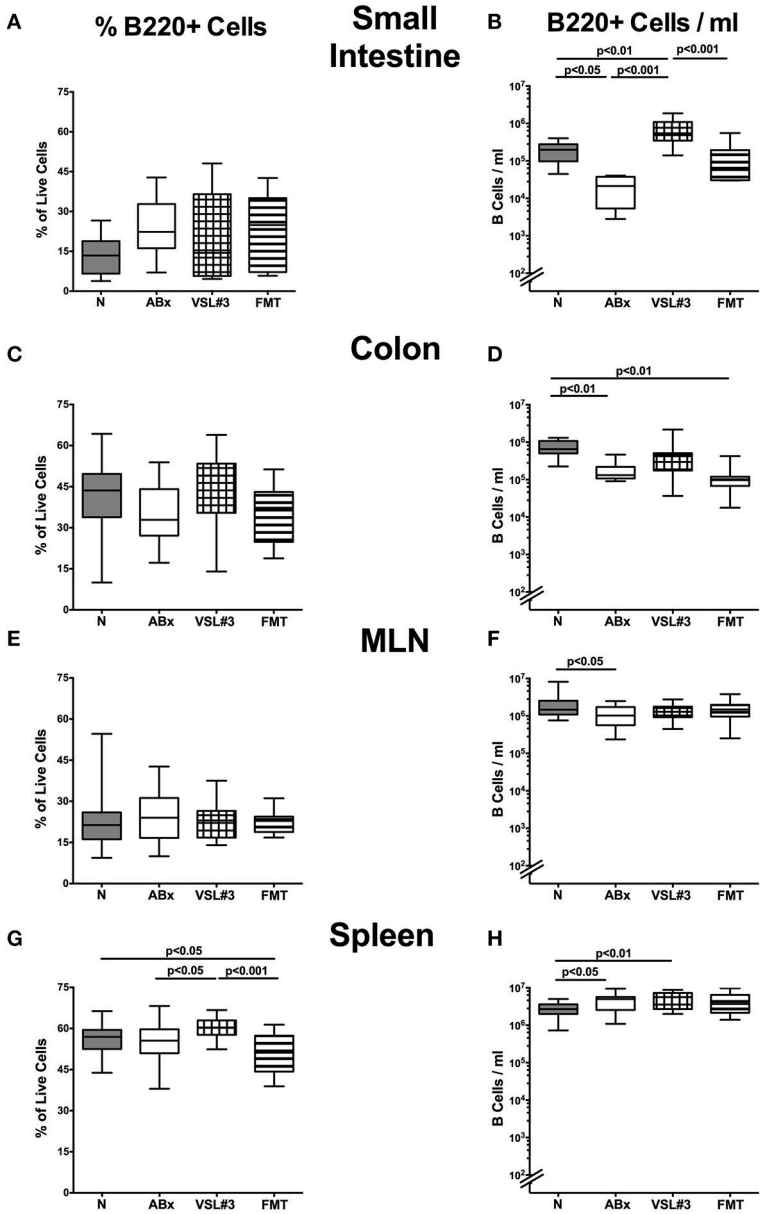

FIGURE 6 | B220+ cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. The percentages (left panel **A,C,E,G**) and absolute cell numbers (right panel **B,D,F,H**) of the B220+ lymphocyte population of small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (p-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments (n = 10–15/group).

more abundant in the spleen of VSL#3 as compared to untreated ABx mice ($p < 0.05$; **Figure 6G**). Taken together, our data indicate that an intestinal VSL#3 microbiota is capable of inducing and modulating distinctive immune cell populations, thus antagonizing immunological consequences of antibiotic treatment not only at mucosal site, but, to some extent, also on a systemic level of the immune system.

Regulatory T Cells and Dendritic Cells in Intestinal and Systemic Compartments of Secondary Abiotic Mice Following Recolonization with VSL#3 or Fecal Microbiota Transplantation

In the following, we addressed whether recolonization with VSL#3 or FMT following broad-spectrum antibiotic treatment

was associated with changes in defined T cell subsets and in the activation status of distinct cell populations. We therefore stained CD4+ cells with antibodies against CD25, a surface protein characteristic for Treg. Microbial depletion by antibiotic treatment led to reduced abundances of the CD4+CD25+ subpopulation in all intestinal and systemic immunological compartments under investigation ($p < 0.001$; **Figure 7**). Remarkably, VSL#3 administration as well as FMT enhanced Treg abundances and completely reversed the antibiotics induced effect ($p < 0.01$–0.001 vs. ABx; **Figures 7A,C,E,G**). VSL#3-induced splenic abundances of CD4+CD25+ cells were, however, less distinct than in naive controls ($p < 0.001$; **Figure 7G**). Broad-spectrum antibiotic treatment was further accompanied by a down-regulation of the surface marker CD86,

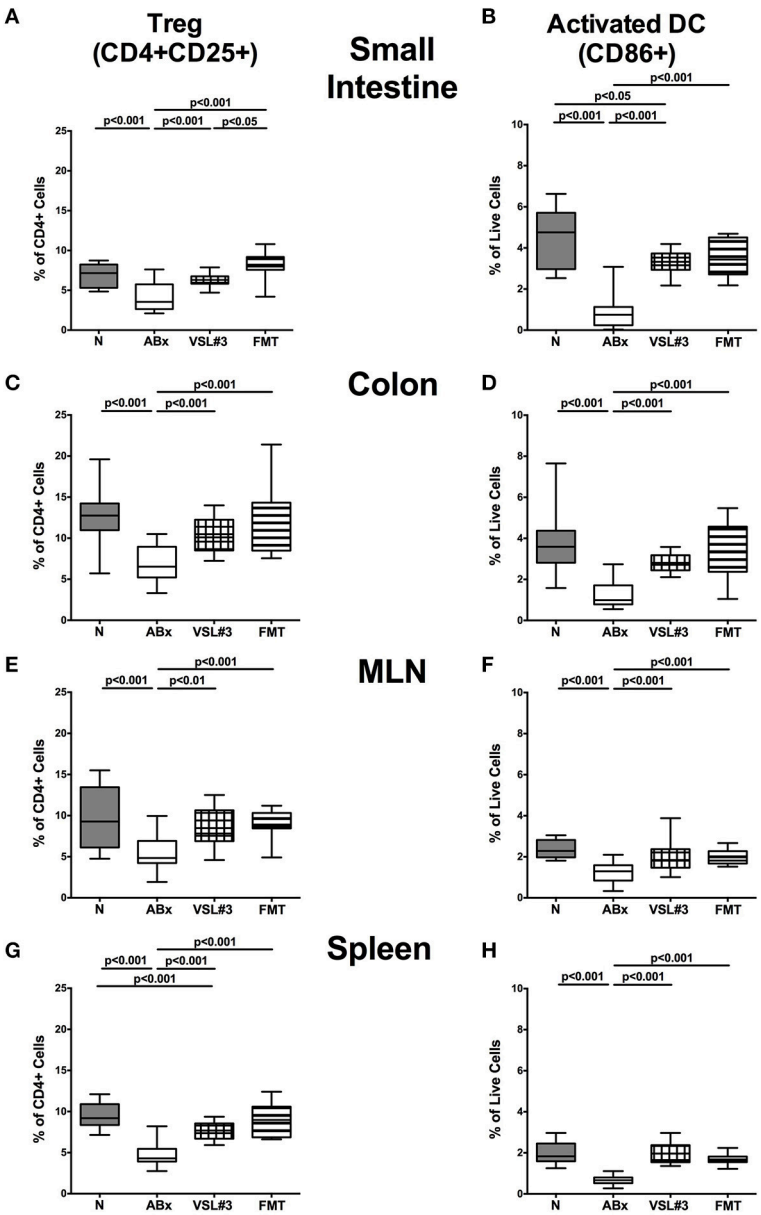

FIGURE 7 | Regulatory T cells and activated dendritic cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. The frequencies of regulatory T cells (Treg, CD4+CD25+, gated on CD4+ cells) (left panel **A,C,E,G**) and activated dendritic cells (CD86+, gated on live CD4-CD8-cells, right panel **B,D,F,H**) in the small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments ($n = 10$–15/group).

a co-stimulatory molecule marking activated DC (Wallet et al., 2005), in small intestine, colon, MLN and spleen of ABx mice, whereas both VSL#3 treatment and FMT virtually reversed these immune-depressive effects ($p < 0.001$; **Figures 7B,D,F,H**). Hence, the activation status of distinct cells in intestinal as well as systemic compartments is down-regulated by broad-spectrum antibiotic treatment, but can be restored upon VSL#3 application or FMT.

Memory/Effector T Cells in Intestinal and Systemic Compartments of Secondary Abiotic Mice Following Recolonization with VSL#3 or Fecal Microbiota Transplantation

We then investigated the impact of VSL#3 and FMT on the memory/effector CD4+ and CD8+ cells by evaluating high expression of CD44, a surface marker expressed (on both, CD4+ and CD8+ cells) upon previous antigen contact (Sprent and Surh, 2002). ABx mice exhibited a significant reduction in abundances of both, CD4+CD44+ and CD8+CD44+ cells in all examined intestinal and systemic lymphoid organs ($p < 0.001$; **Figure 8**). In the small and large intestines, VSL#3 recolonization and FMT resulted in a strong up-regulation of CD44 expression on CD4+ as well as CD8+ cells ($p < 0.05$–0.001; **Figures 8A–D**). The same held true for CD8+ cells in MLN and spleen ($p < 0.001$; **Figures 8F,H**), whereas FMT alone resulted in higher frequencies of memory CD4+ cells in MLN and spleen as compared to antibiotic treatment ($p < 0.001$; **Figures 8E,G**). Hence, microbial depletion resulted in reduced abundances of memory/effector T cells in intestinal and systemic lymphoid compartments, that could, however, at least in part be restored by VSL#3 treatment or FMT.

Pro- and Anti-Inflammatory Cytokine Production in Intestinal and Systemic Compartments of Secondary Abiotic Mice Following Recolonization with VSL#3 or Fecal Microbiota Transplantation

We further assessed the cytokine producing properties of CD4+ T lymphocytes following VSL#3 administration or FMT in ABx mice. Therefore, we determined the frequencies of IFN-γ and IL-10 (**Figure 9**) as well as of IL-17 and IL-22 (**Figure 10**) producing CD4+ cells in small and large intestines, MLN, and spleens. Gating strategies are depicted in **Figures S3G–I** and representative dot plots shown in **Figure S4**. Small intestinal IFN-γ producing CD4+ cells were depressed in ABx mice ($p < 0.01$–0.001; **Figure 9A**) and could not be fully recovered by either bacterial recolonization regimen. Notably, small intestinal CD4+IFN-γ+ cells were more frequently abundant following FMT as compared to ABx mice ($p < 0.05$; **Figure 9A**). Moreover, IFN-γ producing CD4+ cells were less abundant in large intestines of ABx and VSL#3 treated, but not with fecal microbiota transplanted mice ($p < 0.05$–0.001; **Figure 9C**), whereas CD4+IFN-γ+ cells did not differ between respective groups in MLN and spleen (n.s.; **Figures 9E,G**). A strong reduction of CD4+ lymphocytes producing the anti-inflammatory cytokine IL-10 could be determined in all immunological sites following antibiotic therapy ($p < 0.001$; **Figures 9B,D,F,H**). Notably, reassociation with either VSL#3 or complex murine microbiota could fully restore the frequencies of CD4+IL10+ cells in all compartments ($p < 0.001$; **Figures 9B,D,F,H**) and thus reestablish the pre-antibiotic (naive) status. Interestingly, intestinal as well as systemic CD4+ cells producing the pro-inflammatory cytokines IL-17 or IL-22 were less abundant upon antibiotic and also subsequent VSL#3 treatment ($p < 0.05$–0.001; **Figures 10A–H**), but not upon FMT, except for small intestinal CD4+IL17+ cells ($p < 0.05$; **Figure 10A**).

These findings were further underlined by results obtained from mRNA analysis of respective cytokines measured in ileal and colonic *ex vivo* biopsies (**Figure 11**). IL-10 as well as IL-17, IL-22, and IFN-γ mRNA were all down-regulated in both small and large intestines of ABx mice ($p < 0.05$–0.001; **Figure 11**). In the small intestine, FMT ($p < 0.05$), but not VSL#3 treatment could sufficiently up-regulate IL-10 expression back to naive levels, whereas the other way around, recolonization with VSL#3 ($p < 0.001$), but not with complex SPF microbiota reversed antibiotics-induced colonic IL-10 down-regulation (**Figure 11A**). Furthermore, IL-17 and IL-22 mRNA expression were down-regulated upon antibiotic treatment and also subsequent VSL#3 administration ($p < 0.001$; **Figures 11B,C**), whereas respective cytokine levels were comparable in mice subjected to FMT and naive controls, which also held true for ileal IFN-γ mRNA expression (**Figure 11D**). Notably, colonic IFN-γ mRNA levels were highest in mice following FMT ($p < 0.05$), but did not differ between secondary abiotic, VSL#3 treated and naive mice (**Figure 11D**).

Taken together, peroral probiotic VSL#3 treatment and FMT are both sufficient to induce regulatory, anti-inflammatory mechanisms of the peripheral and central immune system and to restore intestinal as well as systemic immunological collateral damages of broad-spectrum antibiotic treatment.

DISCUSSION

With increasing robust evidence regarding the indispensability of gut inhabiting bacteria in host physiology and their contributions to a plethora of pathologies, potentially beneficial modulations of intestinal microbiota composition have raised interest in clinical research and application (McCarville et al., 2016). Various environmental factors can lead to alterations of the intestinal microbiota composition, including diet, pathogens, toxins, and drugs (Carding et al., 2015). One of the most prominent factors causing perturbation of this well-balanced and sensitive ecological system is the widespread usage of antibiotics worldwide. Though being very effective in curing infectious diseases and having contributed tremendously to the increased life expectancy, the long-term adverse effects of antimicrobial compounds have been also recognized and explored (Becattini et al., 2016). Yet the underlying mechanisms of the complex crosstalk between microbiota, host, and potential disruptive factors including antibiotic compounds are incompletely understood. Hence, it remains of utmost interest

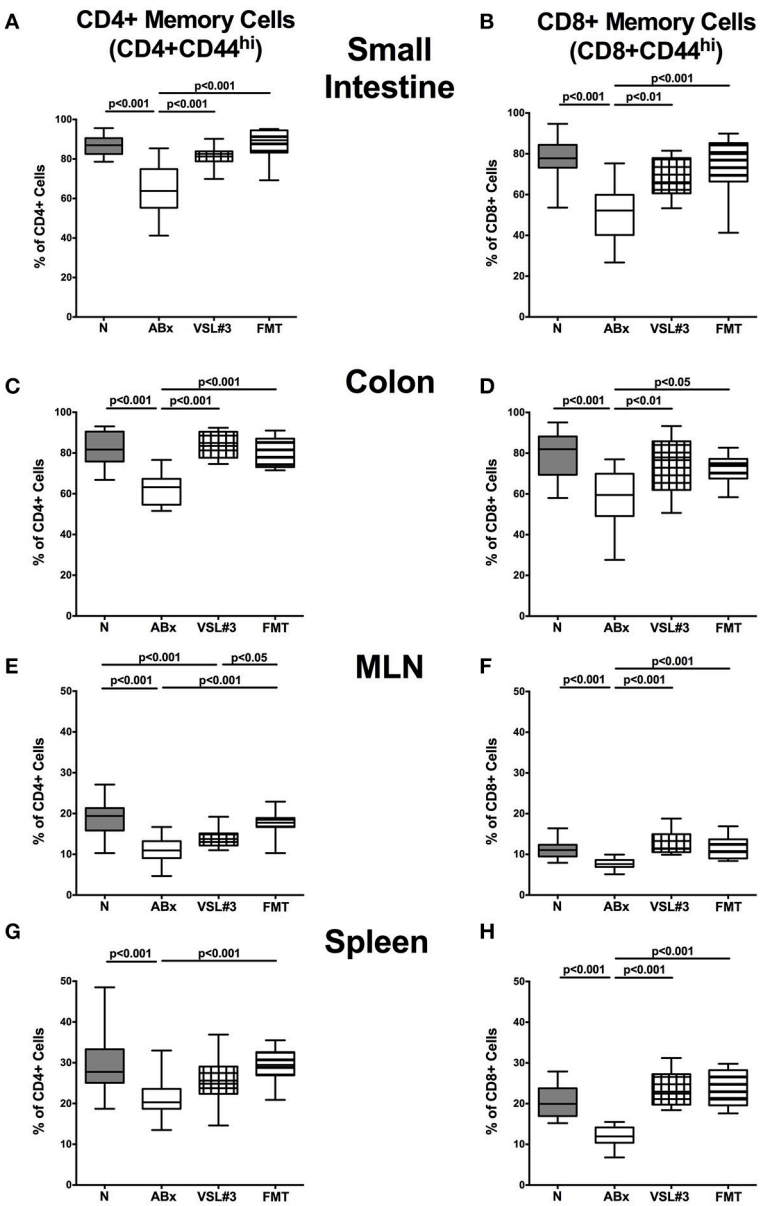

FIGURE 8 | Memory and effector T cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. The proportions of CD4+ memory/effector cells (CD4+CD44^{hi}, gated on CD4+ cells, left panel **A,C,E,G**) and CD8+ memory/effector cells (CD8+CD44^{hi}, gated on CD8+ cells, right panel **B,D,F,H**) in the small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments ($n = 10$–15/group).

not only to further elucidate them, but also to develop novel therapeutic approaches to alleviate the potential harm exerted by antibiotics. In context of the latter, the study of probiotics, their impact, efficacy, but also limitations is still a challenging and not fully explored field of research.

In the present study we focused on VSL#3, a well-known and clinically approved commercially available probiotic compound, and its impact on the immune system of conventional mice and assessed its efficacy in reversing immunological effects of microbiota depletion as compared to reassociation with a complex murine microbiota. Both complex SPF microbiota and VSL#3 bacteria were able to stably colonize the murine intestinal tract of ABx mice, further supporting the suitability of the microbiota depleted mouse model to explore the complex

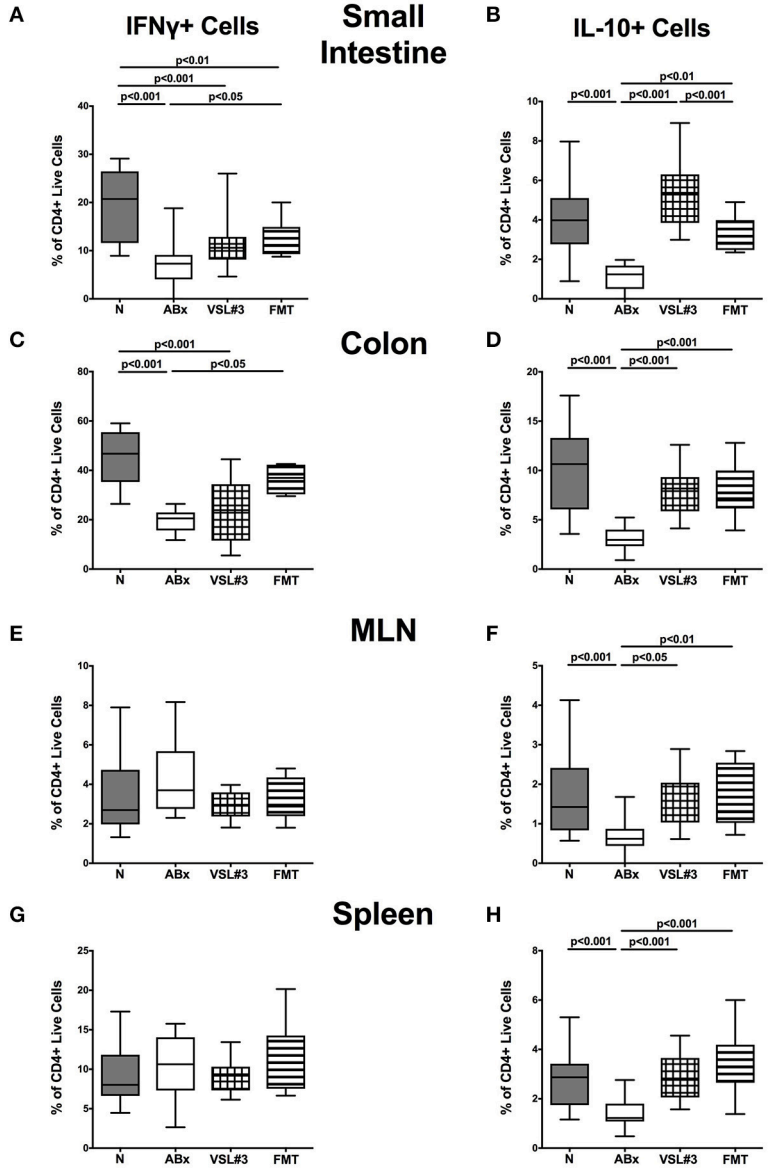

FIGURE 9 | IFN-γ and IL-10 producing CD4+ cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. Lymphocytes were isolated from small intestinal and colonic lamina propria, MLN, and spleen and stimulated with PMA/ionomycin in presence of brefeldin A and subsequently analyzed by flow cytometry. The percentages of IFN-γ (left panel **A,C,E,G**) and IL-10 (right panel **B,D,F,H**) producing CD4+ cells in the small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments ($n = 10–15$/group).

relationship between the innate and adaptive immune system, antibiotics, and recolonization with defined intestinal bacteria as reviewed by Fiebiger et al. (2016). To assure that the observed immunological responses were merely due to the applied probiotic bacterial species, we performed highly sensitive 16S rRNA based molecular microbiota analyses before and after VSL#3 challenge. In fact, only respective probiotic species and no intestinal bacterial commensals that might have regrown after cessation of antibiotic pretreatment were able to establish in the intestinal tract. Notably, conventionally colonized mice displayed unchanged compositions of their microbiota before and after VSL#3 treatment (our unpublished experimental observations), thus further underlining the physiological colonization resistance that prevents the host from stable pathogenic, but also commensal bacterial colonization (Besselink et al., 2008). Of note, administration of viable microorganisms in an

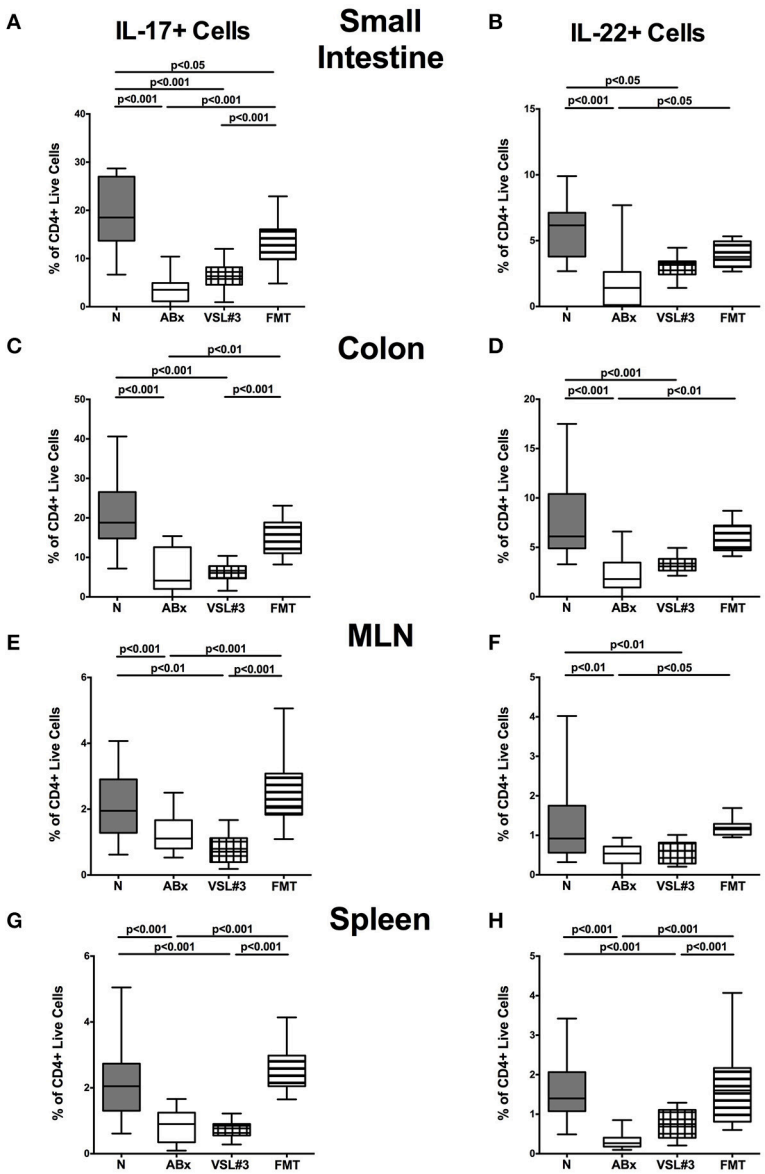

FIGURE 10 | IL-17 and IL-22 producing CD4+ cells in intestinal and systemic compartments of secondary abiotic mice following recolonization with VSL#3 or complex murine microbiota. Lymphocytes were isolated from small intestinal and colonic lamina propria, MLN, and spleen and stimulated with PMA/ionomycin in presence of brefeldin A and subsequently analyzed by flow cytometry. The percentages of IL-17 (left panel **A,C,E,G**) and IL-22 (right panel **B,D,F,H**) producing CD4+ cells in the small intestine **(A,B)**, colon **(C,D)**, MLN **(E,F)**, and spleen **(G,H)** in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) were determined on day 28 following peroral reassociation. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments ($n = 10$–15/group).

experimental context, especially in studies conducted with immunocompromised individuals, rises safety issues for the prophylactic or therapeutic usage of these compounds. This is supported by former findings of higher mortality in a probiotic intervention group as assessed by a study with critically ill patients suffering from acute pancreatitis (Besselink et al., 2008). However, in our study neither antibiotic treatment nor reassociation with VSL#3 or complex microbiota resulted in any clinical adverse effects in mice such as diarrhea, occurrence of fecal blood or weight loss, nor in microscopic sequelae including apoptosis, indicating that both VSL#3 bacteria and complex microbiota administration are safe and do not cause intestinal inflammation in our applied murine model.

