Gut Microbial Dysbiosis Due to Helicobacter Drives an Increase in Marginal Zone B Cells in the Absence of IL-10 Signaling in Macrophages


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It is clear that IL-10 plays an essential role in maintaining homeostasis in the gut in response to the microbiome. However, it is unknown whether IL-10 also facilitates immune homeostasis at distal sites. To address this question, we asked whether splenic immune populations were altered in IL-10−/− mice in which differences in animal husbandry history were associated with susceptibility to spontaneous enterocolitis that is microbiome dependent. The susceptible mice exhibited a significant increase in splenic macrophages, neutrophils, and marginal zone (MZ) B cells that was inhibited by IL-10 signaling in myeloid, but not B cells. The increase in macrophages was due to increased proliferation that correlated with a subsequent enhancement in MZ B cell differentiation.

Cohousing and antibiotic treatment studies suggested that the alteration in immune homeostasis in the spleen was microbiome dependent. The 16S rRNA sequencing revealed that susceptible mice harbored a different microbiome with a significant increase in the abundance of the bacterial genus Helicobacter. The introduction of Helicobacter hepaticus to the gut of nonsusceptible mice was sufficient to drive macrophage expansion and MZ B cell development. Given that myeloid cells and MZ B cells are part of the first line of defense against blood-borne pathogens, their increase following a breach in the gut epithelial barrier would be protective. Thus, IL-10 is an essential gatekeeper that maintains immune homeostasis at distal sites that can become functionally imbalanced upon the introduction of specific pathogenic bacteria to the intestinal tract.

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Interleukin-10 is an important anti-inflammatory cytokine produced in the gut by a variety of immune cells, including B cells, T cells, and macrophages, as well as nonhematopoietic cells (1). Genome-wide association studies revealed a linkage between IL-10 polymorphisms and susceptibility to inflammatory bowel disease in humans (2, 3). Humans harboring loss-of-function mutations in IL10RA and IL10RB, whose gene products encode for the heterodimer IL-10R1 and IL-10R2 subunits, respectively, succumb to early-onset colitis (4–6). In mice, spontaneous onset of severe intestinal inflammation also occurs in mice deficient in IL-10 or its receptor (7, 8), and is prevented by IL-10 signaling in macrophages (9, 10). Collectively, these studies illuminate the essential role for IL-10 in dampening intestinal inflammation and the maintenance of gut homeostasis.

It has become clear that the gut microbiome plays an essential role in the health of the host and that microbial dysbiosis can contribute to disease (11). For example, increases in Enterobacteriaceae and Gram-negative anaerobes, including Bacteroides, have been associated with inflammatory bowel disease in humans (12–14). A decrease in presumed beneficial bacteria, such as Bifidobacteria species, has also been reported in inflammatory bowel disease (14, 15). In mice, spontaneous enterocolitis in Il10−/− mice only occurs in some animal colonies in a microbiome-dependent manner (7, 16, 17). Mice lacking Helicobacter do not succumb to spontaneous colitis in the absence of IL-10 (18). In addition, Helicobacter hepaticus-induced colitis susceptibility has been shown to differ between mouse colonies with markedly different microbiomes (16). Although the gut microbiome can influence immune responses at distal sites (11, 19), the role of specific bacterial species in systematic inflammation remains only partially understood.

The Il10−/− mouse has not been reported to have defects in hematopoietic lineage development, but at 8 wk an elevation in IgM, IgG1, IgG2b, IgG2a, and IgA was reported (7). Whether this is due to altered B cell function is not known, and a comprehensive
analysis of B cell subsets has not been performed in the \( \text{II}10^\text{a/b} \) mouse. Whereas T-dependent immune responses in \( \text{II}10^\text{a/c} \) mice were reported to be normal \((7)\), the slight elevation in IgM is suggestive that T-independent immune responses may be altered. T-independent Ab responses are in part mediated by marginal zone (MZ) B cells that respond very rapidly to blood-borne pathogens due to their localization within the splenic MZ \((20)\). The MZ is situated between the red and white pulp through which most of the blood that enters the spleen flows through before re-entering the circulation \((20)\). Thus, a breach in the epithelial layer during gut inflammation allowing bacterial translocation would potentially prompt a MZ B cell response. In \( \text{II}10^\text{a/c} \) mice, bacteria were found to penetrate the colon mucus layer and directly contact the epithelial barrier, and \textit{Enterococcus faecalis}, through the production of a metalloprotease, was shown to compromise the barrier \((21, 22)\). These effects on the barrier are thought to contribute to inflammation within the gut, but whether they alter immune cell function and homeostasis at distal sites is unknown.