The indispensability of microbial gut stimulation for maintaining epithelial colonic proliferation rates has already been suggested (Reikvam et al., 2011). Our data indicate that VSL#3 treatment of secondary abiotic mice was as effective as reassociation with a complex murine microbiota in stimulating

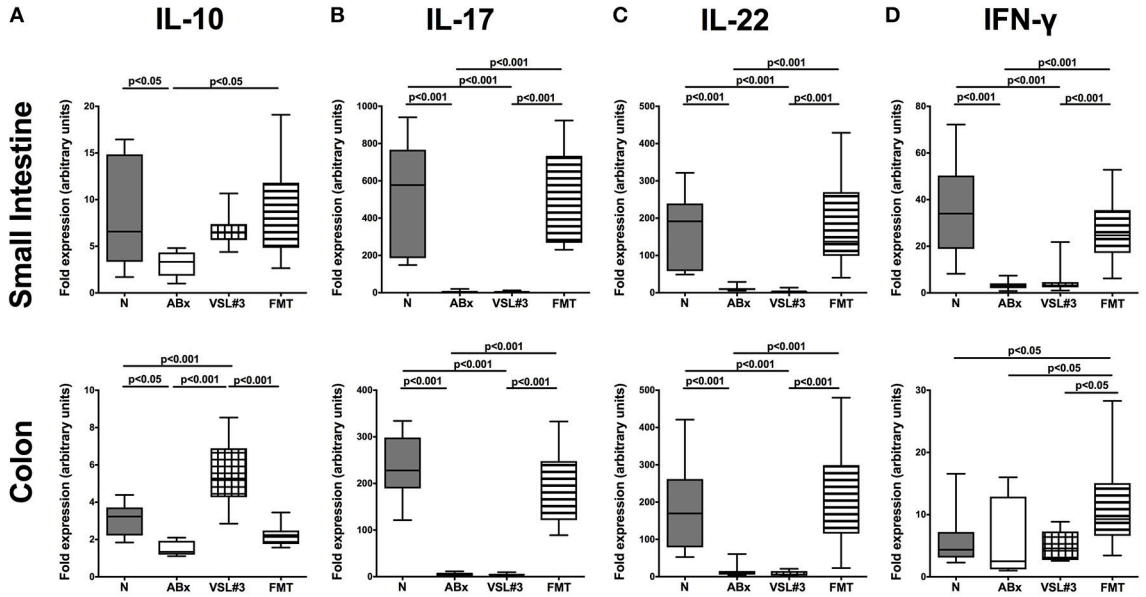

FIGURE 11 | mRNA analysis of pro- and anti-inflammatory cytokines in small intestinal and colonic tissues. RT-PCR results of **(A)** IL-10, **(B)** IL-17, **(C)** IL-22, and **(D)** IFN-γ expression in small intestinal (upper panel) and colonic (lower panel) tissues derived from naive conventional mice (N), from by antibiotic treatment generated secondary abiotic mice (ABx), and from mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT) on day 28 following peroral reassociation are depicted. Box plots represent the 75th and 25th percentiles of the medians (black bar inside the boxes). Total range and significance levels (*p*-values) determined with one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are indicated. Data shown were pooled from two independent experiments (n = 10–15/group).

recovery of colonic and ileal epithelial proliferative properties, as indicated by comparably increased Ki67+ cell numbers in intestinal epithelia that were decreased upon quintuple antibiotic therapy. This might be of importance, given that the proliferation of enterocytes is an essential physiological process for tissue repair and maintenance of gut homeostasis, whereas decreased proliferation rates may ultimately result in loss of epithelial integrity (Potten et al., 1997).

To gain an incipient insight into the role of viable VSL#3 bacteria in modulating intestinal immune cells, we quantitatively assessed distinct immune cell populations by applying immunohistochemical analyses of small and large intestinal paraffin sections *in situ.* Interestingly, the mere withdrawal of the antibiotic compounds did not result in restoring small and large intestinal Treg, T and B lymphocytes, implying that the observed immunological responses following VSL#3 or FMT application were attributable to the respective bacterial reassociation.

Overall, our data revealed that the immunomodulatory properties of VSL#3 recolonization were more pronounced in the colon than in the ileum of mice, whereas antibiotic treatment had resulted in decreased small and large intestinal cell numbers of Treg, T and B lymphoctes. While being able to restore the Treg population of the ileum, VSL#3 microbiota could neither induce T nor B lymphocytes in this compartment. In the colon, however, mice harboring VSL#3 bacteria displayed similar numbers of Treg, T and B lymphocytes similar to their naive conventionally colonized or with a complex microbiota recolonized counterparts. These data reemphasize the importance of considering small and large intestine as two distinctive immunological sites with different properties and mechanisms as previously postulated (Mann et al., 2016). Considering that the bacterial loads within the ileum of mice and men range from 10^4 to 10^8 CFU per ml luminal content (Quigley and Quera, 2006; Bereswill et al., 2010; Heimesaat et al., 2012), whereas the colonic VSL#3 colonization densities were up to six orders of magnitude higher, might at least in part explain this phenomenon. However, these findings seem to be in contrast to former evidence suggesting the capability of probiotics to modulate ileal immunological responses (Smelt et al., 2013) and prevent from ileitis (Pagnini et al., 2009). Given that *in situ* immunohistochemistry has its spatial limitations and does not provide complete information regarding more complex intestinal immune responses, we performed FACS analysis including not only lymphocytes from the lamina propria of the small and large intestines, but also from the MLN and systemic compartment, namely the spleen, in order to more comprehensively address VSL#3 mediated modulations of immune cell populations.

Remarkably, VSL#3 treatment was more effective in recovering CD4+ and CD8+ lymphocyte numbers in the lamina propria of both mucosal sites as compared to reassociation with complex murine microbiota. Whether this was due to distinct species mediated effects or other underlying mechanisms remains to be further investigated. Moreover, VSL#3 treated mice displayed higher B cell numbers in the small intestinal lamina

propria as compared to mice from the other cohorts. Most B cells in the intestinal mucosa are known to be IgA secreting plasma cells (Hill and Artis, 2010). Increased frequencies of small intestinal IgA-expressing B cells have already been demonstrated upon treatment of BALB/c mice with *L. casei* (Galdeano and Perdigon, 2006). Furthermore, a study conducted with intensive care unit patients frequently displaying multiple organ dysfunction as a major cause of mortality (Antonelli et al., 1999) which is pathophysiologically linked to a breakdown of intestinal barrier function and increased translocation of bacteria and bacterial components into the systemic circulation (Hassoun et al., 2001), revealed that serum IgA levels were normalized upon VSL#3 treatment (Alberda et al., 2007). It is therefore tempting to speculate that VSL#3 contributes beneficial effects to host health via IgA mediated mechanisms. Notably, neither reassociation of mice with VSL#3 nor with a complex murine microbiota could reverse the increased splenic CD4+, CD8+, and B220+ cell numbers pointing toward systemic microbiota-independent immunological consequences of long-term broad-spectrum antibiotic therapy. In fact, immunomodulatory properties of distinct antibiotic classes such as quinolones (Dalhoff and Shalit, 2003) and macrolides (Kanoh and Rubin, 2010) have previously been described. In terms of activation status of immune cell populations we could observe that VSL#3 recolonization was as efficient as complex microbiota recolonization, given that VSL#3 application resulted in a complete recovery of Treg, activated DC, and CD8+ memory/effector cells in all included intestinal and systemic immunological sites and of the CD4+ memory/effector cells in the small and large intestinal lamina propria. The latter population, however, could only be fully restored in MLN and spleen upon recolonization with a complex murine microbiota. Similarly, VSL#3 recolonization led to an increase of IL-10 producing CD4+ cell population in all organs, but did not induce the production of pro-inflammatory cytokines such as IL-17, IL-22, and IFN-γ in any of them. In fact, mice harboring VSL#3 bacteria exerted lower intestinal pro-inflammatory cytokine expression levels than their naive or with SPF microbiota recolonized counterparts. To further substantiate these findings, we additionally performed mRNA expression analysis of respective cytokines in ileal and colonic *ex vivo* biopsies and obtained similar results. Hence, our data suggest that VSL#3 dominated microbiota selectively induces activation of memory/effector T cells, activated DC and Treg, and the key anti-inflammatory cytokine IL-10 without driving pro-inflammatory Th1 or Th17 type immune responses. The concept of VSL#3 inducing regulatory immune responses in Th1 or Th17 mediated immune diseases has already been proposed (Di Giacinto et al., 2005) and is, in fact, very attractive for clinical application. Another probiotic mixture consisting of *Lactobacillus acidophilus*, *L. casei*, *L. reuteri*, *Bifidobacterium bifidium*, and *Streptococcus thermophilus*, has also been demonstrated to induce regulatory DC and Treg and, in turn, to suppress experimental immune disorders such as TNBS colitis, experimental atopic dermatitis and rheumatoid arthritis (Kwon et al., 2010). Given the important role of the Th1 and Th17 cell compartments in the protection against bacterial and fungal pathogens (Aujla et al., 2007), the lack of recovery of IL-17, IL-22, and IFN-γ expressing CD4+ cells upon VSL#3 reassociation raises the question, whether mice harboring probiotic bacteria were only more susceptible to pathogenic infections. The fact that probiotics have been shown to exert inflammation ameliorating effects in with antibiotics pre-treated patients suffering from *Clostridium difficile* toxin associated diarrhea, for instance, does not support this hypothesis (Selinger et al., 2013). The mechanisms underlying health-beneficial probiotic bacterial actions are manifold. Firstly, probiotics have been shown to inhibit growth, metabolism and adhesion of enteropathogenic bacteria (Bernet-Camard et al., 1997; Hudault et al., 1997; Gopal et al., 2001). Furthermore, in competition for nutrients and niches they prevent the host from stable pathogenic colonization (Wagner et al., 2009). Moreover, VSL#3 bacteria have been shown to restore epithelial barrier functions and to stimulate intestinal epithelial TNF production under inflammatory conditions (Pagnini et al., 2009). It is thus rather plausible that, while not inducing inflammatory immune responses in healthy mice (as shown here), VSL#3 may still sufficiently induce pro-inflammatory immune responses against invading pathogens.

In summary, in the present study we provide evidence, that beyond the already proposed immunomodulatory effects of VSL#3 on intraepithelial innate immunity (Pagnini et al., 2009), respective probiotic bacterial species modulate innate and adaptive immune cell populations not only at mucosal sites, but also in the peripheral (i.e., MLN) and central (i.e., spleen) immune system. Moreover, when compared to complex SPF microbiota, VSL#3 is capable of equally restoring distinct immune cell populations following microbiota depletion and strongly regulating anti-inflammatory immune responses.

We conclude that the probiotic compound VSL#3 may be regarded as an effective therapeutic tool to restore immune functions following antibiotic therapy. However, future research is needed to elucidate the distinct molecular mechanisms underlying the interactions between host microbiota, its modulations by antibiotic and/or probiotics and immunity.

AUTHOR CONTRIBUTIONS

IE: Performed experiments, analyzed data, wrote paper. EK and UF: Performed experiments, analyzed data, co-edited paper. CN: Suggested critical parameters in design of experiments, supplied antibodies. PB: Suggested critical parameters in design of experiments, supplied antibodies, co-edited paper. AS: Provided advice in design and performance of experiments. SB: Provided advice in design and performance of experiments, co-edited paper. MH: Designed and performed experiments, analyzed data, co-wrote paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00167/full#supplementary-material

Figure S1 | Representative photomicrographs of apoptotic and proliferating epithelial cells as well as of adaptive immune cell subsets in small and large intestines *in situ* following broad-spectrum antibiotic treatment (ABx) and recolonization with VSL#3 or fecal microbiota transplantation (FMT; 100× magnification, scale bar 100 μm).

Figure S2 | Adaptive immune cell subsets in small and large intestines *in situ* following quintuple antibiotic therapy. The average numbers of T lymphocytes (positive for CD3, **A,D**), B lymphocytes (positive for B220, **B,E**), and regulatory T cells (positive for FOXP3, **C,F**) in the small intestinal (upper panel, **A–C**) and colonic (lower panel, **D–F**) tissue of mice following long-term broad-spectrum antibiotic therapy (ABx, white circles) and at d28 after antibiotic withdrawal (ABx %, black circles) in at least six representative high power fields (HPF, 400× magnification) per animal were determined. Medians and significance levels (*p*-values) determined with Mann Whitney *U*-test are indicated. Data shown were pooled from two independent experiments.

Figure S3 | Gating strategies of extracellular stainings on one representative splenic sample after gating for lymphocytes and including only single cells. (A) Exclusion of dead cells via LIVE/DEAD Fixable Aqua Dead Cell Stain kit (L/D) in the forward scatter (FSC) **(B)** CD4+ and CD8+ cells gated on living cells, **(C)** B cells and activated DC gated on CD4-CD8- cells, **(D)** Treg and **(E)** CD4+ memory/effector cells gated on CD4+ cells, **(F)** CD8+ memory/effector cells gated on CD8+ cells. Gating strategies of intracellular stainings on one representative ileal sample after gating for lymphocytes and including only single cells are illustrated. **(G)** Identification of CD4+ living cells by exclusion of dead cells via LIVE/DEAD Fixable Aqua Dead Cell Stain kit (L/D) **(H)** IFN-γ and IL-10 gating **(I)** IL-17 and IL-22 gating.

Figure S4 | Representative FACS analysis dotplots of intracellular stainings of pro- and anti-inflammatory cytokines in intestinal and systemic compartments in naive conventional mice (N), by antibiotic treatment generated secondary abiotic mice (ABx), and mice subjected to VSL#3 recolonization or fecal microbiota transplantation (FMT).

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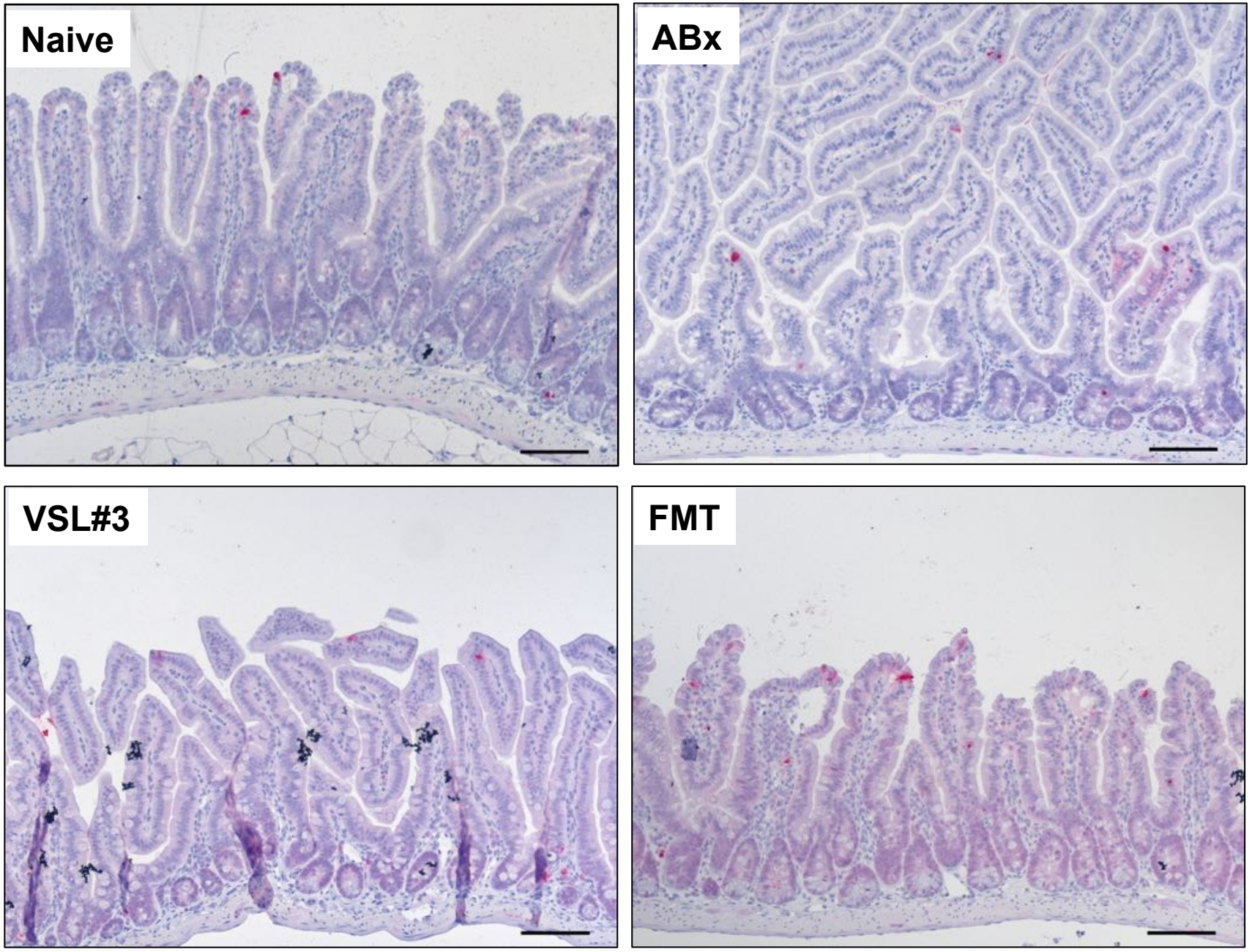
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Apoptotic Cells (Casp3+) – Small Intestine



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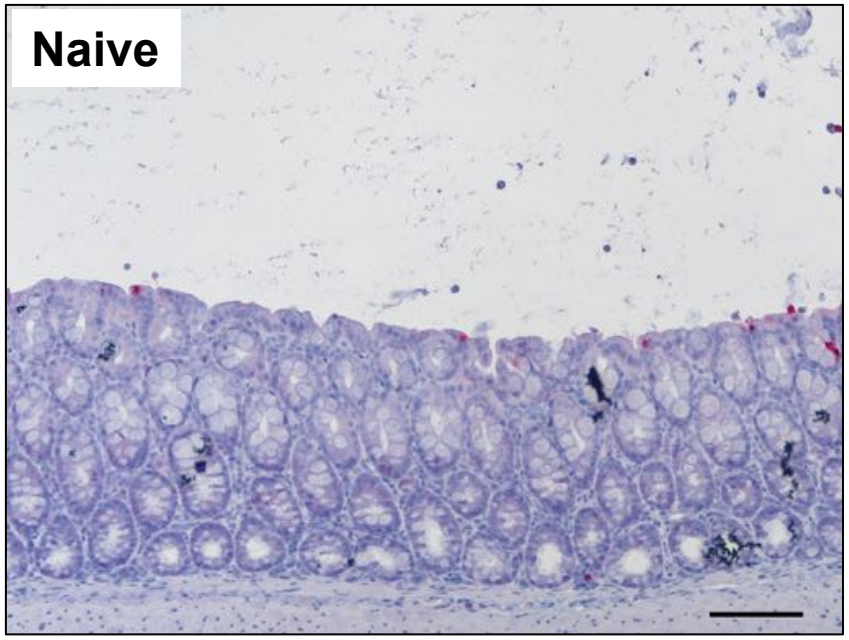
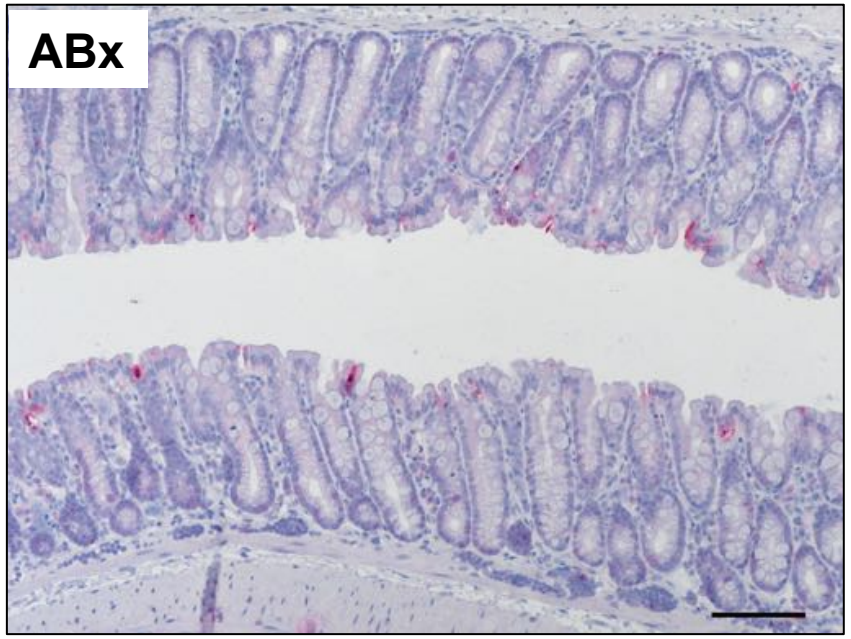
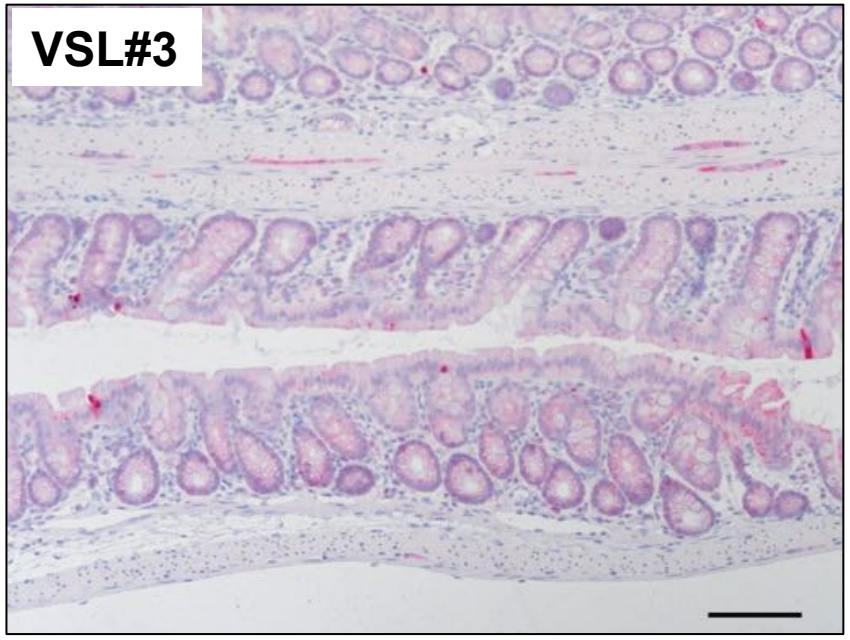
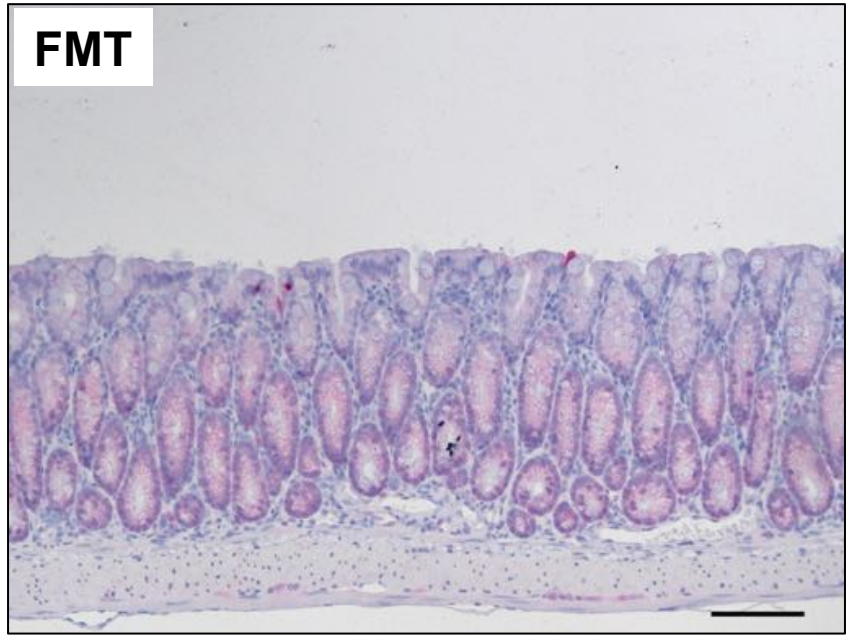
Apoptotic Cells (Casp3+) – Colon









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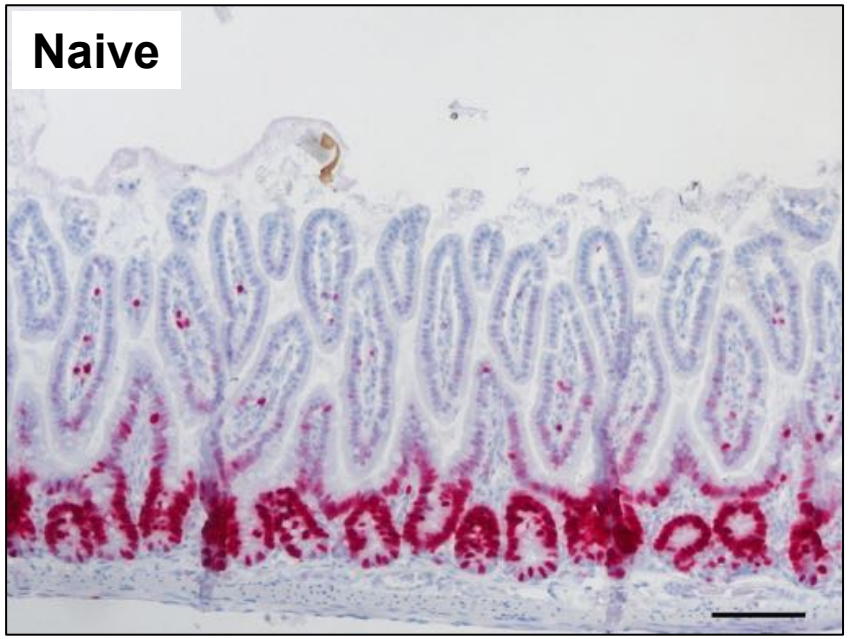
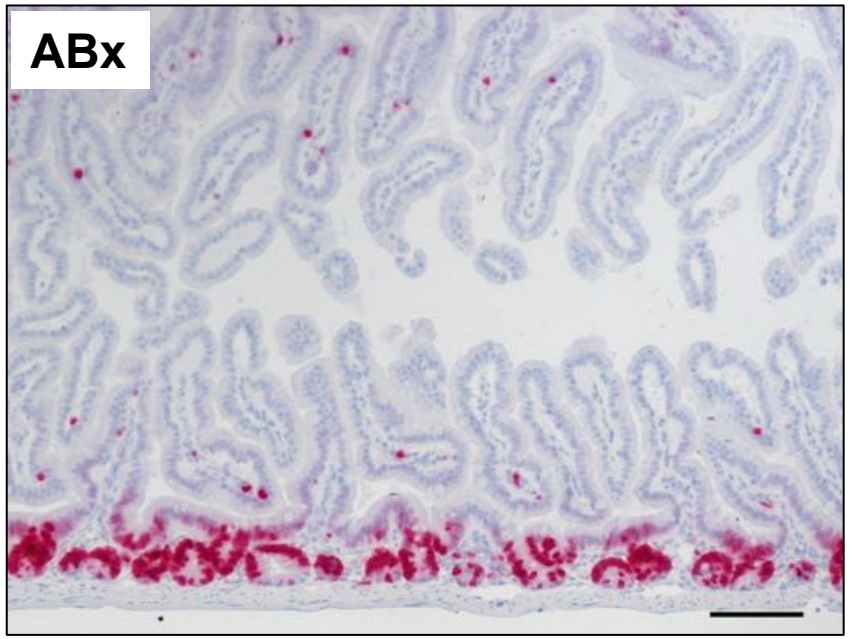
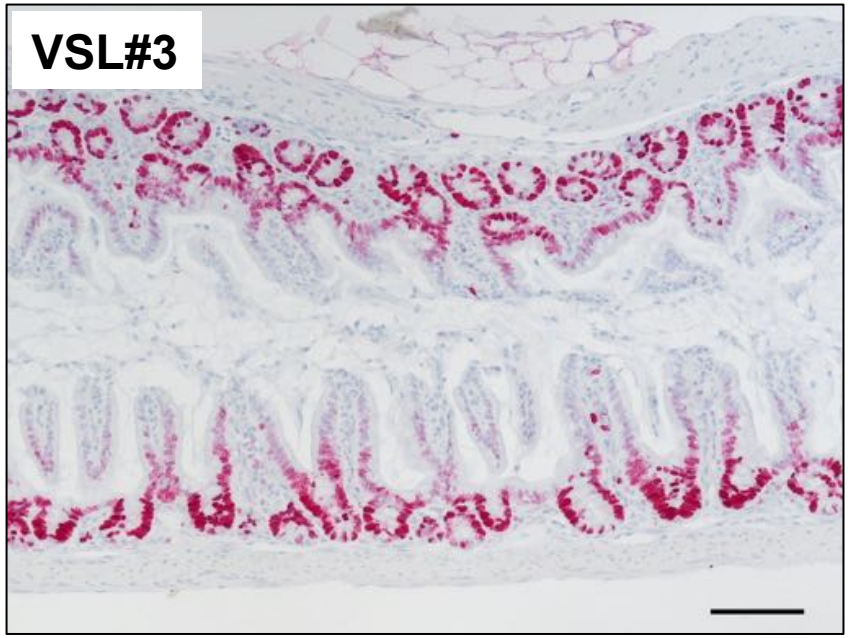
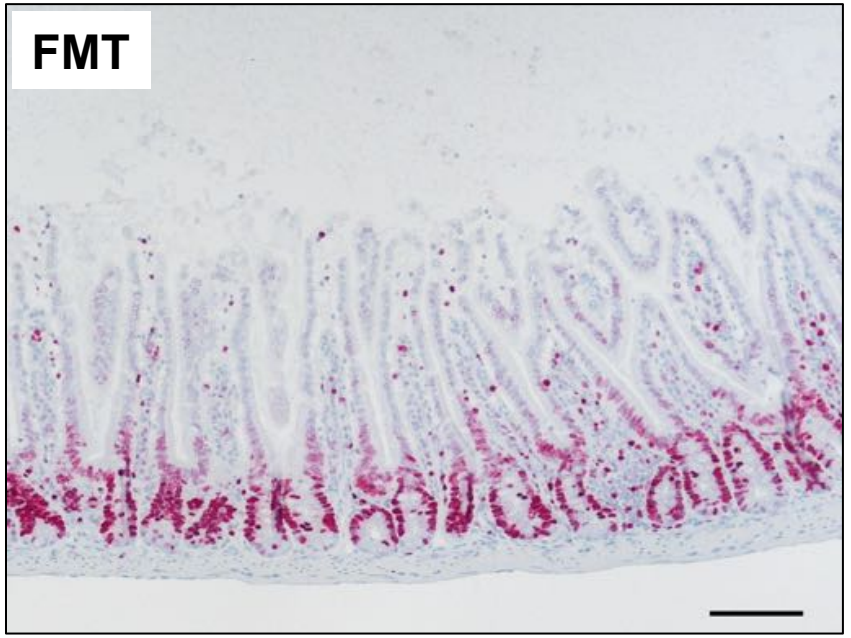
Proliferating Cells (Ki67+) – Small Intestine









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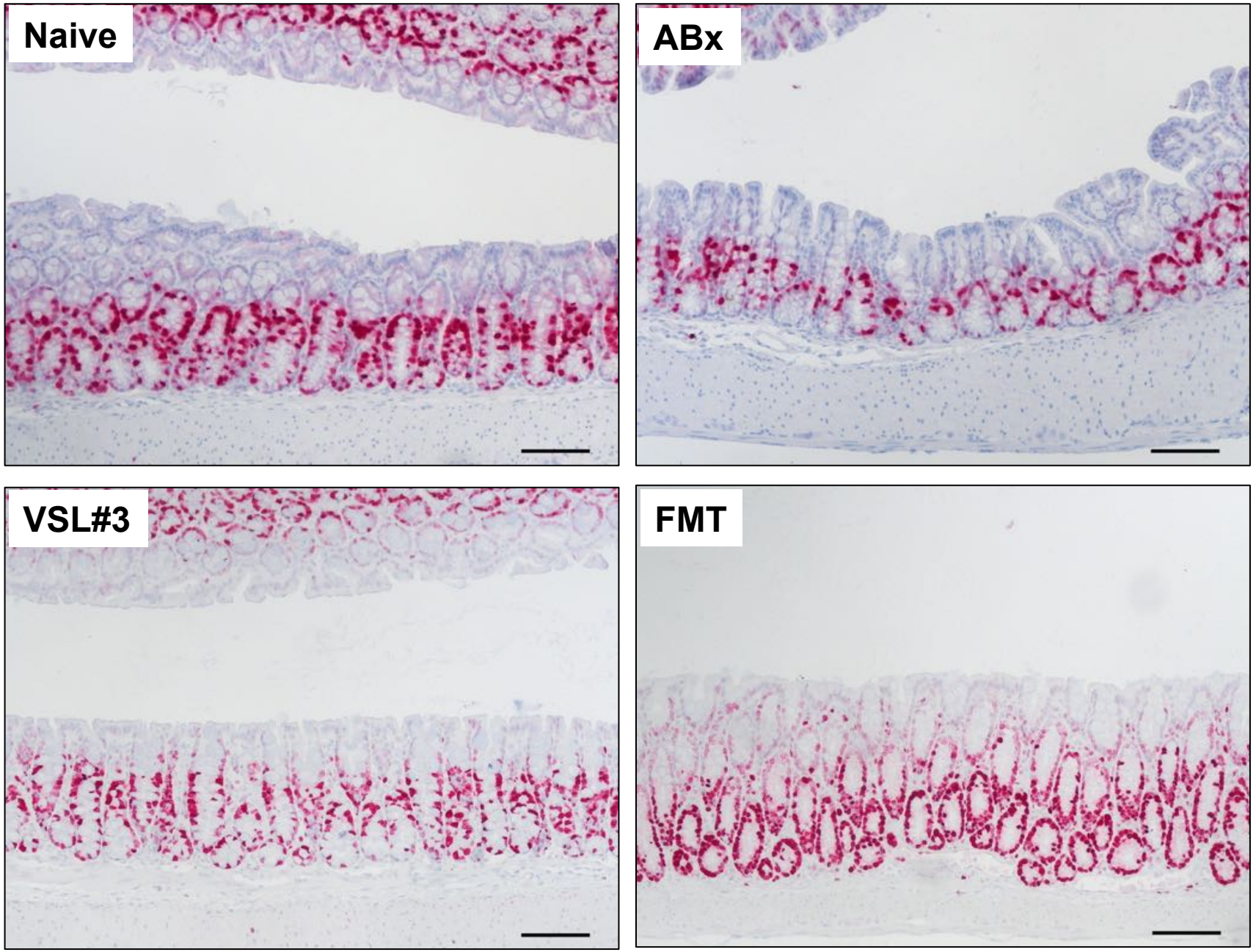
Proliferating Cells (Ki67+) – Colon



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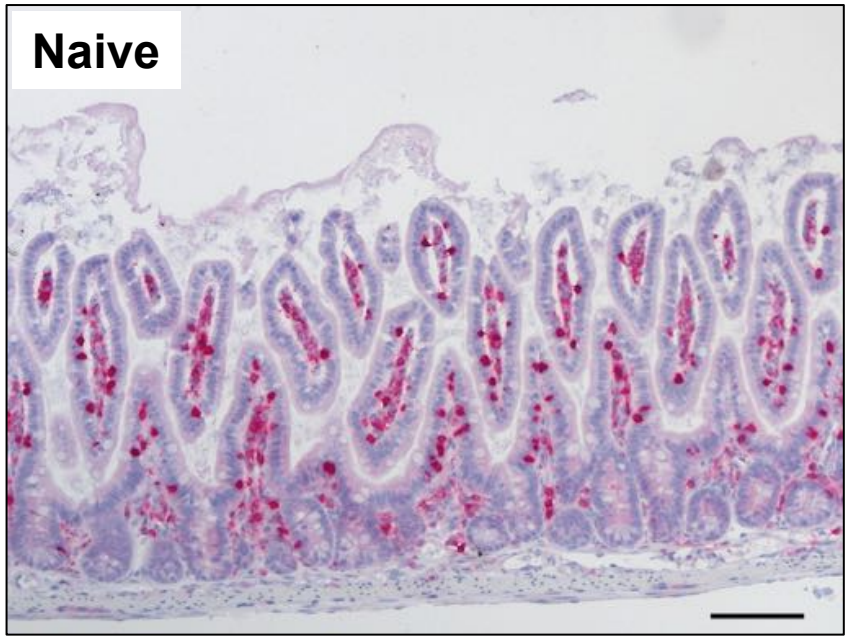
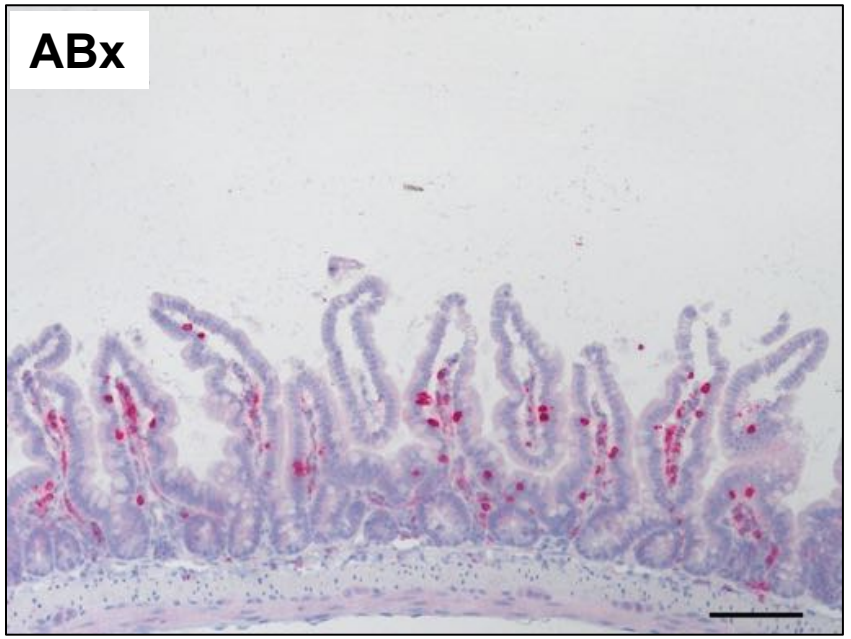
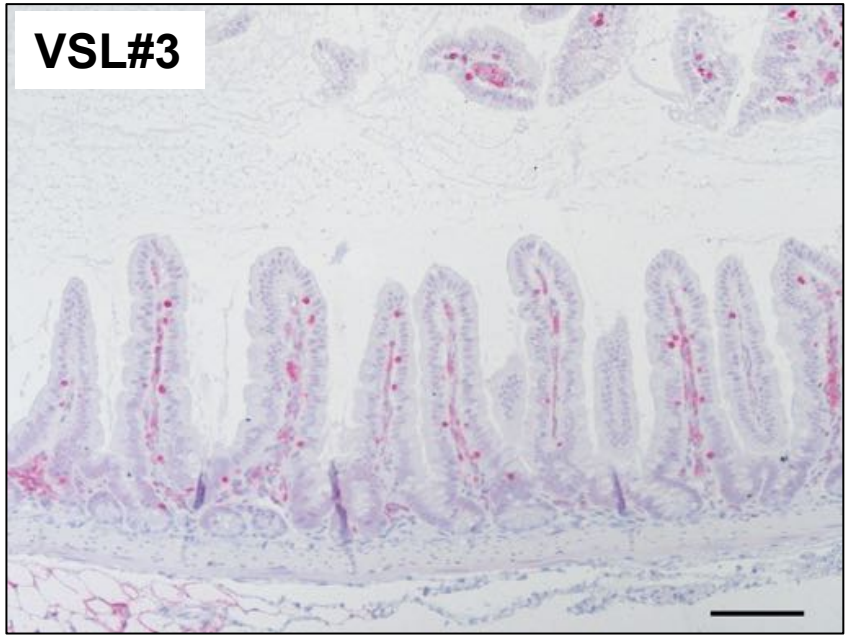
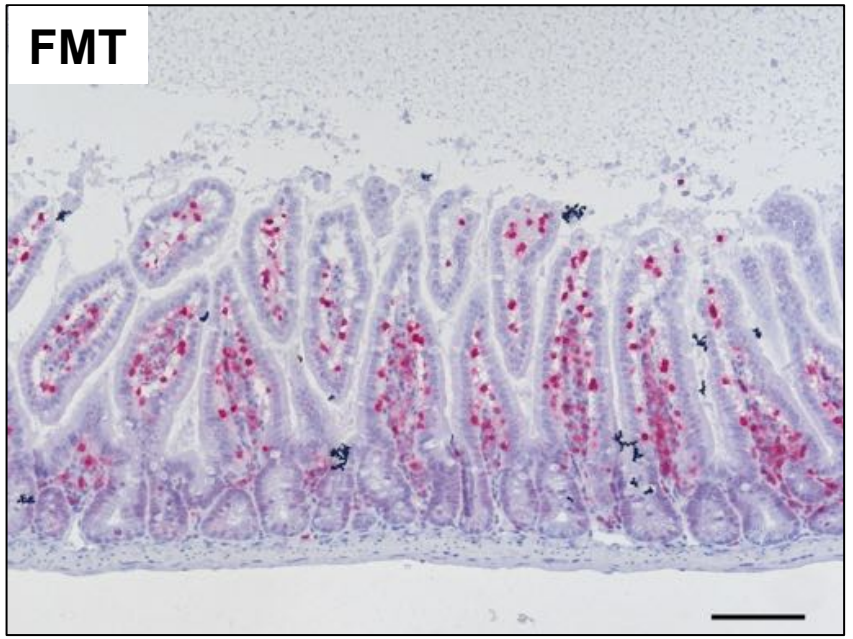
T Lymphocytes (CD3+) – Small Intestine









100 x magnification
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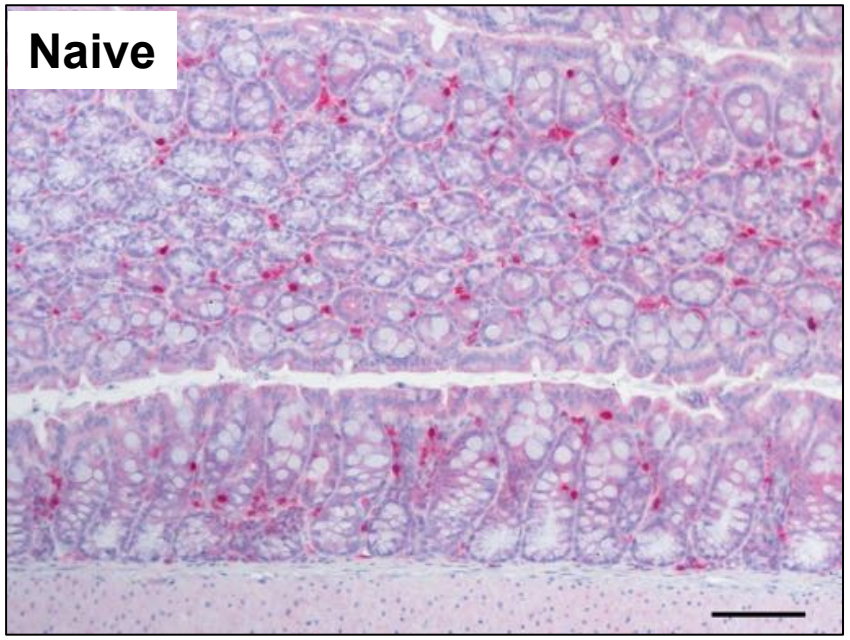
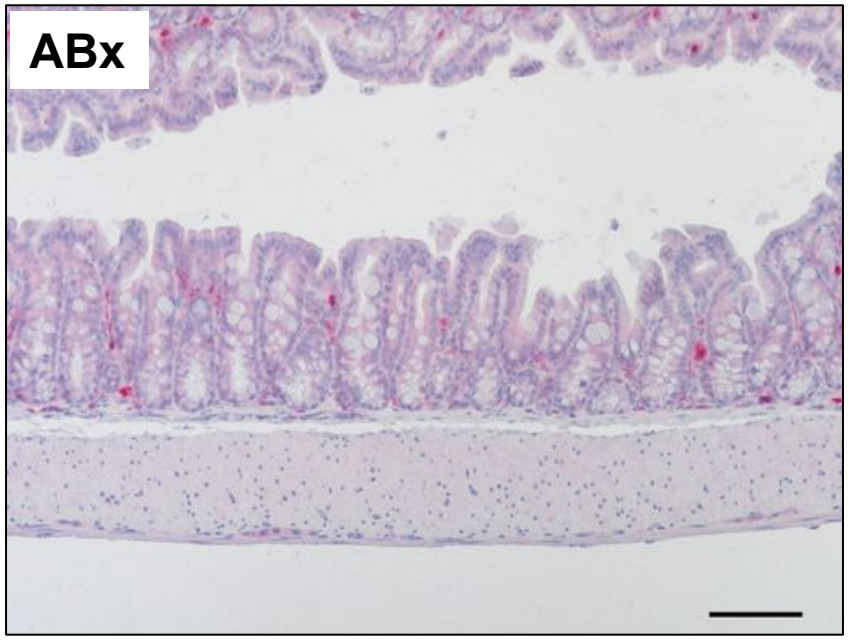
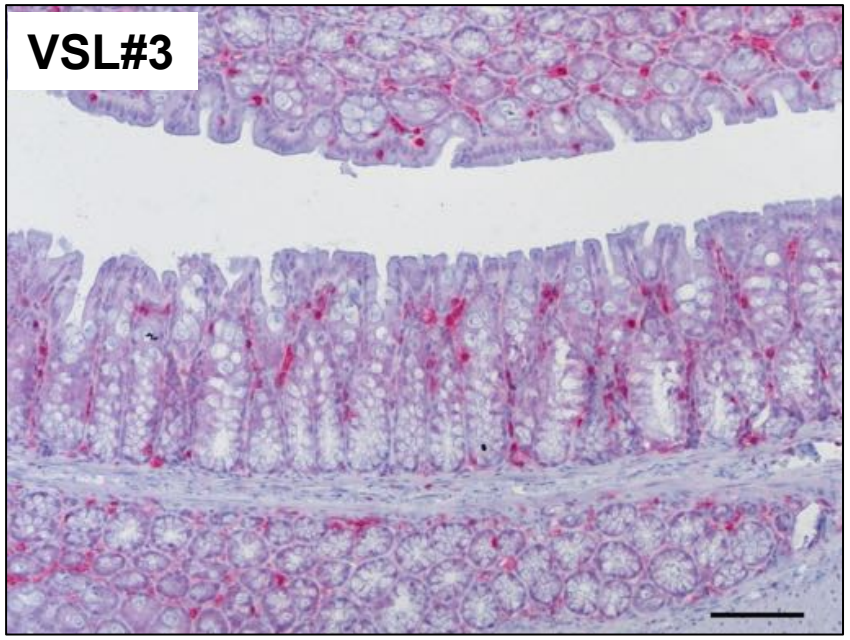
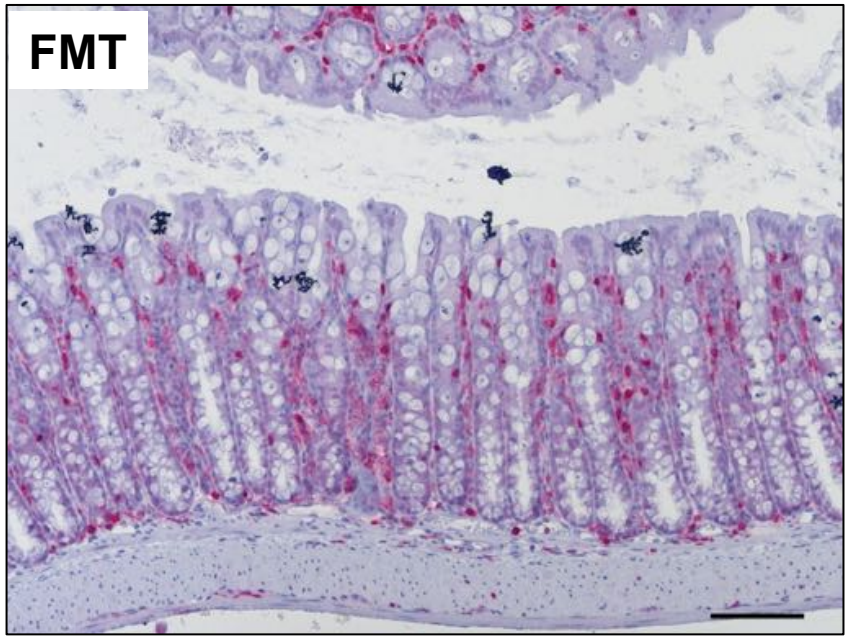
T Lymphocytes (CD3+) – Colon