In this study, we asked whether \( \text{II}10^\text{a/c} \) mice with susceptibility to spontaneous enterocolitis had an imbalance in immune cell homeostasis outside of the gut compared with resistant mice raised in a separate facility. We found that susceptible mice exhibited a significant increase in both MZ B cells and CD11b\(^+\) myeloid cells in the spleen that was inhibited by IL-10 signaling in macrophages. Cohousing, antibiotic treatment, and 16S rRNA sequencing studies indicated that the immune homeostasis alteration was microbiome dependent. In addition, introduction of \textit{H. hepaticus} to nonsusceptible mice was sufficient to drive MZ B cell differentiation and macrophage expansion. These results indicate that introduction of a single bacterial species can produce dysbiosis in the gut and drive a functional imbalance in immune homeostasis in the spleen when the gatekeeper function of IL-10 is compromised.

### Materials and Methods

#### Mice

C57BL/6J and B10.PL \((H-2^b)\) wild-type (WT) mice and B6.129P2-\(\text{II}10^{a/b}\) \(\text{II}10^{a/c} \) mice \((\text{II}10^{a/d})\) were purchased from The Jackson Laboratory \((Jax; Bar Harbor, ME). IL-10R\(^{a/b}\)CD19\(^{a/c}\) and IL-10R\(^{a/b}\) CD19\(^{a/c}\) mice were provided by T. Geiger \((\text{St. Jude's Children's Research Hospital, Memphis, TN})\). IL-10R\(^{a/b}\)LysMC\(^{a/c}\) and IL-10R\(^{a/b}\) LysMC\(^{a/c}\) mice were provided by W. Maller \((\text{University of Manchester, Manchester, UK})\). Abs and the LEGENDplex multianalyte flow assay kit were purchased from BioLegend \((\text{San Diego, CA})\). Mice-specific CD45R-PE-Texas Red, CD45-PE, CD5-allophycocyanin, CD86-V450, Ki-67-FITC, caspase 3-FITC, and CD40 Abs were purchased from BD Biosciences \((\text{San Diego, CA})\). Mouse-specific CD21-effluor 450, CD23-effluor 450, CD19-effluor 450, CD10-effluor 450, CD8-effluor 450, CD4-biotin, CD4-FITC, CD4-allophycocyanin-effluor 780, CD8-effluor 450, CD11b-biotin, CD11b-effluor 450, and Foxp3-PE Abs were purchased from eBioscience \((\text{San Diego, CA})\). Mouse-specific CD11b-Allexa Fluor 488, CD106-Alexa Fluor 594, CD80-Alexa Fluor 647, MHC class II-effluor 450, Ly6C-allophycocyanin, Ly6G-allophycocyanin-effluor 780, LysMC\(^{a/c}\) was purchased from BioLegend \((\text{San Diego, CA})\). Mouse-specific Marco-FITC and MOMA-FITC Abs were purchased from AbD Serotec \((\text{Raleigh, NC})\). Anti-B1c-2 and anti-Bcl-x\(_I\) were purchased from Cell Signaling Technology \((\text{Danvers, MA})\). Anti-mouse IgM-FITC was purchased from Southern Biotech \((\text{Birmingham, AL})\). Anti-mouse IgM F(ab\(^\text{3}\)) was purchased from Jackson ImmunoResearch Laboratories \((\text{West Grove, PA})\). Streptavidin-PE-Cy5.5 was purchased from eBioscience. Anti-BrdU-Allophycocyanin was purchased from BD Biosciences. CFSE and DAPI were purchased from Molecular Probes \((\text{Eugene, OR})\). LPS was obtained from Sigma-Aldrich \((\text{St. Louis, MO})\), and Cpg\(_G\) from Invivogen \((\text{San Diego, CA})\). Ampicillin and neomycin were purchased from LKT Laboratories \((\text{St. Paul, MN})\), and metronidazole and vancomycin were obtained from Sigma-Aldrich \((\text{St. Louis, MO})\).

#### Cell isolation, flow cytometry, and cell sorting

Single-cell suspensions were prepared from bone marrow \((\text{BM})\), thymus, Peyer’s patches, inguinal lymph nodes, and spleens. Peritoneal cavity cells were isolated, as previously described \((25)\). A total of \(1 \times 10^6\) cells was incubated with anti-CD16/CD32 \((\text{Fc block})\) \((\text{clone 2.4G2})\) for 15 min, followed by cell surface staining with specific mAb. Intracellular Ki-67 was performed using the anti-mouse/rat Foxp3 staining buffer set from eBioscience. Cells were acquired on a LSR II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software \((\text{Tree Star, Ashland, OR})\). Splenic B cell subsets were characterized, as described \((26)\). For in vitro culture and real-time PCR, B cell subsets were sorted using a FACSAria cell sorter \((\text{BD Biosciences})\), as described \((27)\).

#### Immunohistology

Spleens from 8-wk-old mice were embedded in Tissue-Tek OCT compound \((\text{Sakura Finetek, Torrance, CA})\) and snap frozen. The 7-μm sections were stained with anti-CD22-PE and anti-CD138-PE-Cy5.5, and images were captured using a Nikon Eclipse TE2000 inverted fluorescence microscope, as described \((28)\). Sections stained with B220-Alexa Fluor 594, CD11b-Alexa Fluor 488, and LysG-Alexa Fluor 647 were imaged by confocal microscopy with an Olympus Fluoview FV1000 MPE Multiphoton Scanning Microscope.