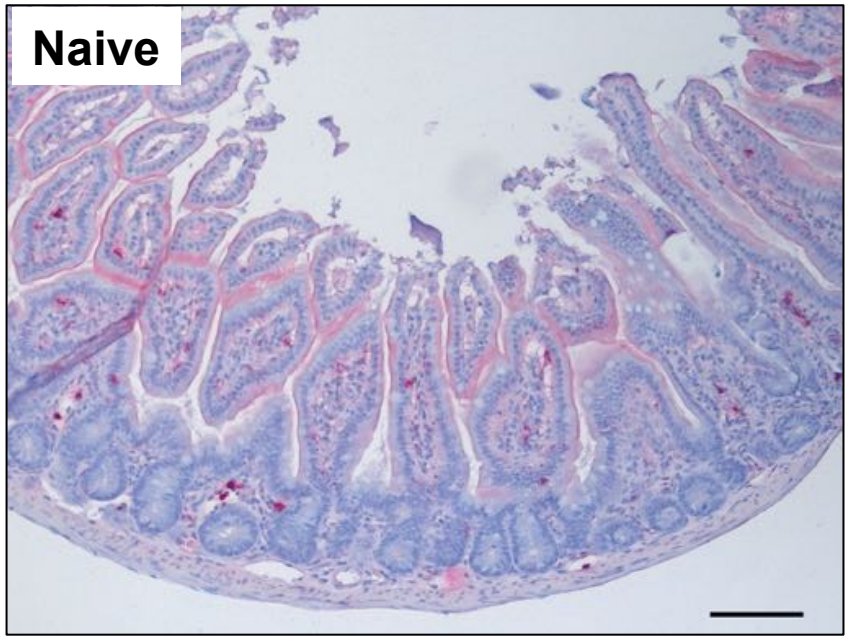


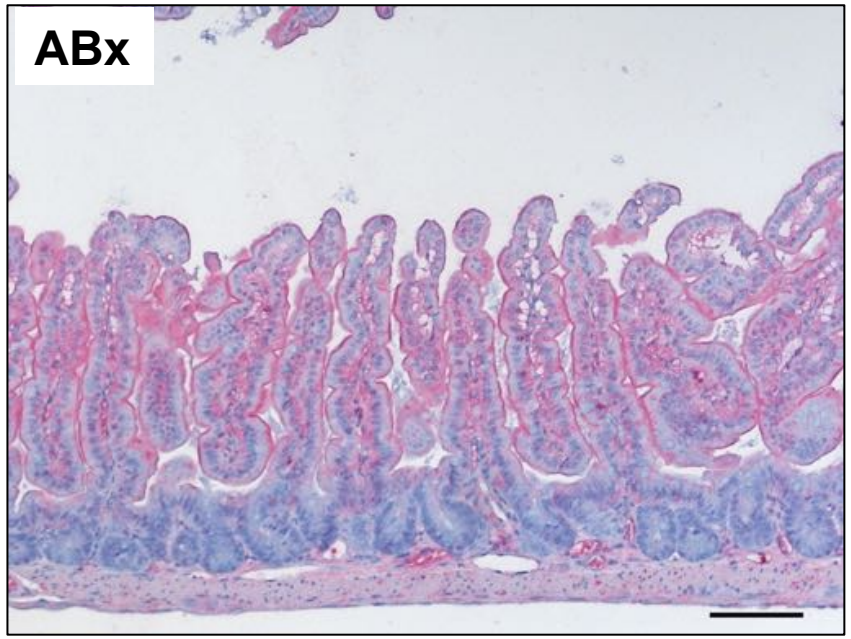


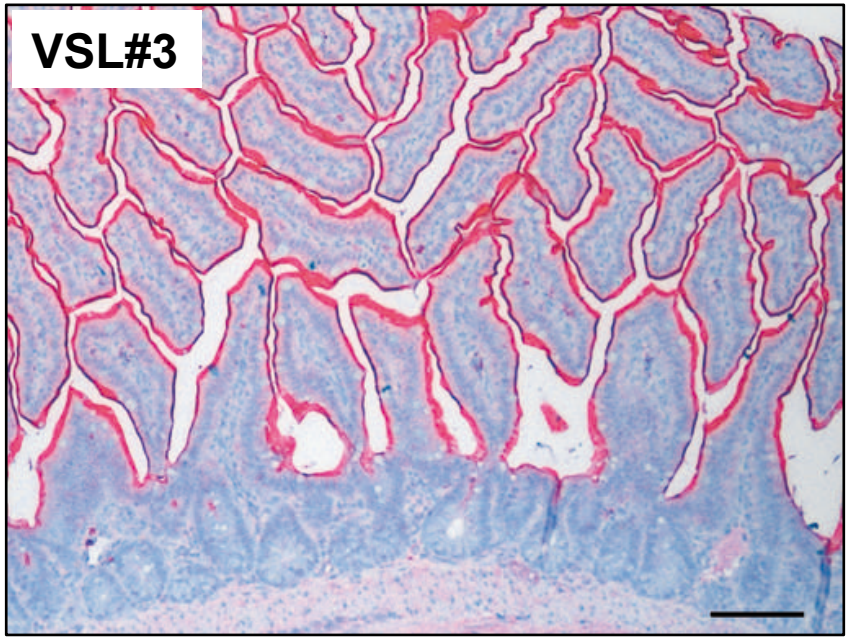


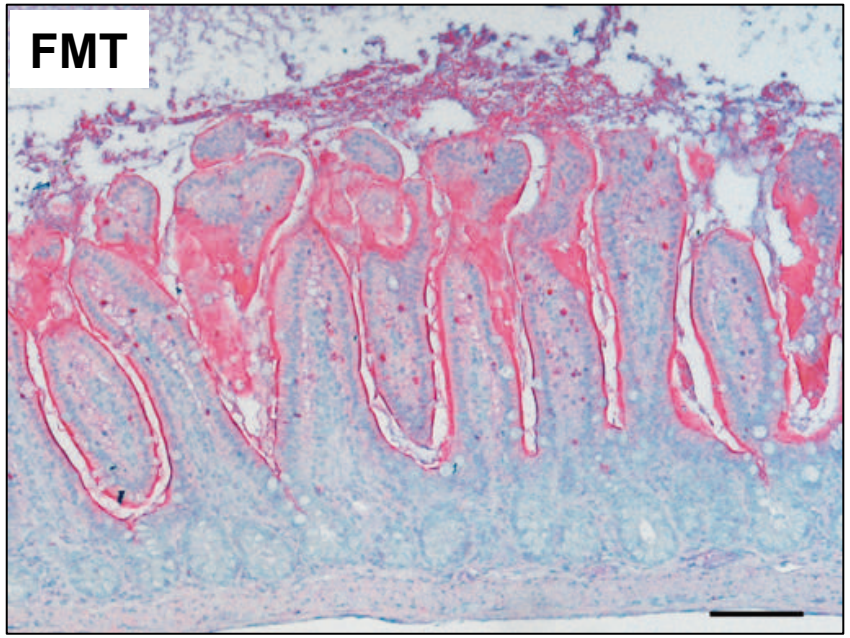
100 x magnification
Scale bar: 100 µm

B Lymphocytes (B220+) – Small Intestine



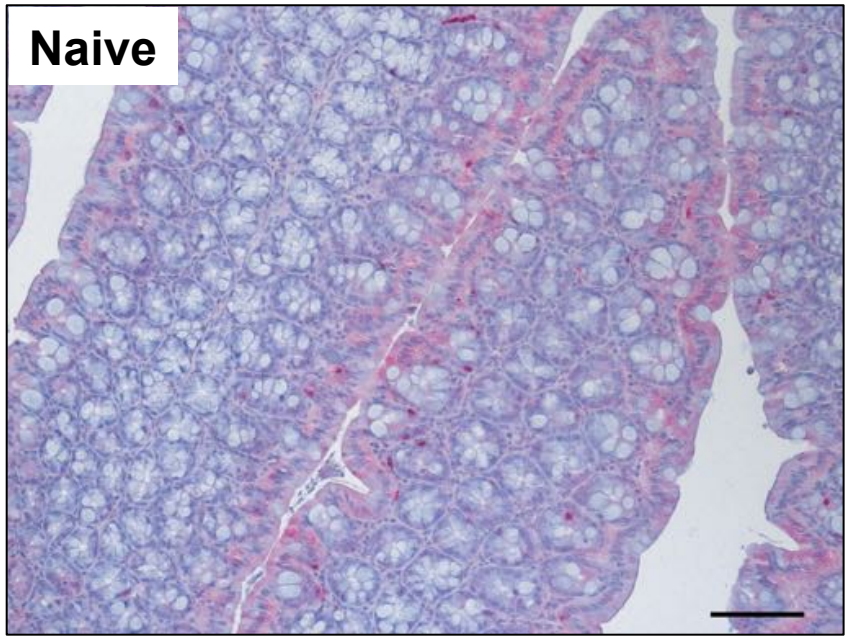


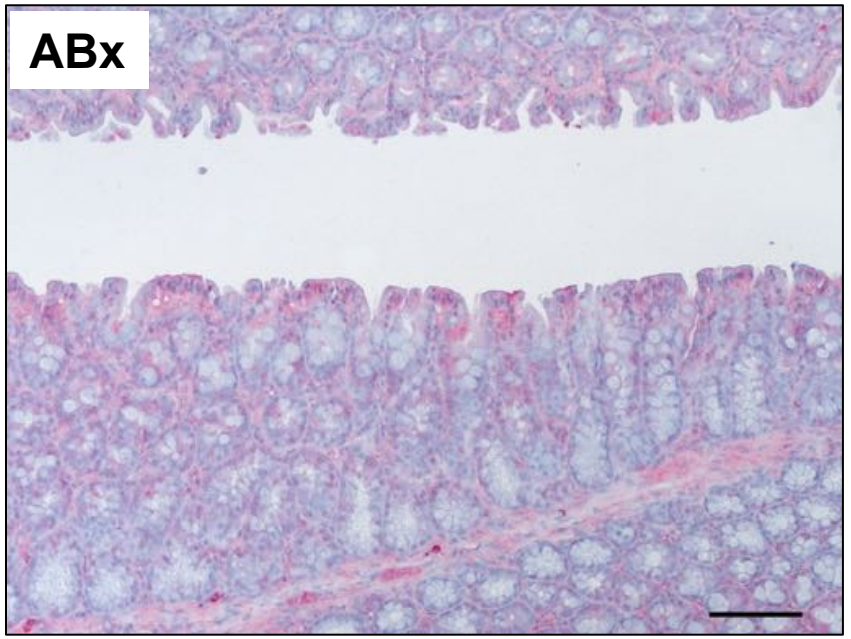


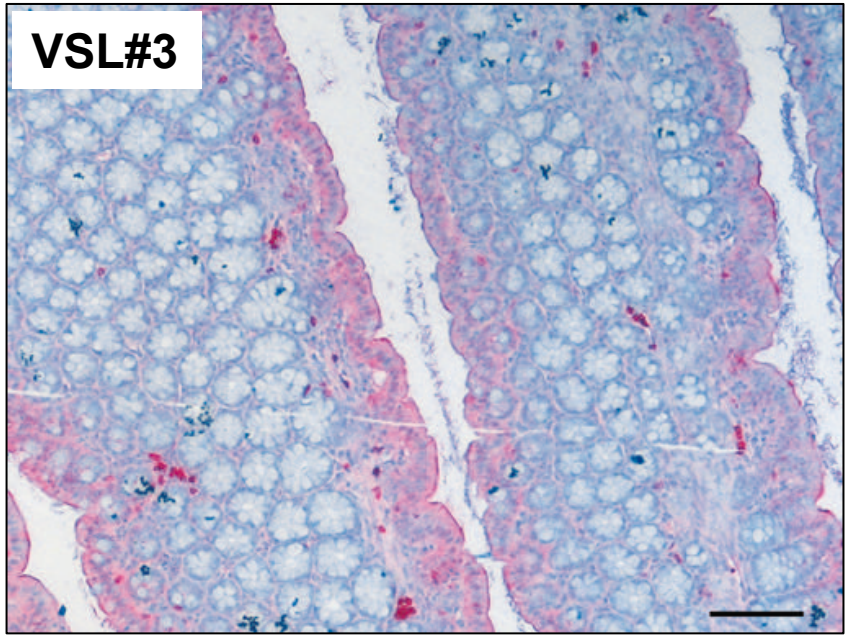


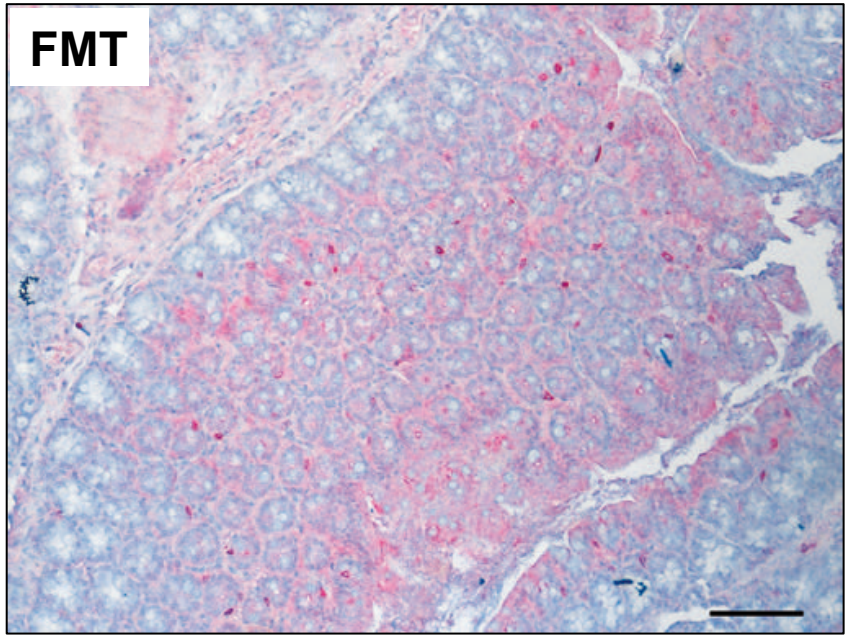
100 x magnification
Scale bar: 100 µm

B Lymphocytes (B220+) – Colon



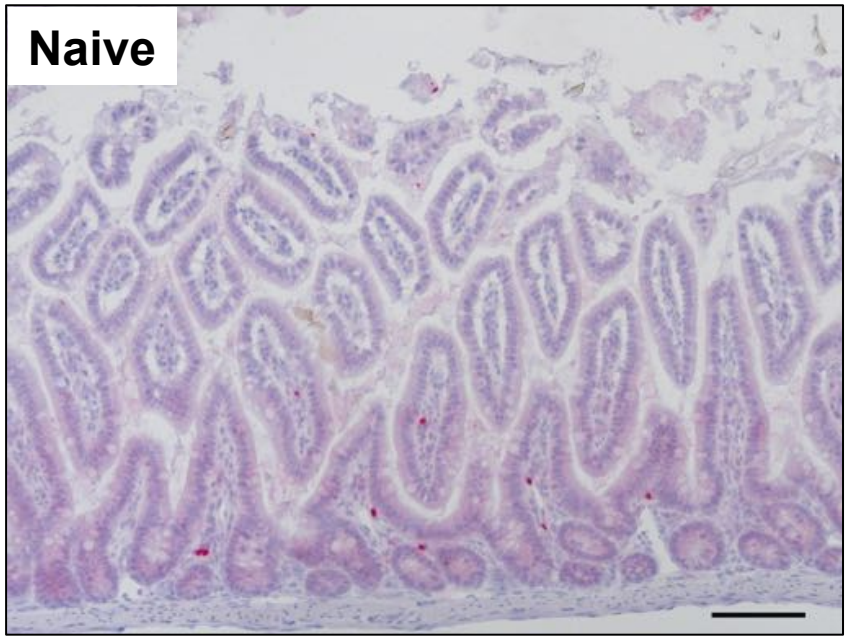


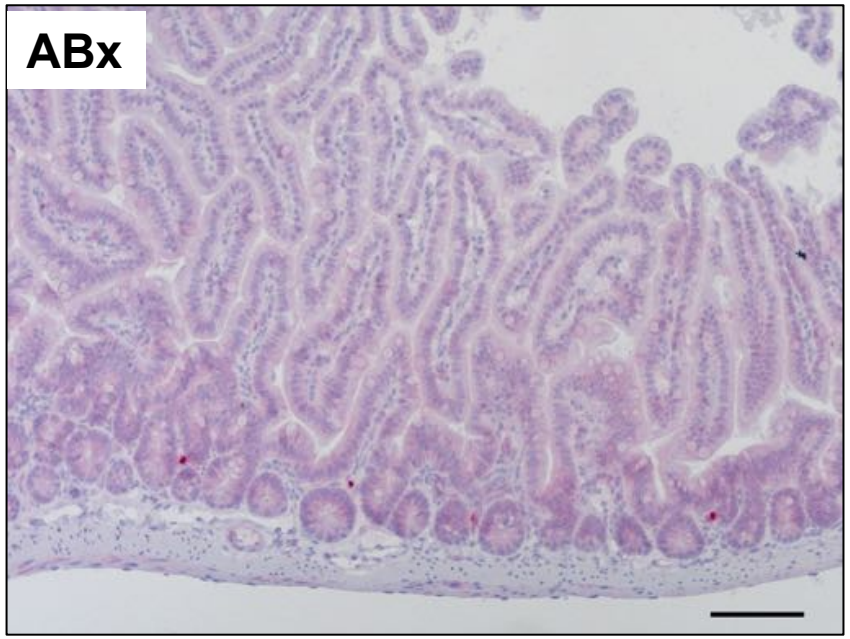


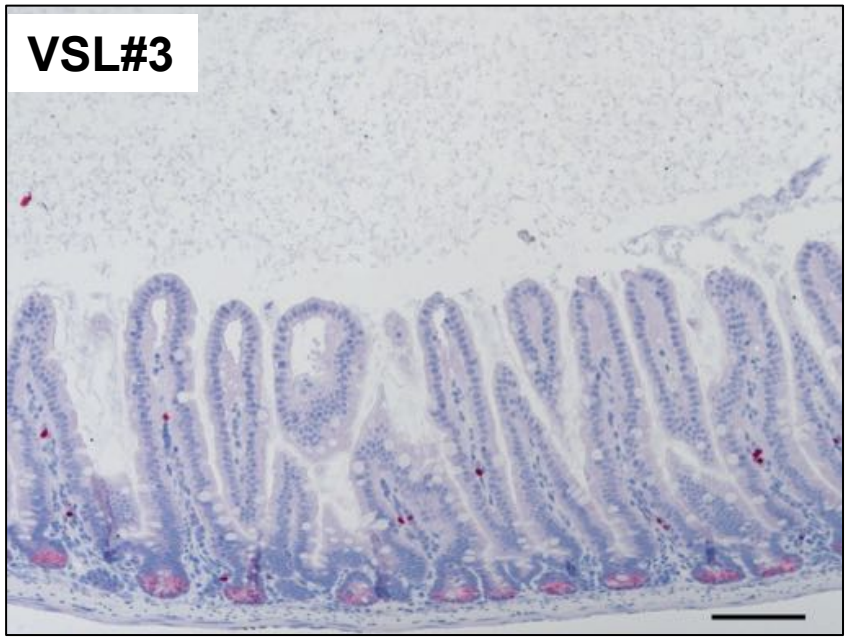


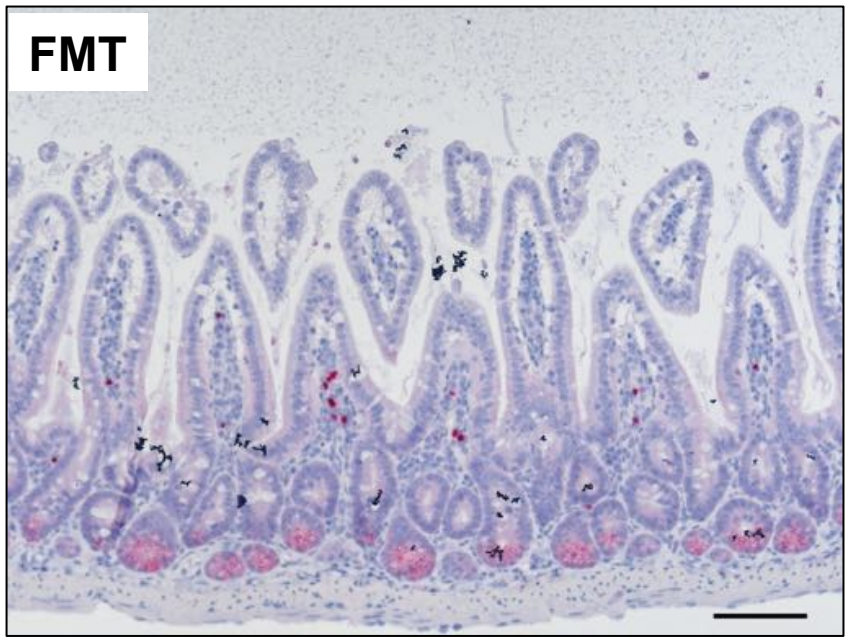
100 x magnification
Scale bar: 100 µm

Regulatory T Cells (Treg, FOXP3+) – Small Intestine



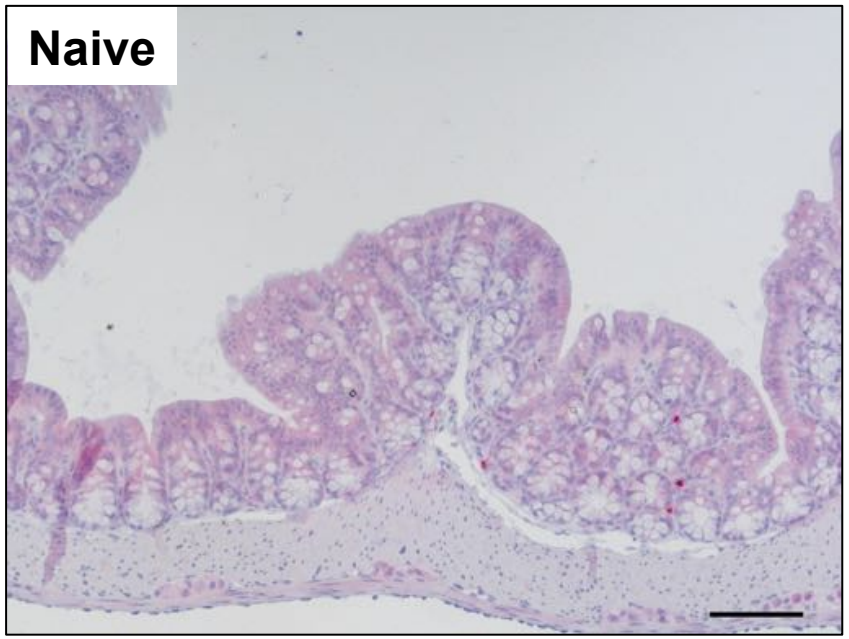


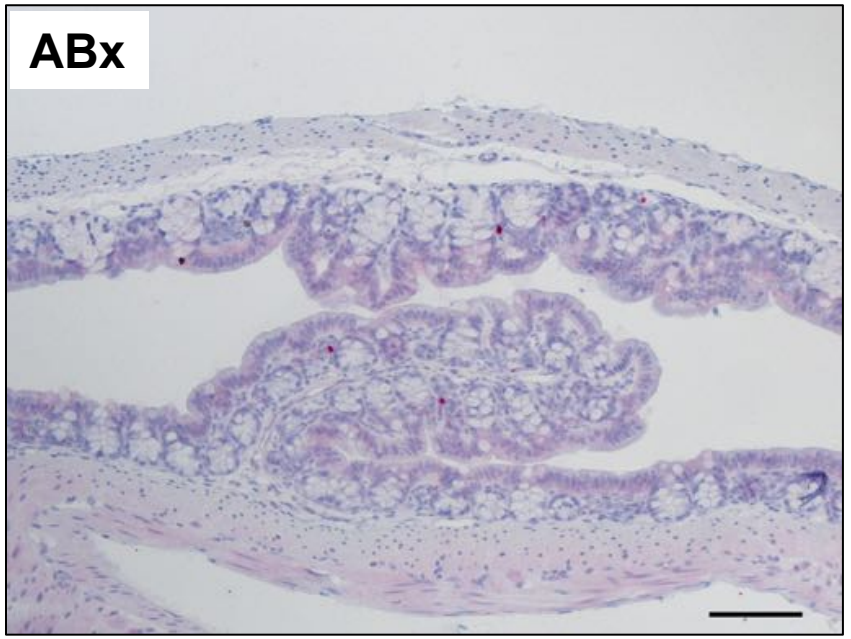


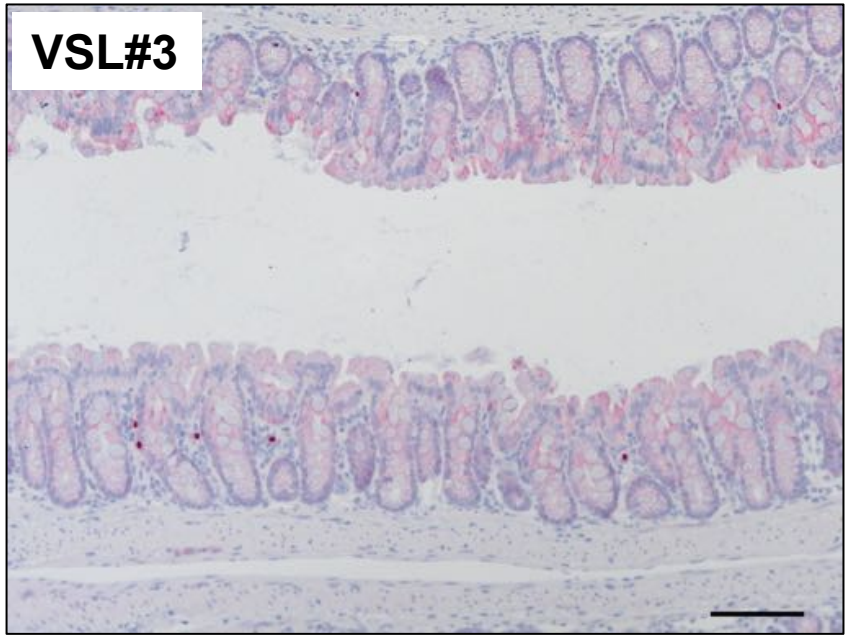


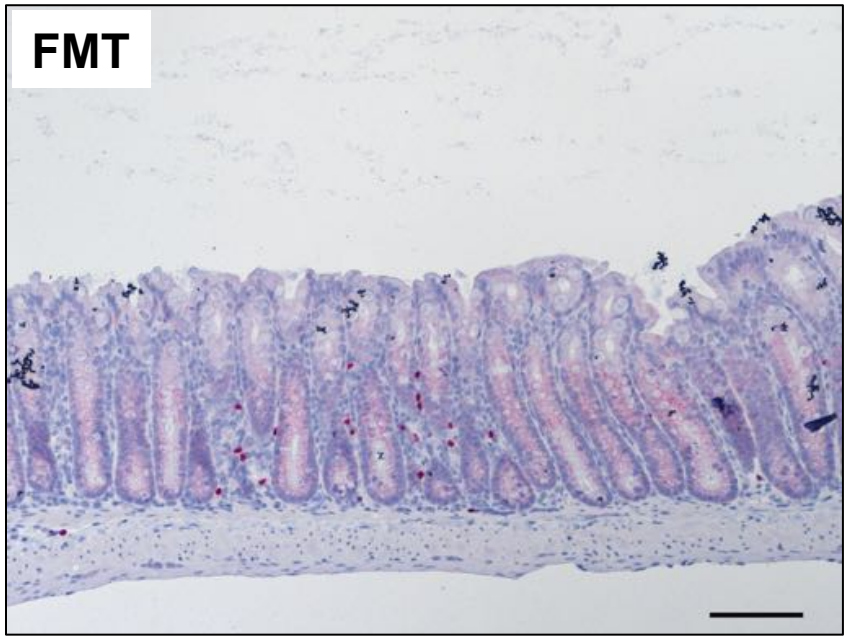
100 x magnification
Scale bar: 100 µm

Regulatory T Cells (Treg, FOXP3+) – Colon



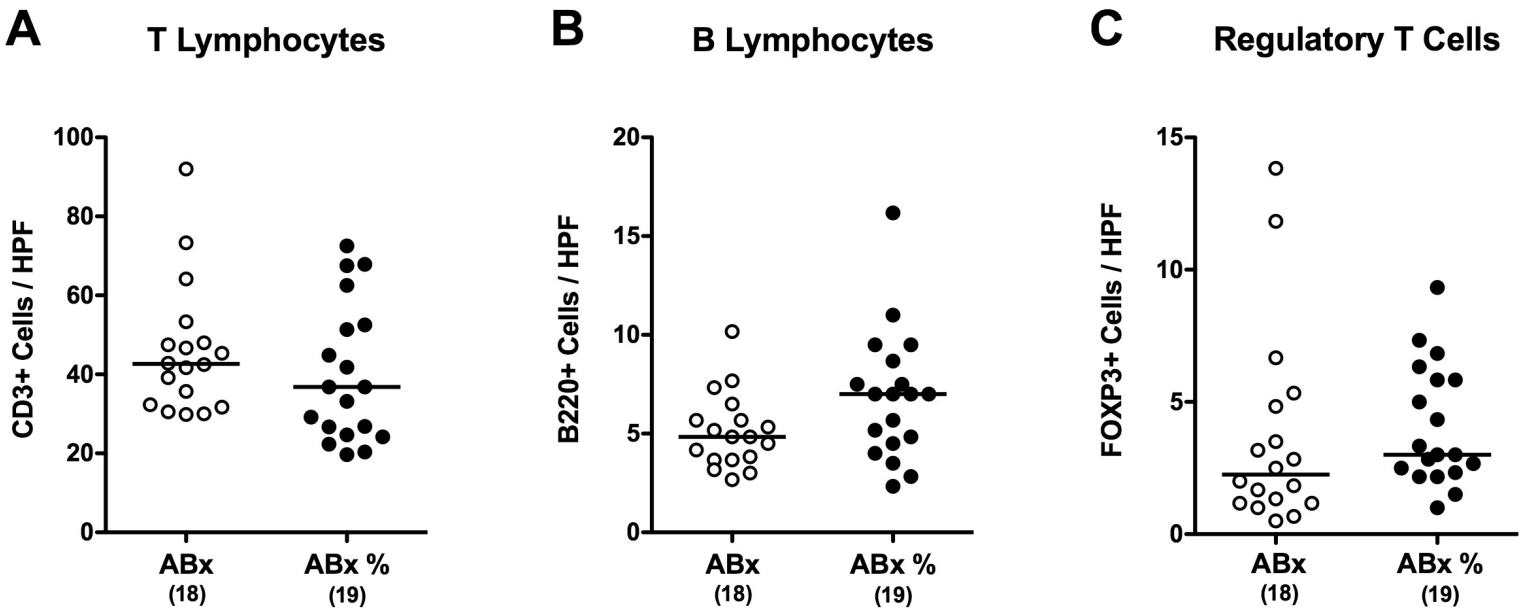






100 x magnification
Scale bar: 100 µm

Small Intestine



Colon

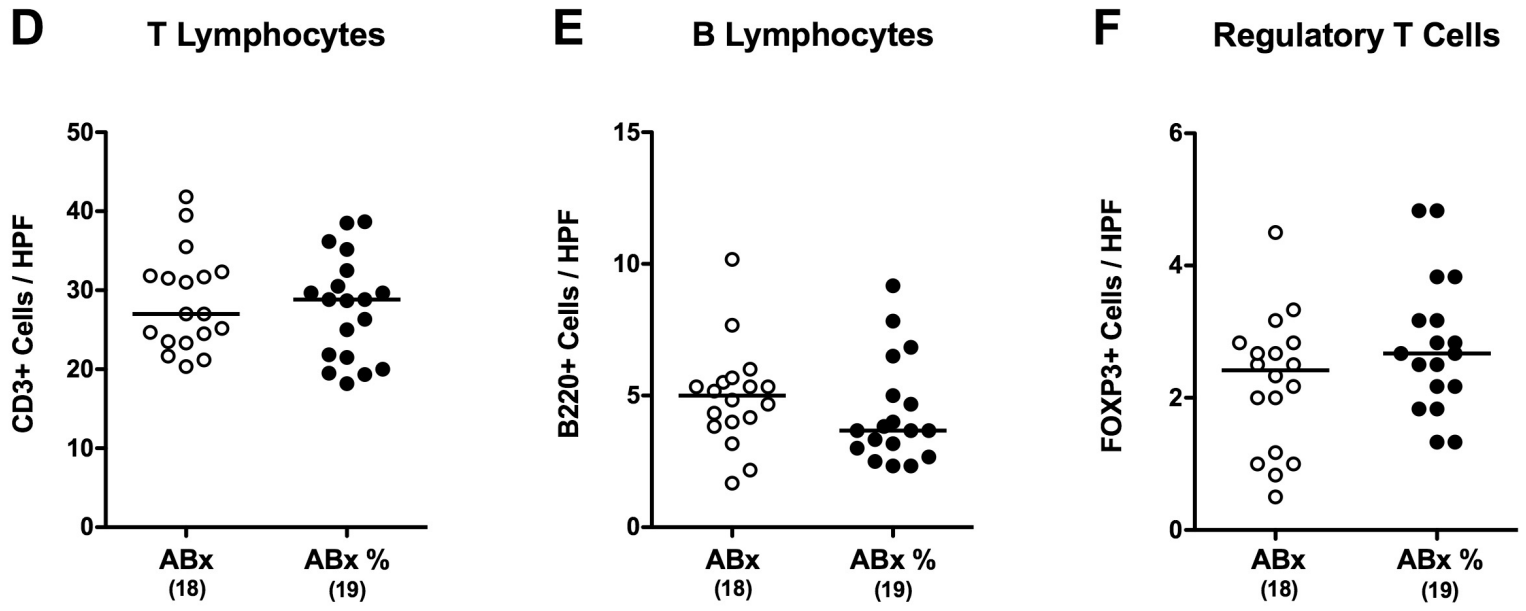


Figure S3

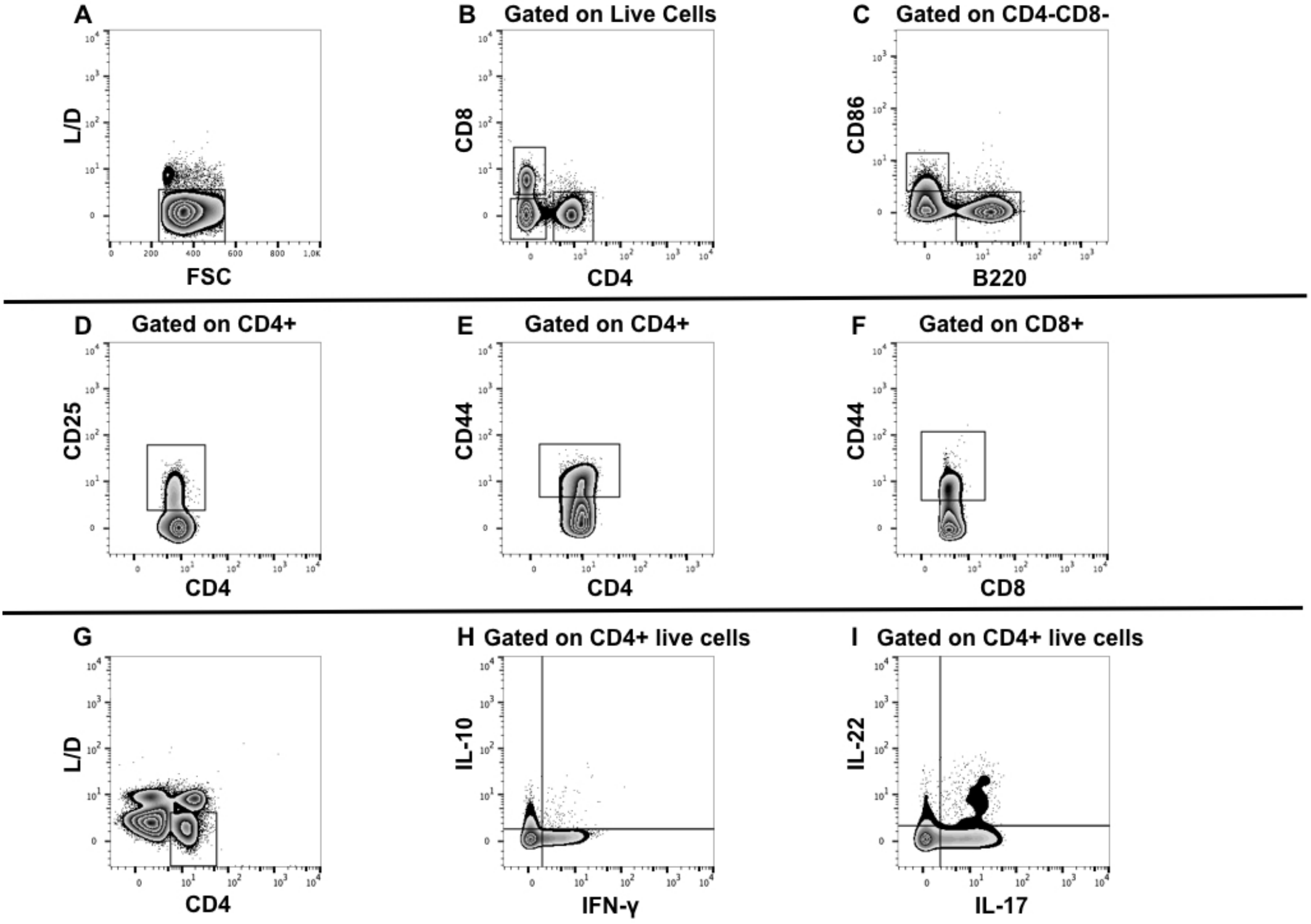
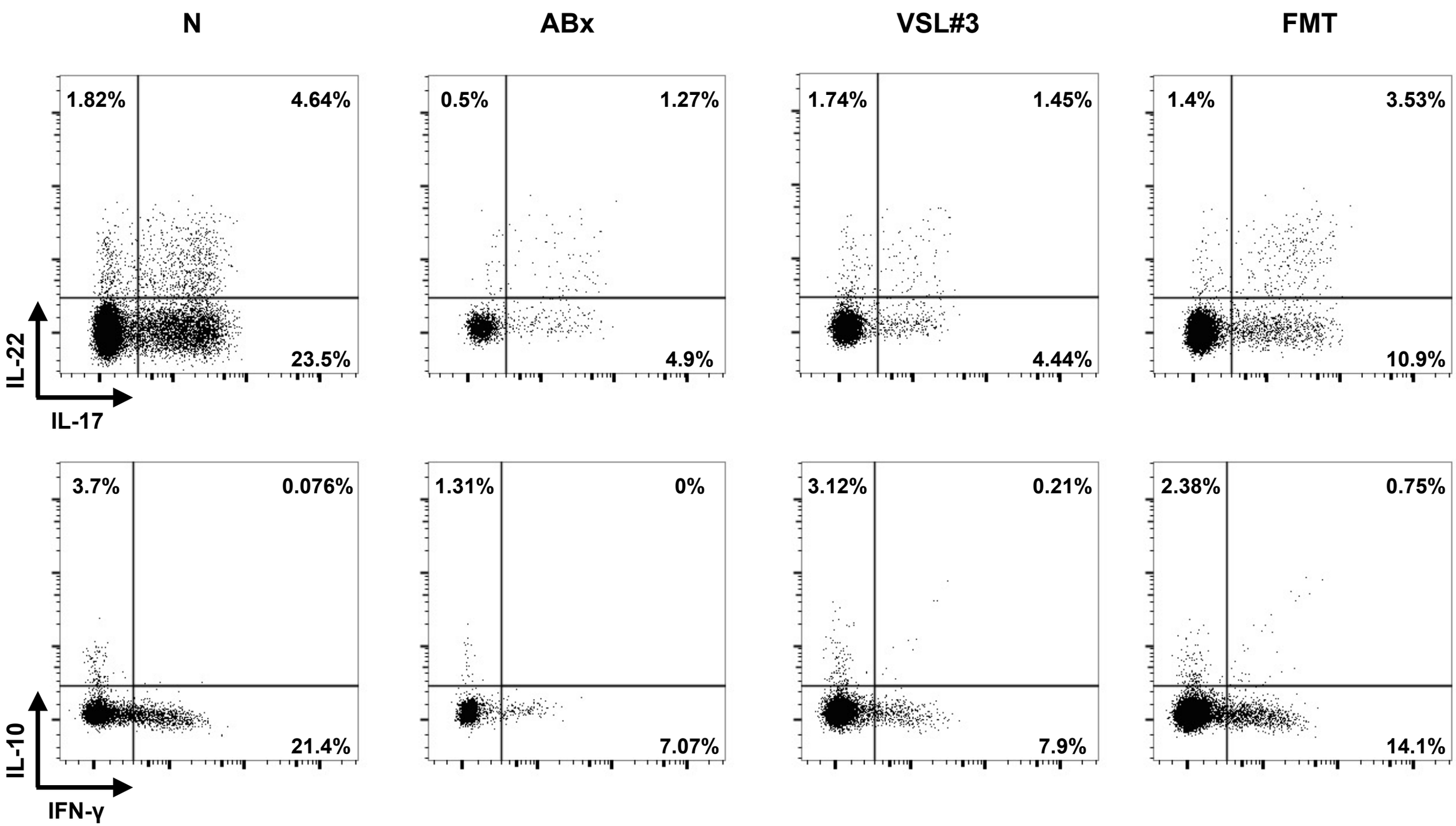
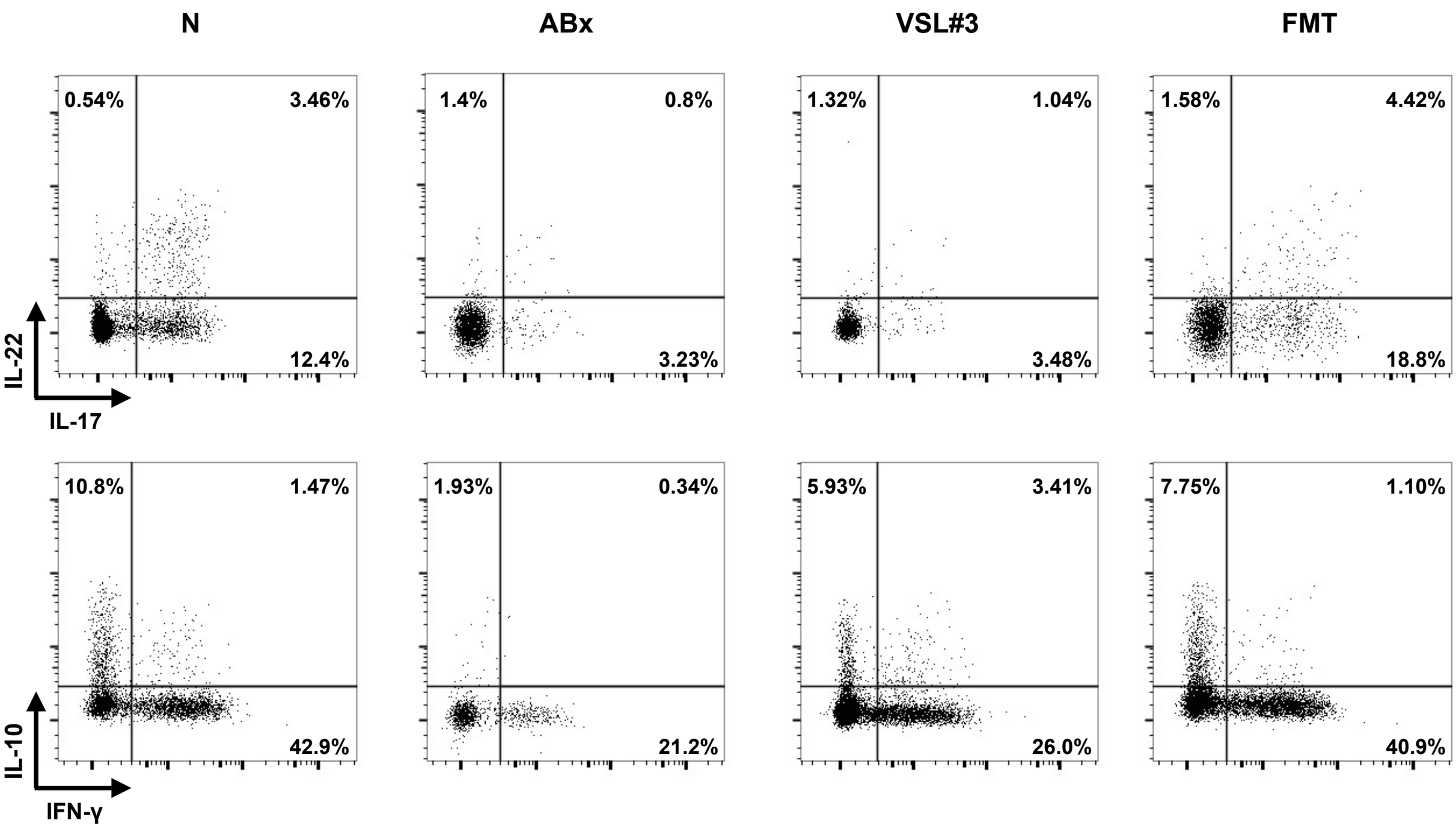

A
L/D
FSC
B Gated on Live Cells
CD8
CD4
C Gated on CD4-CD8-
CD86
B220
D Gated on CD4+
CD25
CD4
E Gated on CD4+
CD44
CD4
F Gated on CD8+
CD44
CD8
G
L/D
CD4
H Gated on CD4+ live cells
IL-10
IFN-γ
I Gated on CD4+ live cells
IL-22
IL-17

Figure S4

Small intestine

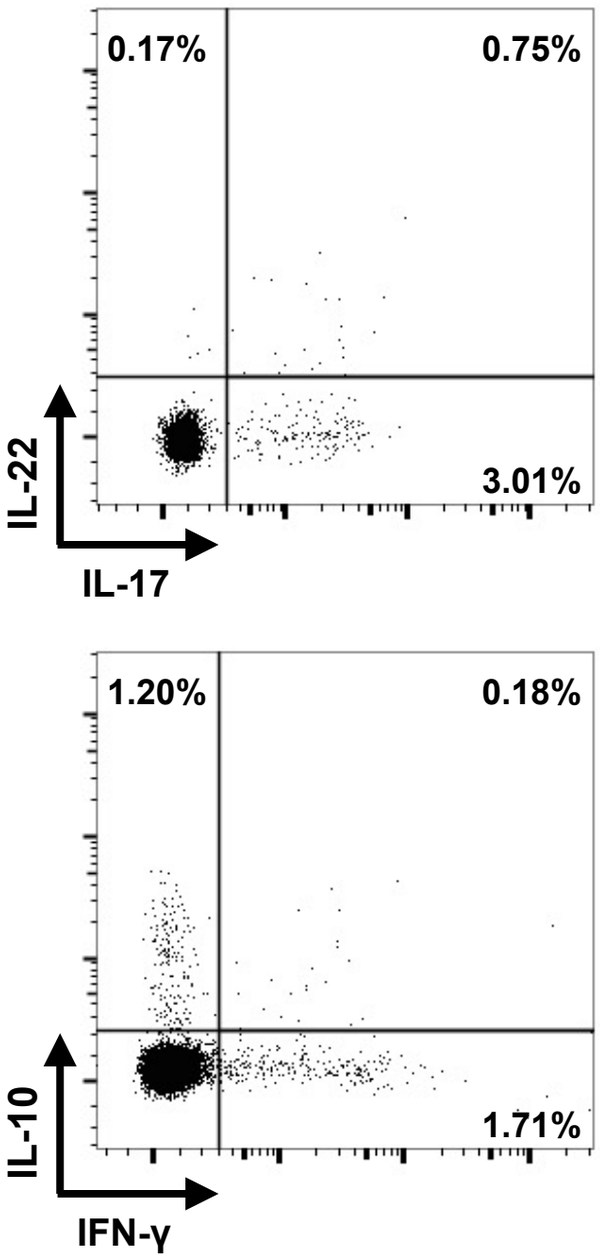


Colon

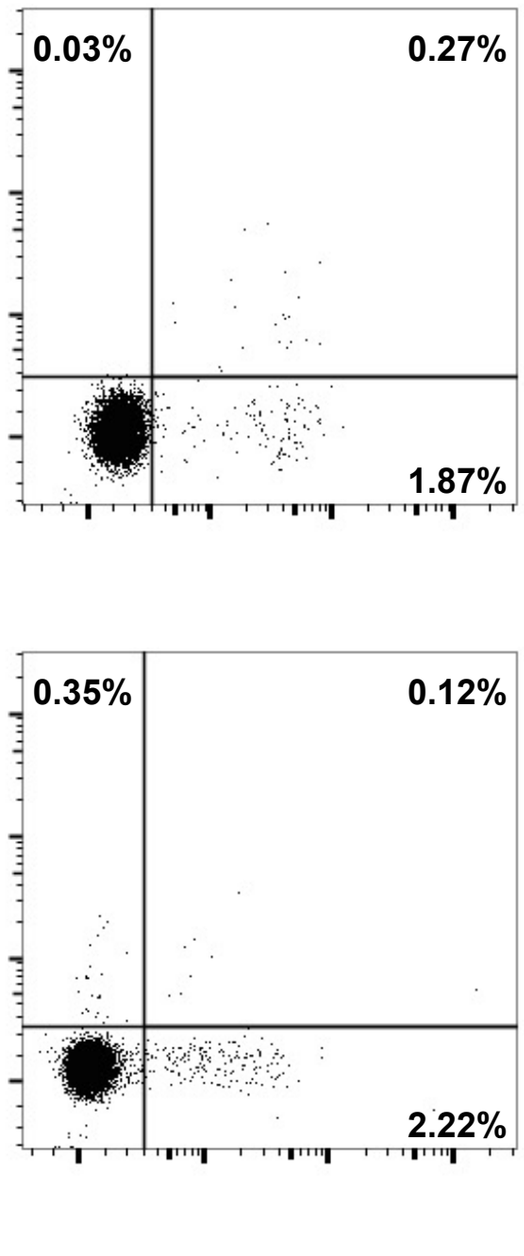

N
ABx
VSL#3
FMT
0.54%
3.46%
12.4%
1.4%
0.8%
3.23%
1.32%
1.04%
3.48%
1.58%
4.42%
18.8%
IL-22
IL-17
10.8%
1.47%
42.9%
1.93%
0.34%
21.2%
5.93%
3.41%
26.0%
7.75%
1.10%
40.9%
IL-10
IFN-γ

MLN

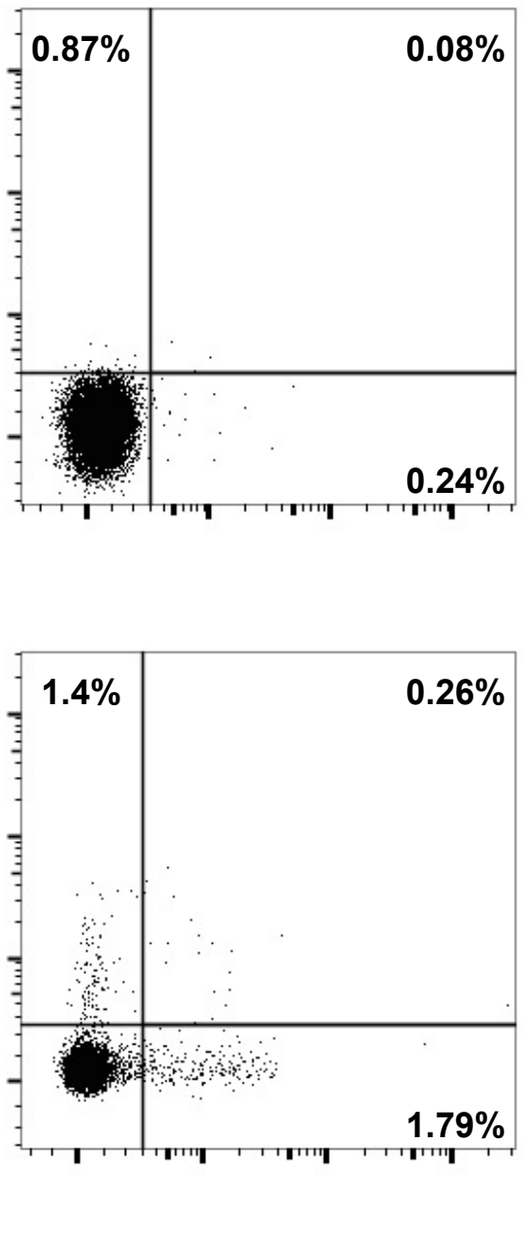
N



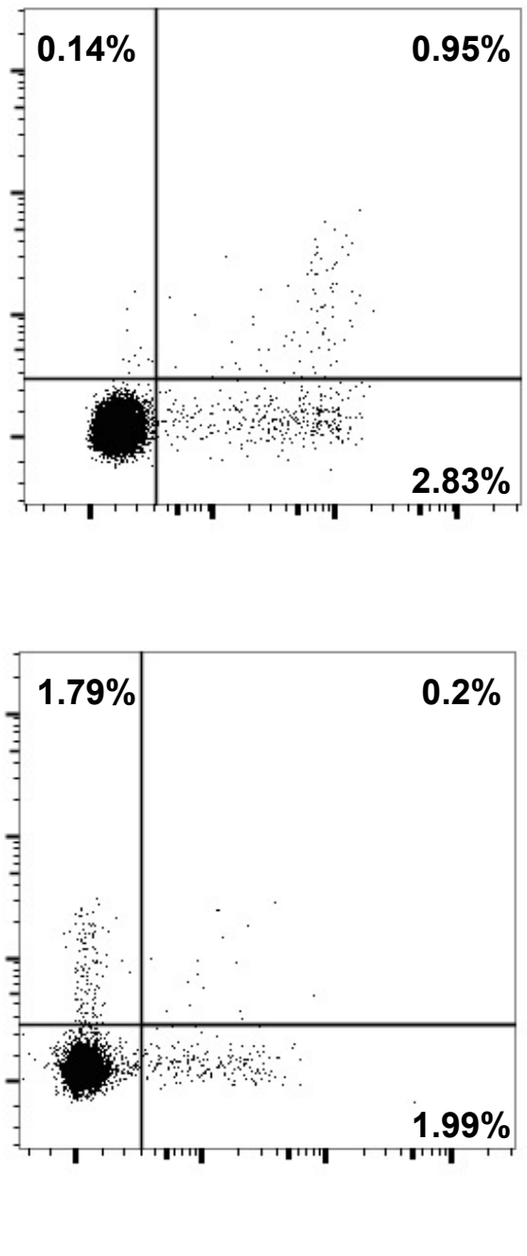
ABx



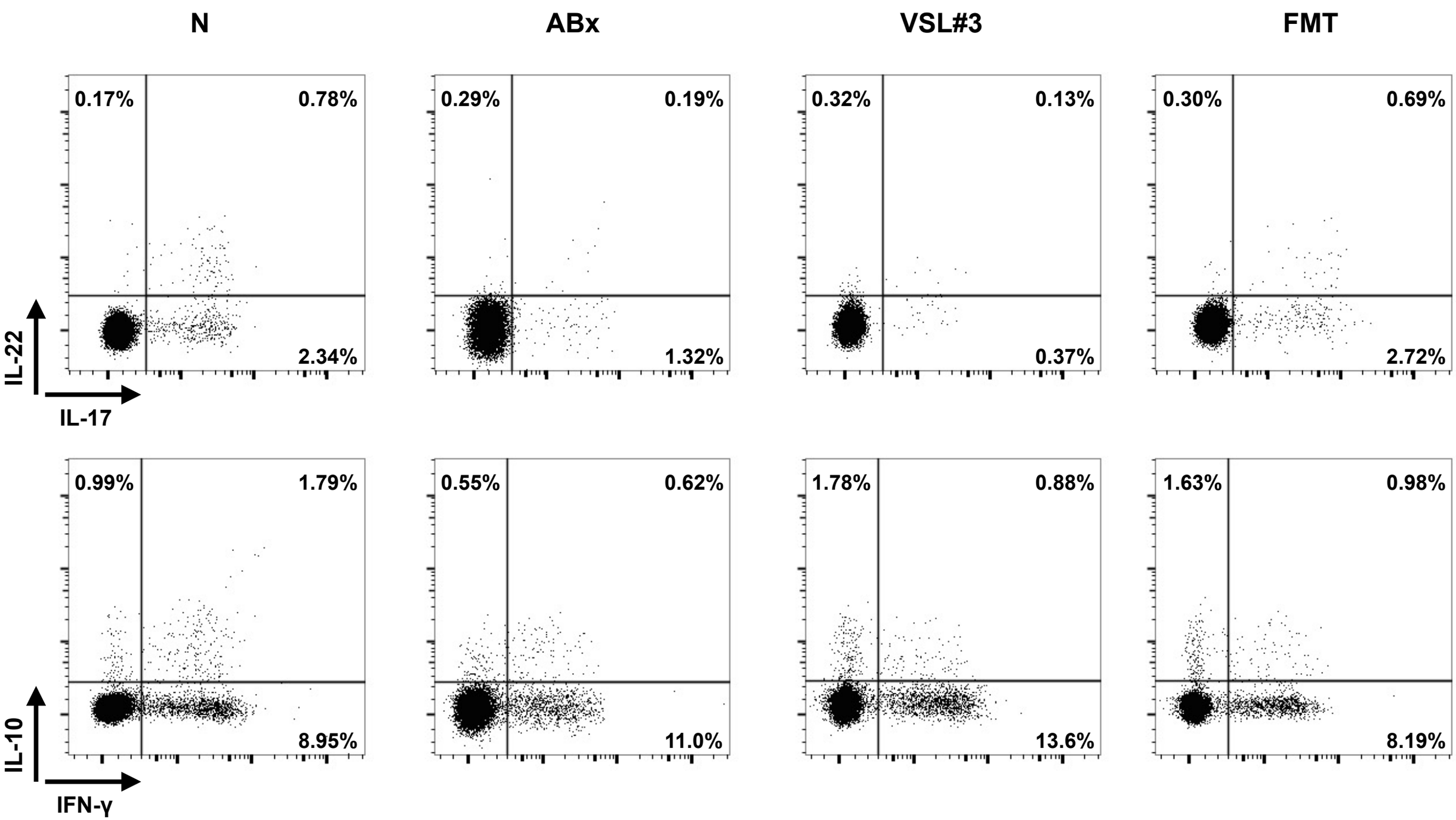
VSL#3



FMT



Spleen


N
ABx
VSL#3
FMT
0.17%
0.78%
2.34%
0.29%
0.19%
1.32%
0.32%
0.13%
0.37%
0.30%
0.69%
2.72%
IL-22
IL-17
0.99%
1.79%
8.95%
0.55%
0.62%
11.0%
1.78%
0.88%
13.6%
1.63%
0.98%
8.19%
IL-10
IFN-γ

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Gut Pathogens

RESEARCH

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Amelioration of intestinal and systemic sequelae of murine *Campylobacter jejuni* infection by probiotic VSL#3 treatment

Ira Ekmekciu[1], Ulrike Fiebiger[1], Kerstin Stingl[2], Stefan Bereswill[1] and Markus M. Heimesaat[1*]

Abstract

Background: The incidence of human *Campylobacter jejuni* infections is progressively increasing worldwide. Probiotic compounds might open up valuable tools to decrease pathogen burden and subsequent pro-inflammatory immune responses, but in vivo data are scarce.

Methods and results: Secondary abiotic mice generated by broad-spectrum antibiotic treatment were perorally challenged with the commercial probiotic compound VSL#3 consisting of *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*) either 5 days before (i.e. prophylactic regimen) or after (i.e. therapeutic regimen) peroral *C. jejuni* strain 81–176 infection, and analyzed 3 weeks following the initial bacterial re-association. Upon challenge, mice were colonized with the probiotic bacteria and/or *C. jejuni* at comparable intestinal loads, but co-colonization did not result in reduction of the pathogen burden. Remarkably, prophylactic as well as therapeutic VSL#3 treatment of *C. jejuni* infected mice ameliorated intestinal apoptosis and pro-inflammatory immune responses as indicated by lower numbers of innate and adaptive immune cell populations in the murine colon upon probiotic prophylaxis or treatment and reduced colonic concentrations of pro-inflammatory mediators including IL-6 and MCP-1. Importantly, concentrations of anti-inflammatory mediators such as IL-10 were significantly elevated in the colon of probiotics treated mice as compared to untreated controls. Strikingly, prophylactic VSL#3 treatment attenuated *C. jejuni* induced systemic pro-inflammatory responses as indicated by less TNF and IL-12p70 secretion in the spleen of VSL#3 pre-treated as compared to non-treated mice.

Conclusion: Administration of probiotic formulations such as VSL#3 might open up valuable strategies for prophylaxis and/or treatment of *C. jejuni* induced intestinal and systemic sequelae in vivo by the suppression of pro-inflammatory and induction of anti-inflammatory responses.

Keywords: Probiotic compound, VSL#3, Secondary abiotic mice, Gnotobiotic mice, Bacterial in vivo competition, Pathogen–commensal bacteria–host interaction, Apoptosis, Innate and adaptive immune cells, Pro-inflammatory cytokines, Anti-inflammatory cytokines, Extra-intestinal and systemic sequelae of infection

Background

The enteric bacterial pathogen *Campylobacter jejuni* is regarded as a commensal within the intestinal tract of wild and domestic animals, but highly virulent in humans acquiring the pathogen usually by consumption of contaminated products derived from livestock animals or contaminated surface water via the peroral route [1–3]. Whereas *Campylobacter* infections are on the rise worldwide [4–6], patients present with gastroenteritis of varying degree ranging from mild malaise and watery diarrhea to severe ulcerative colitis with inflammatory, bloody diarrhea [7]. In the vast majority of cases, intestinal disease resolves spontaneously, whereas

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systemic post-infectious sequelae including peripheral neuropathies such as Guillain-Barré-syndrome, Miller-Fisher syndrome or reactive arthritis might develop with a latency of weeks to months [8–10]. Due to the lack of suitable experimental in vivo models of campylobacteriosis, our understanding of the molecular mechanisms underlying *Campylobacter*-host interactions has been hampered for a long time [3, 11]. Conventionally colonized mice, for instance, are protected from *C. jejuni* infection due to the host specific microbiota composition exerting physiological colonization resistance [3, 12]. Previous results from our own experiments revealed that modification of the murine intestinal microbiota facilitated *C. jejuni* infection [12, 13]. Upon virtual eradication of the intestinal microbiota by broad-spectrum antibiotic treatment secondary abiotic mice became highly susceptible to *C. jejuni* colonization and exhibited key features of human campylobacteriosis such as apoptosis and inflammation in the colon [12]. Notably, colonization resistance was restored in secondary abiotic mice recolonized with a murine microbiota. Thus, both secondary abiotic mice and secondary abiotic animals re-colonized with a murine microbiota are well suited to unravel the triangular relationship between intestinal pathogens, bacteria and the host immune system in vivo [12, 14].

Given the importance of the distinct intestinal microbiota composition in rendering the vertebrate host resistant against enteric pathogens including *C. jejuni*, interest in the potential of "beneficial" modulations of the microbiota composition in humans as well as in livestock animals has arisen. One promising strategy is the application of probiotics, defined as live microorganisms which, when administered in adequate concentrations, bestow health benefits to the host [15]. There are numerous indications from both in vitro and in vivo studies pointing out the efficacy of probiotics in therapy and prevention of enteric infections. Strains of probiotic microorganisms such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus gasseri*, and *Bifidobacterium lactis*, for instance, have been shown to inhibit growth, metabolism and adhesion of enteropathogenic bacteria including *C. jejuni*, *Salmonella*, *Shigella*, enterotoxigenic *Escherichia coli* or *Vibrio cholerae* to intestinal cells [16–20]. Furthermore, effects of probiotics have been examined in clinical studies for a number of gastrointestinal diseases. For instance, randomized trials suggest that co-administration of VSL#3, a probiotic compound consisting of eight different bacterial strains [21], or *Saccharomyces boulardii* [22] significantly decrease the incidence of antibiotics associated diarrhea (AAD). Moreover, episodes of infectious diarrhea in both adults and children can be shortened by the use of probiotics [23]. A meta-analysis of 74 experimental studies, 84 clinical trials and more than 10,000 patients revealed that probiotics were effective in the therapy and prevention of several gastrointestinal diseases including AAD, *Clostridium difficile* toxin induced acute enterocolitis (the most severe form of AAD), infectious diarrhea, pouchitis and irritable bowel syndrome, but not of travelers' diarrhea or necrotizing enterocolitis [24]. However, the underlying mechanisms of the probiotic effect are yet not fully understood. Proposed mechanisms of action include, for instance, modification of the intestinal microbiota [25], enhancement of colonization resistance [26] and intestinal barrier functions [27], as well as modulation of innate and adaptive immune functions [28].

In the present study, we examined the beneficial effects exerted by prophylactic and therapeutic treatment of *C. jejuni* infected mice with the probiotic compound VSL#3. We addressed, whether peroral VSL#3 application would lower intestinal pathogenic burden in the host, down-regulate *C. jejuni* induced pro-inflammatory sequelae and/or conversely, up-regulate anti-inflammatory immune responses not only locally (i.e. in the intestinal tract), but also in extra-intestinal compartments including systemic compartments.

Methods

Generation of secondary abiotic mice

Female C57BL/6j mice were bred and maintained within the same specific pathogen free (SPF) unit in the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité-University Medicine Berlin). Secondary abiotic mice virtually lacking an intestinal microbiota were generated by broad-spectrum antibiotic treatment for 8 weeks as described previously [29]. In brief, 8–10 week old mice were transferred to sterile cages and treated with a quintuple broad-spectrum 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. Absence of cultivable bacteria in feces samples (applying thioglycolate enrichment broths; Oxoid, Wesel, Germany) for at least three consecutive weeks was used as a quality control for the successful depletion of the gut microbiota [29].

Probiotic treatment and *C. jejuni* infection of secondary abiotic mice

Three days prior to bacterial re-colonization or infection experiments the quintuple antibiotic cocktail was withdrawn and replaced by autoclaved tap water. Mice

were perorally infected with 10^9 colony forming units (CFU) *C. jejuni* strain 81–176 in 0.3 mL sterile phosphate buffered saline (PBS) by gavage as described earlier [12]. For probiotic re-colonization, mice received a suspension of the commercial formulation VSL#3 (probiotic food supplement; Manufacturer: SIIT S.r.l, Trezzano sul Naviglio, Italy; distributed by Actial Farmaceutica, Funchal, Madeira, Portugal) consisting of the following eight bacterial species: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*). A total of 4.5×10^{11} probiotic bacteria were dissolved in 50 mL sterile PBS. By gavaging 0.3 mL (either five days before or after *C. jejuni* infection), each mouse received 10^9 viable probiotic bacteria as confirmed by cultural analyses of the suspensions. Mice infected either with the pathogen or re-colonized with the probiotic formulation alone as well as naive uninfected mice served as controls. Mice were continuously kept in a sterile environment (autoclaved food and drinking water) and were handled under strict aseptic conditions to prevent from contaminations.

Sampling procedures

Tissue samples from colon, mesenteric lymph nodes (MLN) and spleen were removed under sterile conditions. Colonic ex vivo biopsies were collected in parallel for microbiological and immunological analyses. Immunohistopathological changes were determined in colonic samples that had been immediately fixed in 5% formalin and embedded in paraffin. Sections (5 µm) were stained with respective antibodies for in situ immunohistochemistry as described earlier [30].

Quantitative analysis of *C. jejuni* or probiotic bacterial colonization

Viable *C. jejuni* strain 81–176 were detected in feces or at time of necropsy in luminal samples taken from the colon, dissolved in sterile PBS and serial dilutions cultured on Karmali- and Columbia-Agar supplemented with 5% sheep blood (Oxoid, Wesel, Germany) for two days at 37 °C under microaerobic conditions using CampyGen gas packs (Oxoid) as described earlier [12]. Probiotic bacteria of the formulation VSL#3 were quantitated in serial dilutions streaked onto Columbia-Agar supplemented with 5% sheep blood and Columbia-CNA Agar supplemented with colistin and nalidixic acid (both Oxoid) in parallel and incubated under aerobic (with 5% CO_2), microaerophilic (in jars using CampGen gas packs; Oxoid) and obligate anaerobic (in jars using Anaerogen gas packs; Oxoid) conditions for at least 2 days. Bacterial species were identified according to their typical morphological appearances and confirmed by 16S rRNA based sequencing. The total probiotic bacterial loads in intestinal samples were assessed by the sum of identified CFU derived from the respective culture conditions. The detection limit of viable bacteria was ≈100 CFU per g.

Immunohistochemical stainings of colonic ex vivo biopsies

In situ immunohistochemical analysis of colonic paraffin sections was performed as described previously [12, 31, 32]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200), Ki67 (TEC3, Dako, Glostrup, Denmark, 1:100), CD3 (#N1580, Dako, Denmark, dilution 1:10), FOXP3 (FJK-16s, eBioscience, San Diego, CA, USA, 1:100), B220 (eBioscience, 1:200) and F4/80 (# 14-4801, clone BM8, eBioscience, 1:50) were used. The average numbers of positively stained cells within at least six high power fields (HPF, 400× magnification) were determined for each animal microscopically by an independent blinded investigator.

Cytokine detection in culture supernatants of ex vivo biopsies taken from colon, mesenteric lymph nodes and spleen

Colonic ex vivo biopsies were cut longitudinally and washed in PBS. MLN, spleen or strips of approximately 1 cm^2 colonic tissue were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 µL serum-free RPMI 1640 medium (Gibco, life technologies, Paisley, UK) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL; PAA Laboratories, Pasching, Austria). After 18 h at 37 °C, culture supernatants were tested for TNF, MCP-1, IL-6, IL-12p70, and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences).

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney test (GraphPad Prism v5, La Jolla, CA, USA) as indicated. Two-sided probability (p) values ≤ 0.05 were considered significant.

Results

Intestinal colonization densities in secondary abiotic mice following peroral re-colonization with probiotic bacteria and/or *C. jejuni* strain 81–176 infection

In the present study we investigated the potential of probiotic bacteria in the commercial formulation VSL#3 to reduce pathogen burdens and to ameliorate pro-inflammatory immune responses upon *C. jejuni* infection in vivo. To address this, secondary abiotic mice were generated by broad-spectrum antibiotic treatment of conventionally reared mice. These mice were virtually lacking

intestinal bacteria and hence, physiological colonization resistance was abrogated to assure stable intestinal probiotic bacterial colonization and/or *C. jejuni* infection [11, 12]. Secondary abiotic mice were then perorally challenged with a probiotic suspension (i.e. VSL#3) containing 10^9 viable bacteria in total by gavage either 5 days before (i.e. prophylactic regimen) or after (i.e. therapeutic regimen) peroral *C. jejuni* strain 81–176 infection (with 10^9 CFU) and compared to control mice that were either challenged by probiotic bacteria or *C. jejuni* alone. Uninfected, naive mice served as negative controls. In fact, probiotic bacteria as well as *C. jejuni* could stably colonize the murine intestinal tract, both with high median loads of approximately 10^9 CFU per gram feces, irrespective of the re-colonization regimen (n.s.; Fig. 1). Neither in the therapeutic nor prophylactic re-colonization group, however, probiotic bacteria were able to lower *C. jejuni* burden as indicated by comparably high pathogen loads in fecal samples over time, and the same was true the other way around (n.s.; Fig. 1c, d). If compared to *C. jejuni* mono-infected mice, however, fecal pathogen loads were approximately 0.25 orders of magnitude lower in mice of the probiotic treatment group at day 21 following *C. jejuni* infection ($p < 0.005$; Fig. 2).

Overall, mice could be stably re-associated with probiotic bacteria and/or *C. jejuni*, but co-colonization did not result in a biologically relevant reduction of either bacteria.

Macroscopic and intestinal sequelae of *C. jejuni* infection and probiotic treatment

Given that re-association with probiotic bacteria and/or *C. jejuni* strain 81–176 infection did not macroscopically (i.e. clinically) compromise secondary abiotic mice (not shown), we next investigated potential intestinal sequelae resulting from respective bacterial challenges. Given that apoptosis is a well-established marker for histopathological grading of intestinal inflammation and a key feature

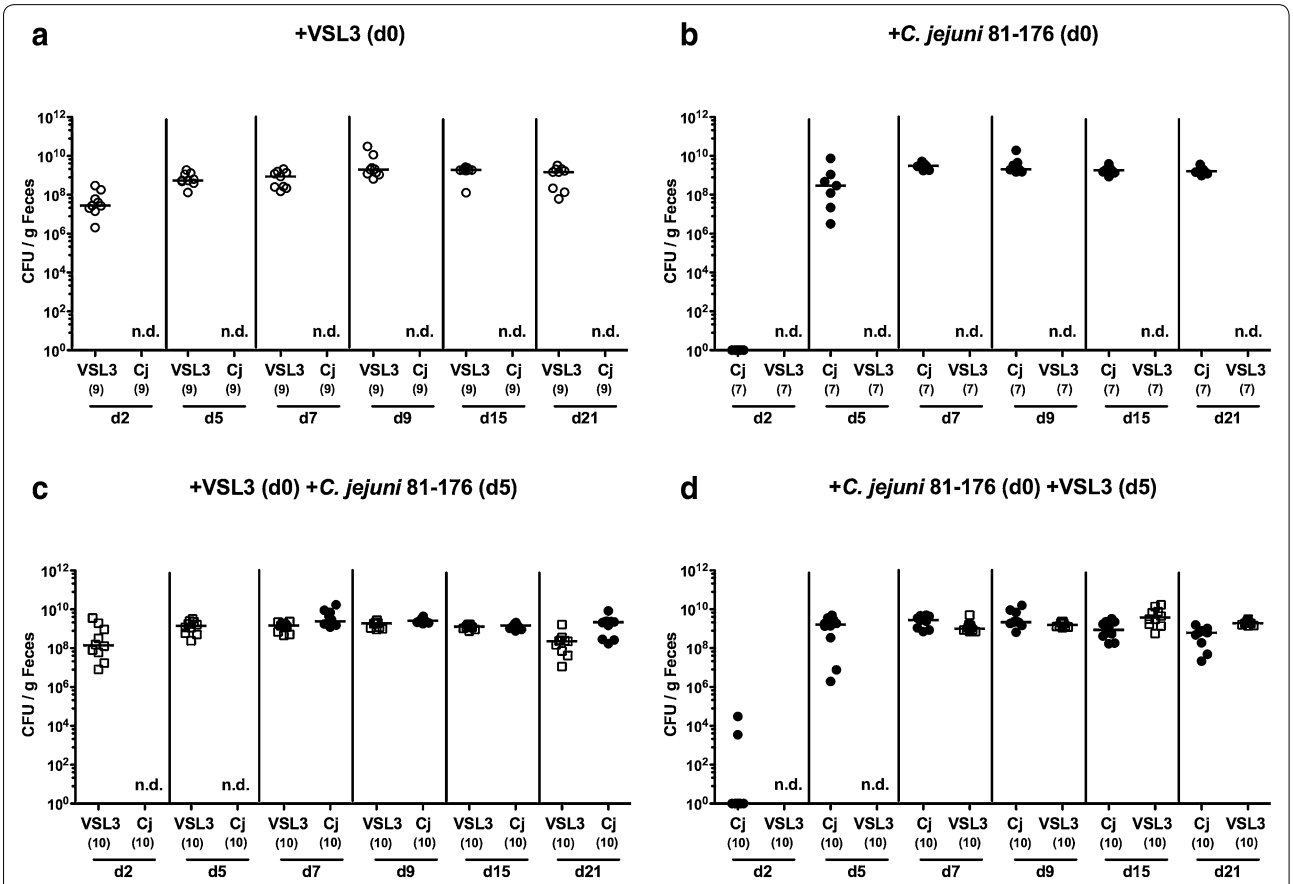

Fig. 1 Kinetic of intestinal probiotic bacterial and/or *C. jejuni* strain 81–176 loads in perorally associated secondary abiotic mice. Secondary abiotic mice were generated by broad-spectrum antibiotic treatment and perorally re-associated with **a** the probiotic compound VSL#3 (*white symbols*) on day (d) 0, **b** *C. jejuni* strain 81–176 (Cj; *black symbols*) on d0, **c** VSL#3 on day 0 and *C. jejuni* strain 81–176 on d5 or **d** *C. jejuni* strain 81–176 (d0) and VSL#3 on d0 as described in "Methods". Bacterial colonization densities were assessed in fecal samples (CFU/g, colony forming units per gram) over time upon re-association as indicated by culture. Medians (*black bars*) and levels of significance (p value) determined by Mann–Whitney U test are indicated. *Numbers* of analyzed mice are given in *parentheses*. Data were pooled from three independent experiments. *N.d.* not determined

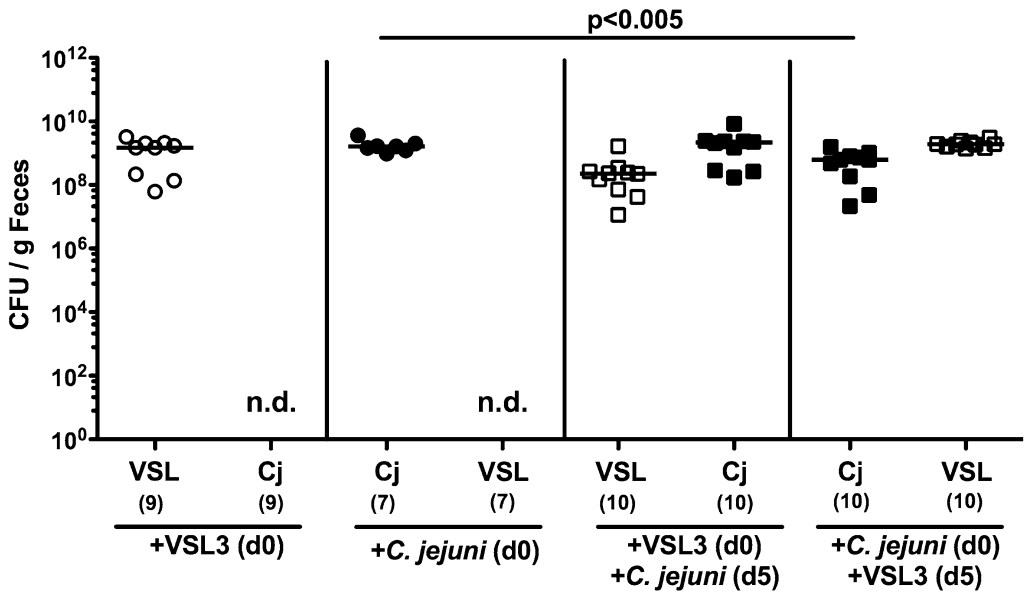

Fig. 2 Fecal bacterial loads in *C. jejuni* strain 81–176 infected and/or VSL#3 associated secondary abiotic mice. Secondary abiotic mice were generated by broad-spectrum antibiotic treatment and perorally re-associated with the probiotic compound VSL#3 (*white symbols*) on day (d) 0, *C. jejuni* strain 81-176 (Cj; *black symbols*) on day 0, VSL#3 (day 0) and *C. jejuni* strain 81–176 (day 5) or *C. jejuni* strain 81–176 (day 0) and VSL#3 (day 5) as described in "Methods". Bacterial colonization densities were assessed in fecal samples (CFU/g, colony forming units per gram) at day 21 upon initial re-association as indicated by culture. Medians (*black bars*) and level of significance (p value) determined by Mann–Whitney U test are indicated. *Numbers* of analyzed mice are given in *parentheses*. Data were pooled from three independent experiments. *N.d.* not determined

of campylobacteriosis [12], we quantitatively assessed large intestinal epithelial caspase3 + cell numbers by in situ immunohistochemistry. Numbers of colonic apoptotic cells were higher in *C. jejuni* infected mice of either group as compared to naive or VSL#3 mono-associated mice ($p < 0.05$–0.001; Fig. 3a). These increases, however, were far less pronounced in VSL#3 co-colonized mice of either regimen as indicated by approximately 50% lower apoptotic cell numbers as compared to *C. jejuni* infected mice ($p < 0.005$–0.001; Fig. 3a). Notably, re-association of mice with the probiotic compound alone was not associated with colonic apoptosis (Fig. 3a). Given that Ki67 comprises a nuclear factor necessary for cellular proliferation [33], we additionally stained colonic paraffin sections with Ki67 antibodies to assess potential proliferative (and thus regenerative) measures of the large intestinal epithelium counteracting apoptosis. Bacterial or pathogenic mono- as well as co-association resulted in increases of Ki67+ colonic epithelial cell numbers ($p < 0.001$; Fig. 3b) with a trend towards highest numbers in mice that were co-colonized with probiotic bacteria and *C. jejuni* (n.s. vs *C. jejuni* alone; $p < 0.05$–0.005 versus VSL#3 alone; Fig. 3b). Hence, prophylactic as well as therapeutic challenge of *C. jejuni* infected mice with the probiotic compound VSL#3 resulted in less pronounced large intestinal apoptotic responses.

Intestinal and systemic pro- and anti-inflammatory responses upon probiotic treatment of *C. jejuni* infected mice

Since recruitment of pro-inflammatory immune cells to the site of infection is a key feature of intestinal inflammation in the course of campylobacteriosis [12], we next quantitatively assessed distinct innate as well as adaptive immune cell subsets in large intestinal ex vivo biopsies, again applying in situ immunohistochemistry. Peroral *C. jejuni* infection, but not VSL#3 re-colonization alone was associated with increases in colonic numbers of T and B lymphocytes, regulatory T cells (Treg) as well as macrophages and monocytes ($p < 0.001$; Fig. 4). These increases, however, were significantly less pronounced in with probiotics treated *C. jejuni* infected mice, irrespective whether VSL#3 was applied prophylactically or therapeutically ($p < 0.05$–0.001; Fig. 4).

We further measured pro- and anti-inflammatory cytokine concentrations in large intestinal ex vivo biopsies. Bacterial mono- as well as co-association were accompanied by increases in colonic pro-inflammatory

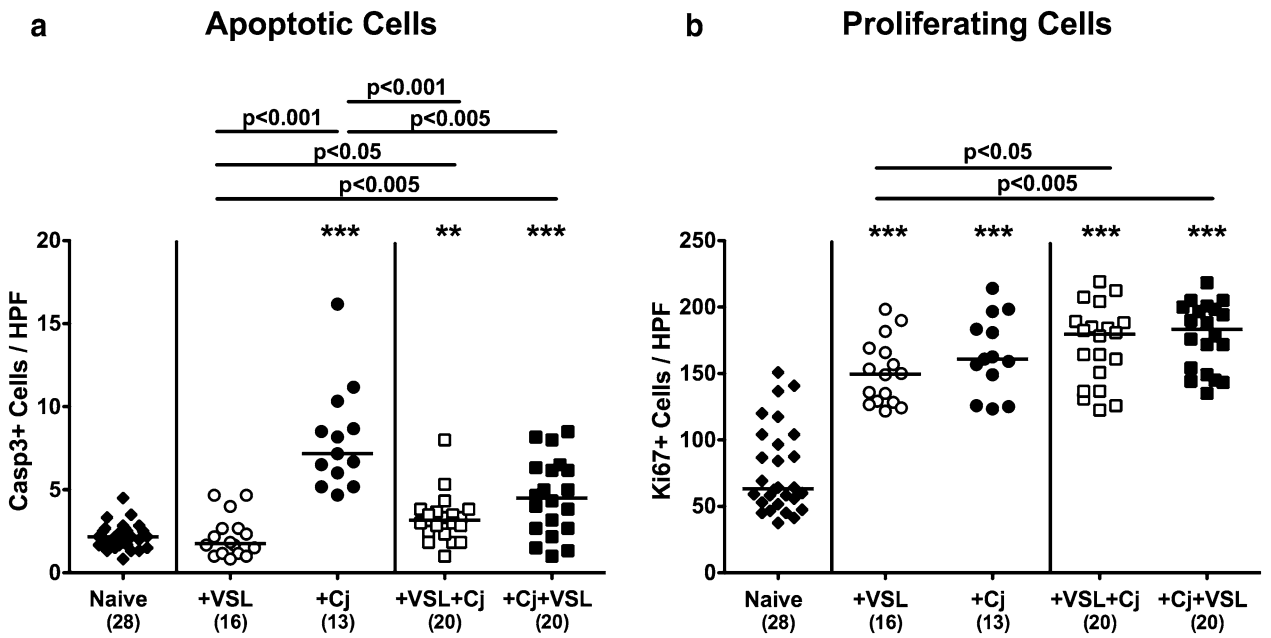

Fig. 3 Apoptotic and proliferating cells in the colonic epithelium of *C. jejuni* strain 81–176 and/or VSL#3 associated secondary abiotic mice. Secondary abiotic mice were perorally associated either with the probiotic compound VSL#3 (+VSL; *white symbols*) or with *C. jejuni* strain 81–176 (+Cj; *black symbols*) on day 0. In bacterial competition experiments VSL#3 associated mice were challenged with *C. jejuni* strain 81–176 5 days thereafter (+VSL + Cj) or, the other way around (+Cj + VSL), *C. jejuni* infected mice additionally associated with the probiotic compound. The average numbers of colonic **a** apoptotic cells (positive for caspase-3, Casp3) and **b** proliferating cells (positive for Ki67) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colonic paraffin sections at day 21 upon initial bacterial association. Naive mice served as uninfected controls (*black diamonds*). Medians (*black bars*), numbers of analyzed animals (in parentheses) and levels of significance (p values) determined by Mann–Whitney U test are given. Significant differences as compared to naive controls are indicated by *asterisks* (**p < 0.01; ***p < 0.001). Data were pooled from four independent experiments

mediators including TNF, MCP-1, and IL-6 (p < 0.05–0.001; Fig. 5a–c). *C. jejuni* induced increases in colonic MCP-1 and IL-6, but not increased TNF concentrations could be dampened by prophylactic probiotic treatment (p < 0.05; Fig. 5a–c). Notably, IL-6 levels were also decreased in large intestines derived from *C. jejuni* infected mice of the therapeutic VSL#3 cohort (p < 0.05; Fig. 5c) and did not differ from IL-6 concentrations measured in naive controls (n.s.; Fig. 5c). Notably, large intestinal concentrations of the anti-inflammatory cytokine IL-10 were increased upon mono- and co-association with the probiotic compound as compared to naive controls (p < 0.05–0.001; Fig. 5d). Moreover, VSL#3 application of either regimen resulted in elevated IL-10 concentrations in *C. jejuni* infected mice (p < 0.05; Fig. 5d).

We next measured cytokine levels in another intestinal compartment. In MLN, concentrations of respective pro- and anti-inflammatory cytokines increased upon bacterial and/or pathogenic re-association (p < 0.05–0.001; Fig. 6). Prophylactic probiotic treatment of *C. jejuni* infected mice, however, resulted in slightly lower IL-10 concentrations when compared to *C. jejuni* mono-associated mice (p < 0.05; Fig. 6d).

We further assessed systemic cytokine responses upon bacterial and/or pathogenic challenges of secondary abiotic mice. *C. jejuni* induced increases in splenic TNF concentrations could be slightly lowered following probiotic pre-treatment (p < 0.05; Fig. 7a), but not if probiotic treatment followed *C. jejuni* infection. In addition, mice of the prophylactic cohort exhibited lower IL-12p70 concentrations in their spleen as compared to *C. jejuni* infected animals (p < 0.05; Fig. 7b), whereas *C. jejuni* infection was associated with increased splenic IL-6 secretion (p < 0.05–0.005; Fig. 7c) that could neither be lowered by prophylactic nor therapeutic probiotic challenges. Notably, splenic IL-10 concentrations were unaffected upon bacterial re-colonization and/or pathogenic infection (n.s.; Fig. 7d).

Taken together, less colonic apoptosis upon probiotic co-administration in *C. jejuni* infected mice was accompanied by lower numbers of innate and adaptive immune cell populations in the large intestinal mucosa and lamina propria and less secretion of pro-inflammatory mediators, whereas anti-inflammatory IL-10 concentrations were increased in the colon upon prophylactic or therapeutic VSL#3 treatment of *C. jejuni* infected

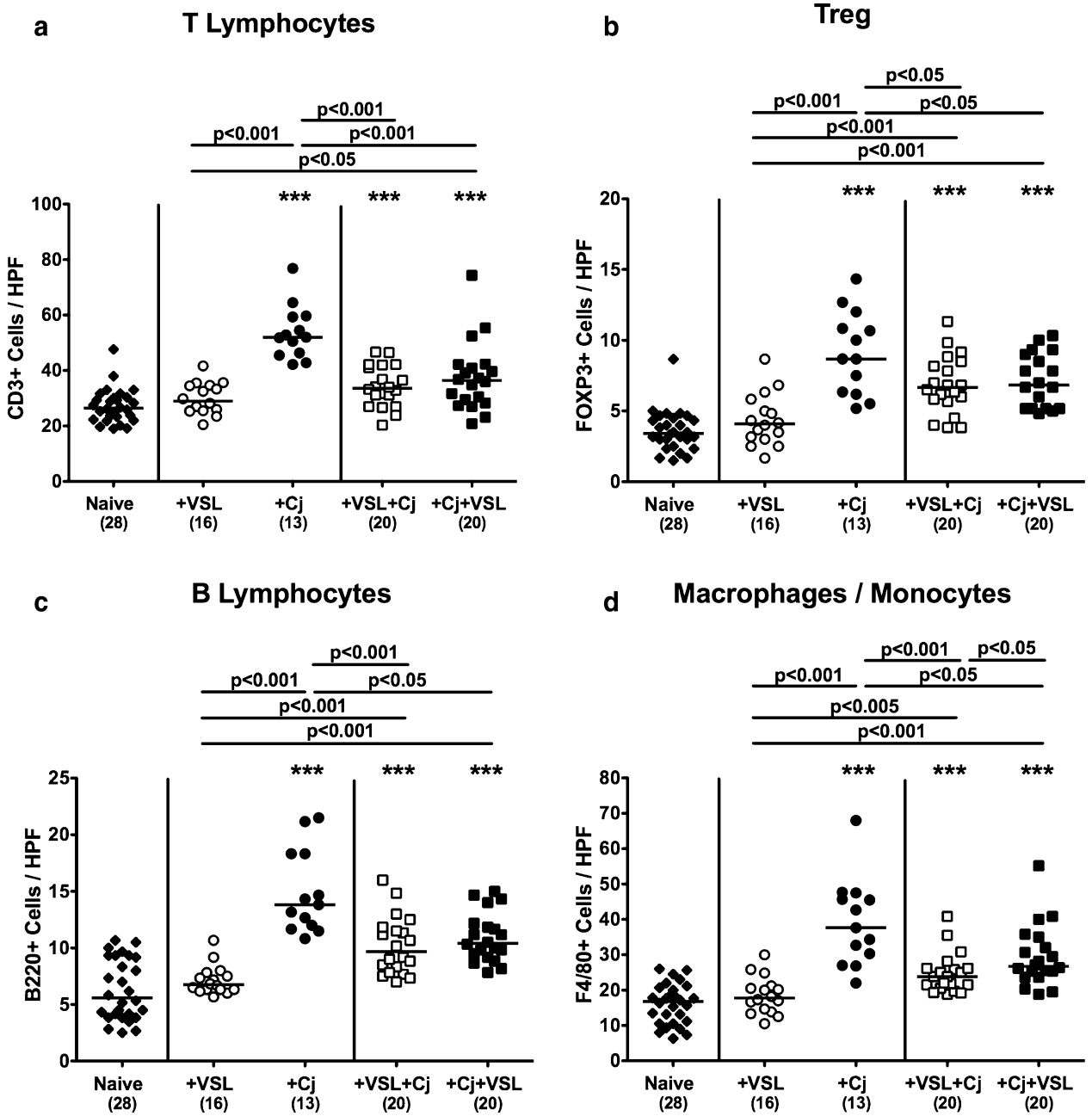

Fig. 4 Colonic immune cell responses in *C. jejuni* strain 81–176 and/or VSL#3 associated secondary abiotic mice. Secondary abiotic mice were perorally associated either with the probiotic compound VSL#3 (+VSL; *white symbols*) or with *C. jejuni* strain 81-176 (+Cj; *black symbols*) on day 0. In bacterial competition experiments VSL#3 associated mice were challenged with *C. jejuni* strain 81–176 5 days thereafter (+VSL + Cj) or, the other way around (+Cj + VSL), *C. jejuni* infected mice additionally associated with the probiotic compound. The average numbers of colonic **a** T lymphocytes (positive for CD3), **b** regulatory T cells (Treg; positive for FOXP3), **c** B lymphocytes (positive for B220), and **d** macrophages and monocytes (positive for F4/80) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colonic paraffin sections at day 21 upon initial bacterial association. Naive mice served as uninfected controls (*black diamonds*). Medians (*black bars*), numbers of analyzed animals (in *parentheses*) and levels of significance (p-values) determined by Mann–Whitney U test are given. Significant differences as compared to naive controls are indicated by *asterisks* (***p < 0.001). Data were pooled from four independent experiments

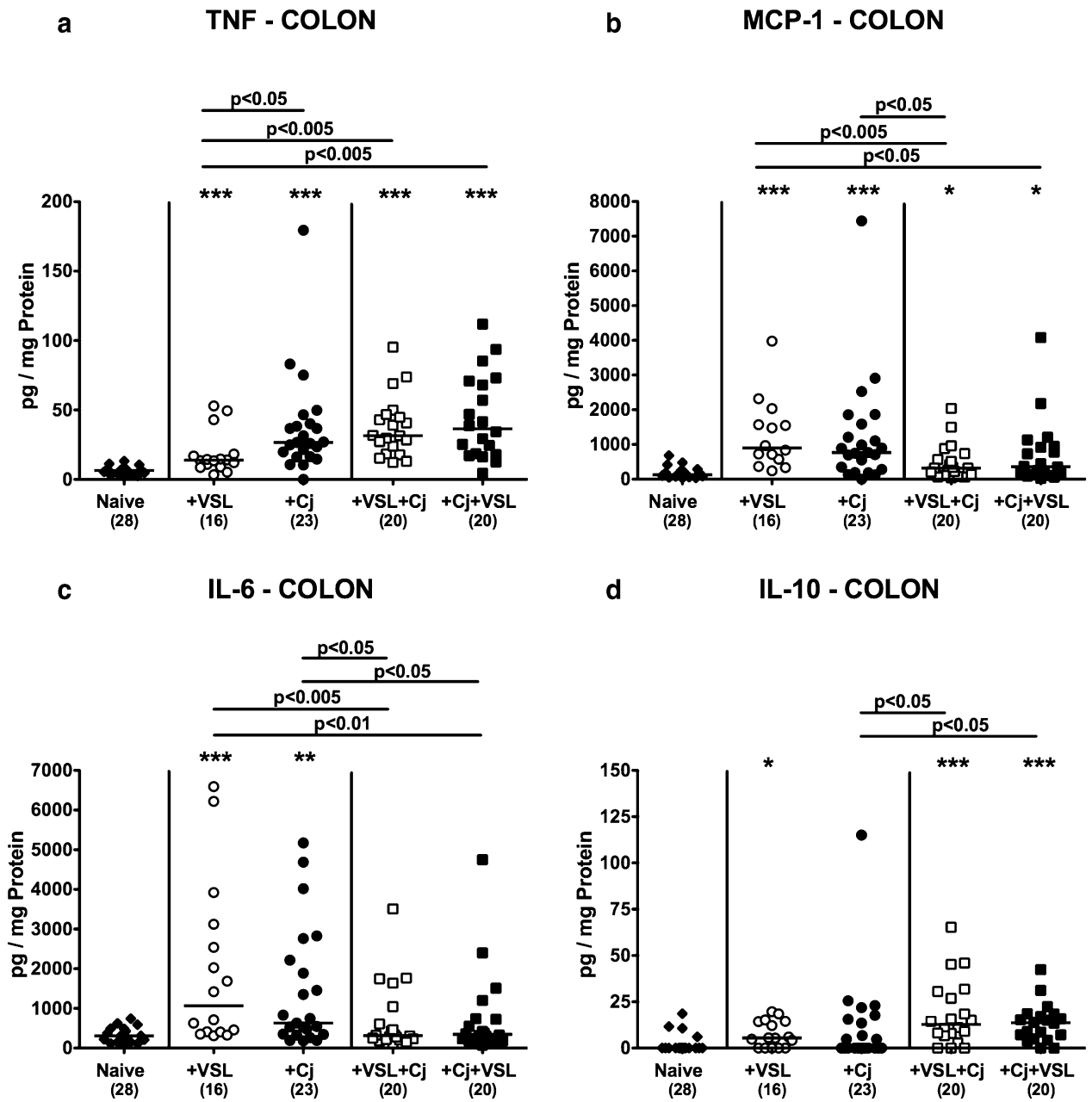

Fig. 5 Colonic secretion of pro- and anti-inflammatory cytokines in *C. jejuni* strain 81-176 and/or VSL#3 associated secondary abiotic mice. Secondary abiotic mice were perorally associated either with the probiotic compound VSL#3 (+VSL; *white symbols*) or with *C. jejuni* strain 81–176 (+Cj; *black symbols*) on day 0. In bacterial competition experiments VSL#3 associated mice were challenged with *C. jejuni* strain 81–176 5 days thereafter (+VSL + Cj) or, the other way around (+Cj + VSL), *C. jejuni* infected mice additionally associated with the probiotic compound. **a** TNF, **b** MCP-1, **c** IL-6 and **d** IL-10 concentrations were determined in colonic ex vivo biopsies at day 21 upon initial bacterial association. Naive mice served as uninfected controls (*black diamonds*). Medians (*black bars*), numbers of analyzed animals (in *parentheses*) and levels of significance (p-values) determined by Mann–Whitney U test are given. Significant differences as compared to naive controls are indicated by *asterisks* (*p < 0.05; **p < 0.01; ***p < 0.001). Data were pooled from four independent experiments

mice. Furthermore, prophylactic VSL#3 challenge could dampen *C. jejuni* induced TNF responses in the systemic (i.e. splenic) compartment.

Discussion

Although *C. jejuni* are the most commonly reported bacterial etiological agents of diarrhea in developed

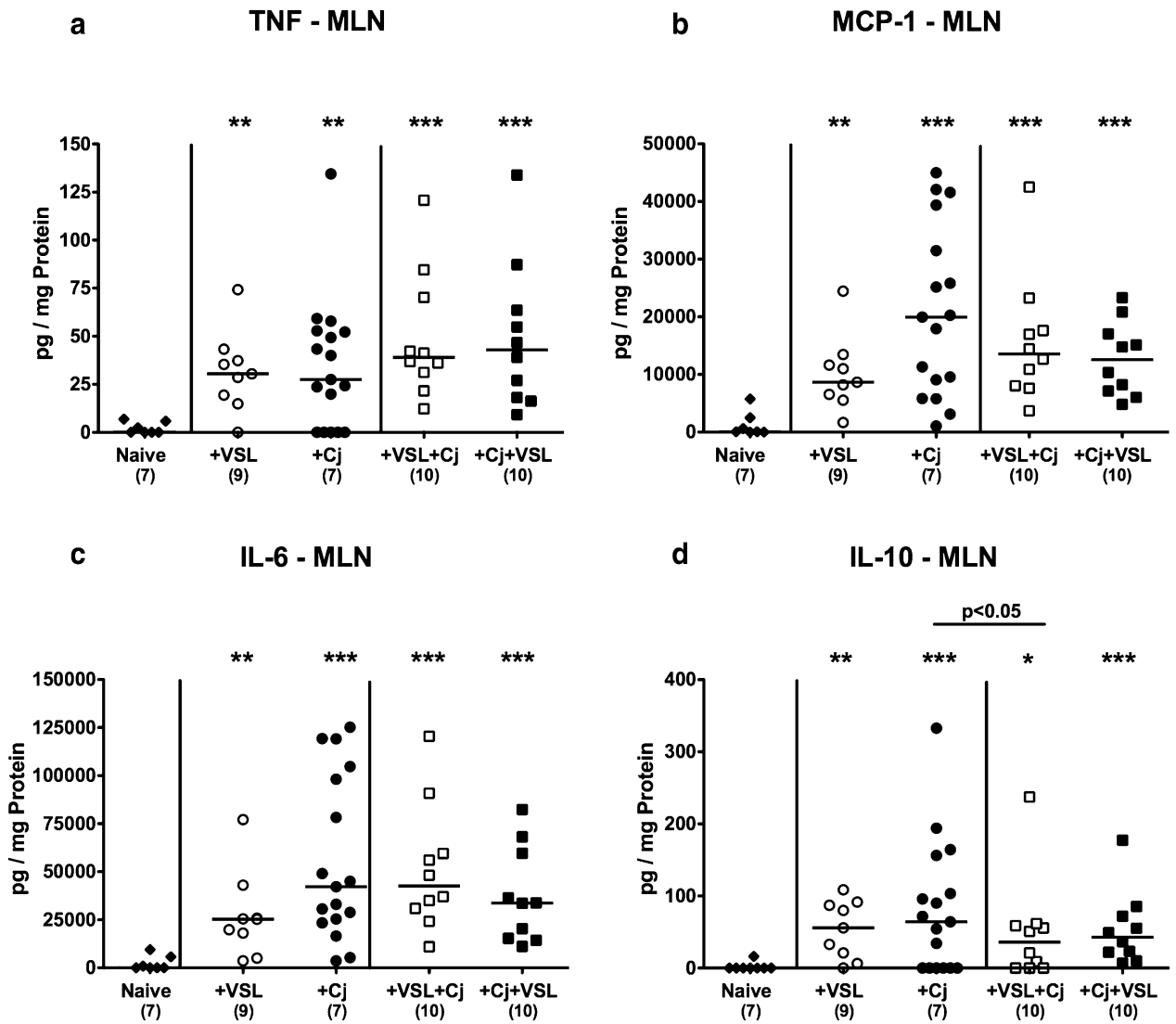

Fig. 6 Secretion of pro- and anti-inflammatory cytokines in mesenteric lymph nodes derived from *C. jejuni* strain 81–176 and/or VSL#3 associated secondary abiotic mice. Secondary abiotic mice were perorally associated either with the probiotic compound VSL#3 (+VSL; *white symbols*) or with *C. jejuni* strain 81–176 (+Cj; *black symbols*) on day 0. In bacterial competition experiments VSL#3 associated mice were challenged with *C. jejuni* strain 81–176 5 days thereafter (+VSL + Cj) or, the other way around (+Cj + VSL), *C. jejuni* infected mice additionally associated with the probiotic compound. **a** TNF, **b** MCP-1, **c** IL-6 and **d** IL-10 concentrations were determined in ex vivo biopsies derived from mesenteric lymph nodes (MLN) at day 21 upon initial bacterial association. Naive mice served as uninfected controls (*black diamonds*). Medians (*black bars*), numbers of analyzed animals (in *parentheses*) and levels of significance (p-values) determined by Mann–Whitney U test are given. Significant differences as compared to naive controls are indicated by *asterisks* (*p < 0.05; **p < 0.01; ***p < 0.001). Data were pooled from three independent experiments

countries [3], in vivo data regarding the molecular mechanisms underlying pathogen-host interactions are still scarce, partly due to lack of suitable mouse models mimicking *C. jejuni* induced immunopathology in humans. We have previously shown that intestinal microbial depletion following antibiotic treatment of mice can overcome physiological colonization resistance against *C. jejuni* that is elicited by the murine host specific microbiota composition [12]. Apart from that, *C. jejuni* infected secondary abiotic mice exhibit immunopathological key features of human campylobacteriosis, thus providing a well-suited model to further unravel interactions between enteropathogens and the vertebrate host [3, 11, 12]. Given the importance of the distinct microbiota composition in disease susceptibility and progression, secondary modulation of the intestinal microbiota by application of probiotic compounds has arisen as an attractive preventive or therapeutic approach. In bacterial

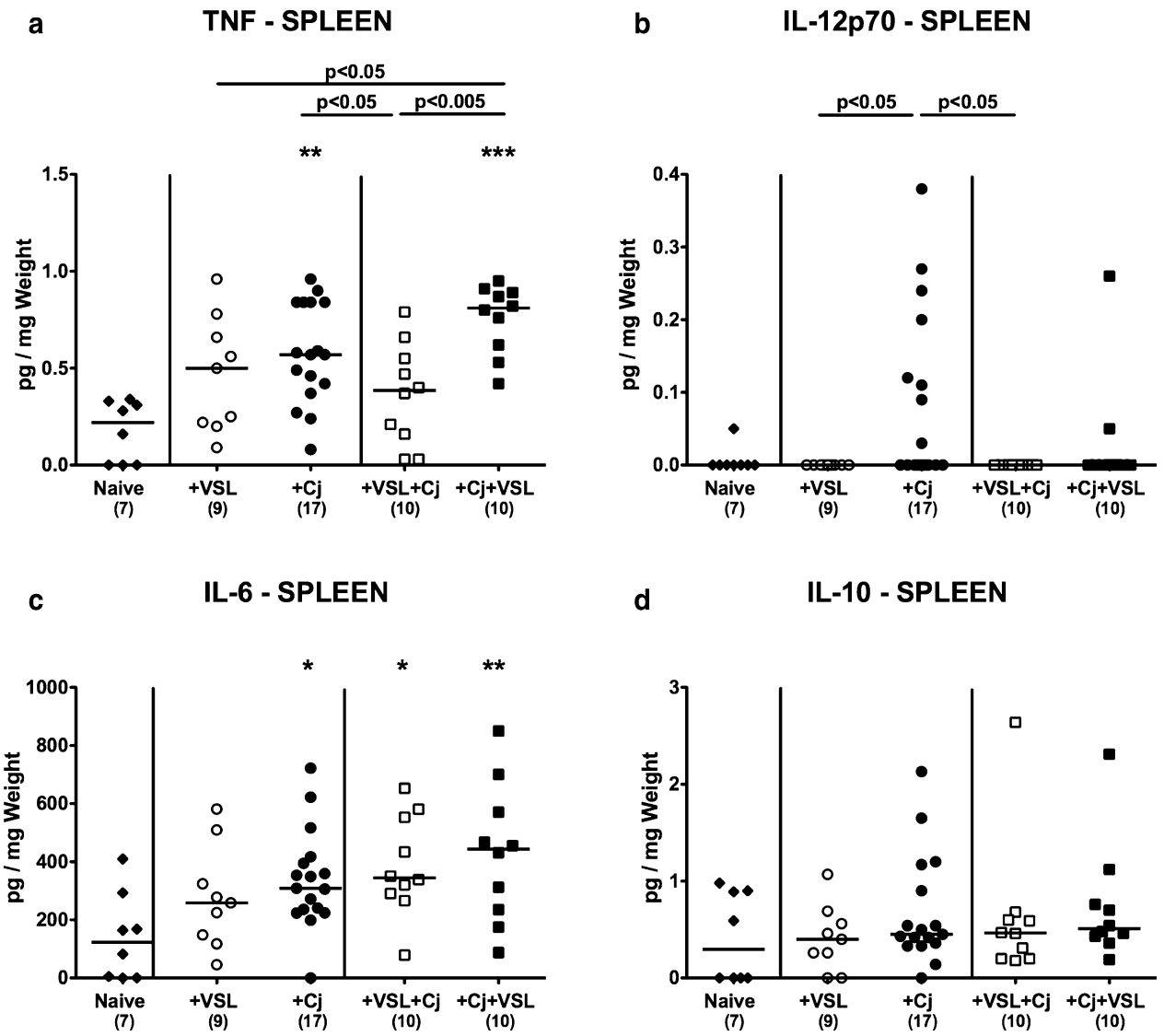

Fig. 7 Splenic secretion of pro- and anti-inflammatory cytokines in *C. jejuni* strain 81-176 and/or VSL#3 associated secondary abiotic mice. Secondary abiotic mice were perorally associated either with the probiotic compound VSL#3 (+VSL; *white symbols*) or with *C. jejuni* strain 81-176 (+Cj; *black symbols*) on day 0. In bacterial competition experiments VSL#3 associated mice were challenged with *C. jejuni* strain 81–176 5 days thereafter (+VSL + Cj) or, the other way around (+Cj + VSL), *C. jejuni* infected mice additionally associated with the probiotic compound. **a** TNF, **b** MCP-1, **c** IL-6 and **d** IL-10 concentrations were determined in ex vivo biopsies derived from spleen at day 21 upon initial bacterial association. Naive mice served as uninfected controls (*black diamonds*). Medians (*black bars*), numbers of analyzed animals (in *parentheses*) and levels of significance (p-values) determined by Mann–Whitney U test are given. Significant differences as compared to naive controls are indicated by *asterisks* (*p < 0.05; **p < 0.01; ***p < 0.001). Data were pooled from three independent experiments

in vivo competition experiments applying our secondary abiotic mouse model, we here investigated changes in intestinal pathogen burden and host immune responses upon peroral *C. jejuni* infection and following pre- or post-treatment with the commercially available probiotic compound VSL#3. Upon comparable and stable pathogenic as well as probiotic bacterial colonization of the intestinal tract, VSL#3 could not sufficiently decrease intestinal *C. jejuni* loads in a biologically relevant manner within 3 weeks following initial bacterial challenge. Lowering the bacterial loads in livestock animals including poultry would be of great benefit in decreasing disease transmission rates to humans via the food chain, while immunomodulatory effects such as attenuation of intestinal inflammation would additionally result in less severe disease progression in the host. Our data are in

contrast to a previous study applying isolator-raised germfree BALB/c mice that had been re-associated with a complex human microbiota and treated with a probiotic mix of five different *Lactobacillus* and three *Bifidobacterium* strains [26]. Following peroral infection with *C. jejuni* the authors observed a complete eradication of *C. jejuni* from the small and large intestines of with probiotics pre-challenged "humanized" mice [26]. One needs to take into consideration, that the observed differences in pathogen-eradicative properties might be due to differences in the used probiotic mixtures and could also be explained by different immunological features of the applied animal models. Due to the lacking contact to any bacterial ligands and subsequent absence of immunological differentiation and stimulation, germfree mice exhibit only poorly-developed intestinal lymphatic tissues [34, 35]. It is thus highly reasonable that the immunological repertoire in formerly isolator raised germfree mice substantially differs from the secondary abiotic mice applied here that had been born, raised and housed under conventional conditions. In addition, reconstitution of secondary abiotic mice with eight different probiotic strains (abundant in the VSL#3 compound) might not be sufficient to reconstitute the complex physiological prerequisites for effective competition with *C. jejuni* for nutrients and niches. Instead, a well-orchestrated interplay of mucosal immunity and the intestinal intraluminal milieu determined by the concert of the complex microbiota plus beneficial probiotic strains might be required to successfully combat and/or prevent from enteropathogenic infection.

Whereas neither antibiotic treatment nor bacterial re-association compromised mice clinically, we detected more pronounced apoptotic responses in colonic epithelia following *C. jejuni* infection as reported by us previously [12, 14, 30, 36–38]. Remarkably, *C. jejuni* induced apoptosis could be alleviated by both therapeutic and prophylactic VSL#3 application. This is well in line with a former study demonstrating the capacity of VSL#3 to attenuate epithelial apoptosis in a murine dextran sodium sulphate (DSS) induced colitis model [39]. Notably, less apoptosis was associated with more than three times increased numbers of colonic epithelial Ki67+ cells as compared to naive counterparts indicative for up-regulated regenerative properties upon therapeutic as well as prophylactic VSL#3 treatment. Given that enhanced cell proliferative activity is essential in tissue repair and cell regeneration, and thus prevents from loss of epithelial integrity [40], this VSL#3 induced measure might counteract and prevent from pathogen-induced apoptosis. This mechanism has already been proposed for other probiotic species including *E. coli* Nissle 1917 [27]. The anti-apoptotic properties exerted by VSL#3 were further paralleled by a dampened *C. jejuni* induced recruitment of pro-inflammatory innate immune cell subsets including macrophages and monocytes as well as adaptive immune cell populations such as T lymphocytes, Treg and B lymphocytes into the large intestinal mucosa and lamina propria. A VSL#3 mediated attenuated influx of pro-inflammatory immune cells into the colonic mucosa has already been shown in murine trinitrobenzene sulfonic acid (TNBS) induced colitis [41]. Intestinal and extra-intestinal cytokine analyses in our study further revealed that both therapeutic and prophylactic application of the probiotic compound resulted in increased colonic secretion of the anti-inflammatory key cytokine IL-10, whereas pro-inflammatory IL-6 concentrations were decreased in large intestines as compared to untreated *C. jejuni* infected mice. In addition, VSL#3 prophylaxis resulted in decreased secretion of colonic MCP-1 upon *C. jejuni* infection. Most strikingly, anti-inflammatory properties of VSL#3 were not restricted to the intestinal tract, but could also be observed systemically, given that prophylactic VSL#3 treatment attenuated *C. jejuni* induced TNF and IL-12p70 secretion in the spleen. At the first glance it appeared somewhat confusing in this context that both mono- as well as prophylactic probiotic bacterial co-colonization resulted in elevated levels of the pro-inflammatory cytokine TNF in colon and MLN. This result was, however, further supported by a former study demonstrating that VSL#3 can in fact stimulate the intestinal epithelium to produce TNF in response, which interestingly resulted in improved epithelial barrier function and prevention of intestinal disease such as experimental ileitis [42]. Furthermore, observations that TNF deficient mice were more susceptible to acute DSS colitis have led to the concept that TNF might have protective functions in normal gut homeostasis and intestinal epithelial integrity [43]. Prophylactic VSL#3 challenge dampened *C. jejuni* induced systemic TNF responses, however. While it may be physiologically important for the host to maintain a certain well-balanced level of local inflammation as a proper response to enteropathogens, avoidance of extra-intestinal and systemic inflammatory sequelae were pivotal for host health integrity. This further supports the dichotomic properties of cytokines depending on the respective (patho-)physiological context and cytokine milieu determining whether the same cytokine acts rather pro- or anti-inflammatory [44, 45]. Our findings here further reinforce former data providing evidence for anti-inflammatory properties of VSL#3. For instance, VSL#3 has been shown to suppress MCP-1 production from human dendritic cells in vitro [46] and to down-regulate colonic MCP-1 mRNA expression also in vivo [47]. Moreover VSL#3 application could ameliorate recurrent Th1-mediated TNBS colitis

in mice by inducing IL-10 and IL-10-dependent regulatory T cells expressing TGF-β [48]. The observed immunomodulatory effects of the probiotic compound might, at least in part, be attributed to the impact of probiotics on Toll-like receptor (TLR) expression [49]. We have previously reported that *C. jejuni* induced immunopathology depends on TLR-4- and TLR-9 signaling [12], whereas in a very recent study VSL#3 was shown to down-regulate TLR-2, TLR-3, TLR-4, and TLR-9 expression in vitro [50]. Whether the observed beneficial effects can be attributed to single bacterial species out of the eight probiotic strains within the compound VSL#3 or the mutualistic/synergistic interaction of distinct strains warrants further investigations.

Conclusion

Our data presented here provide evidence that though not sufficiently enforcing pathogenic clearance, therapeutic as well as prophylactic VSL#3 application can induce anti-inflammatory responses and limit not only intestinal, but also systemic pro-inflammatory sequelae of vertebrate *C. jejuni* infection. The probiotic compound VSL#3 might therefore further open up promising tools for prophylaxis and/or treatment of *C. jejuni* induced sequelae.

Abbreviations

AAD: antibiotics-associated diarrhea; CFU: colony forming units; DSS: dextran sodium sulphate; MLN: mesenteric lymph nodes; PBS: phosphate buffered saline; SPF: specific pathogen free; TNBS: trinitrobenzene sulfonic acid; Treg: regulatory T cells.

Authors' contributions

Conceived and designed the experiments: IE MMH SB. Performed the experiments: IE UF MMH. Analyzed the data: IE UF MMH. Critically discussed the paper: IE KS SB MMH. Wrote the paper: IE MMH. Co-edited the paper: UF KS SB. 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 number G0184/12). Animal welfare was monitored twice daily by assessment of clinical conditions. Mice did not display any clinical symptoms during the entire experiment. Twentyone days following the initial bacterial re-colonization mice were sacrifized by isofluran treatment (Abbott, Greifswald, Germany).

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

5. Publikationsliste

1. 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. Frontiers in immunology. 2017;8:397.
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