#### Detection of chemokines and cytokines in serum and colon tissue

Serum and colon tissue were collected from 7- to 8-wk-old naive mice. Colonos were homogenized in PBS containing 0.1% IGEPA. \(\text{CA-630 (Sigma-Aldrich)}\) and mini protease inhibitor \((\text{Roche, Indianapolis, IN})\), using the gentleMACS Dissociator \((\text{Miltenyi Biotec, San Diego, CA})\). Cytokines and chemokines in serum and colon lysates were determined using LEGENDplex multianalyte flow cytometry assay kit \((\text{BioLegend})\).

#### Immunization and detection of serum Igs

Mice were immunized with 30 μg 4-hydroxy-3-nitrophenylacetyl \((\text{NP})\)-Ficoll i.p., and sera were collected 7 and 14 d later. Serum from 8-wk unimmunized mice were used for detecting resting IgM and IgG3 levels. ELISA was performed to determine NP-specific Ig titers and total resting IgM and IgG3 levels in the serum, as described previously \((28, 29)\).

#### H. hepaticus culture

Culture of the MU-94 \textit{H. hepaticus} strain used in these studies was performed at the University of Missouri in the laboratory of C. L. Franklin \((30, 31)\). The culture suspension was pipetted into 15-ml conical tubes, wraped in bubble wrap, and placed in a foam shipping container lined with ice packs. A previous experiment demonstrated that \textit{H. hepaticus} was viable in these conditions for \(>16\) h (data not shown). Cultures were shipped by overnight courier to ensure arrival in Milwaukee, WI, within 16 h and gavaged immediately upon receipt.

#### Cohousing and \textit{H. hepaticus} gavage

\(\text{II}10^{a/d}\) Jax, IL-10R\(^{a/b}\)CD19\(^{a/c}\), or IL-10R\(^{a/b}\)LysMC\(^{a/c}\) mice were cohoused with \(\text{II}10^{a/d}\) mice from our colony \((\text{B10.PL background})\) in a 1:1 ratio for 4 wk for feces-mediated transfer of the microbiota. \(\text{II}10^{a/d}\) Jax mice were orally gavaged with \(5 \times 10^5\) CFU \textit{H. hepaticus} suspended in \textit{Brucella} broth. Control mice received sterile broth only. Cellular analysis of splenic B cell subsets and CD11b\(^+\) myeloid cells was performed 4 wk after cohousing or bacterial gavage.

#### Antibiotic treatment

The following antibiotics were provided ad libitum in drinking water for 4 wk: ampicillin trihydrate \((1.0 \text{ g/L})\), vancomycin hydrochloride \((0.5 \text{ g/L})\), neomycin sulfate \((1.0 \text{ g/L})\), and metronidazole \((1.0 \text{ g/L})\). This combined...
antibiotic regimen provides effective broad-spectrum coverage against Gram-positive and -negative bacteria (32).

**BrdU incorporation assay**

Mice were i.p. injected with 1 mg BrdU, twice daily, for 3 d. BrdU⁺ B cells were tracked 1, 30, and 45 d after the last BrdU injection using the allophycocyanin BrdU flow kit (BD Biosciences). For the B cell turnover assay, mice were provided with BrdU in the drinking water (0.8 mg/ml) for 9 d, and the next day BrdU⁺ B cells were detected using flow cytometry (33, 34).

**In vitro survival of B cells**

FACS-purified B cells (1 × 10⁶) were cultured in vitro in the presence of LPS (1 and 10 μg/ml), CpG (0.1 and 1 μg/ml), anti-mouse IgM F(ab)² (0.5 and 5 μg/ml), or anti-CD40 (0.1 and 1 μg/ml) for 72 h, and cell survival was determined by flow cytometry gating on live DAPI⁺ cells.

**RNA isolation and real-time PCR**

Total RNA was isolated from FACS-purified cells using the RNAqueous-4PCR kit (Ambion, Austin, TX), and first-strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). The quality of the cDNA was assessed by Gapdh PCR. The expression of Gapdh, Hes1, Dxl1, and Rbpj was quantified by real-time PCR using TaqMan primers and probes from Invitrogen and an ABI Prism 7500 PCR machine (Applied Biosystems, Foster City, CA). The amplification cycle was as follows: 10 min hold at 95°C, and then 40 cycles of 15 s at 95°C and 60 s at 60°C. The expression of the Hes1, Dxl1, and Rbpj genes was normalized to the housekeeping gene Gapdh by calculating the ΔΔCt. The relative expression of each gene is represented as 2⁻ΔΔCt.

**Isolation of microbial DNA, PCR amplification, and sequencing**

Small and large intestines and cecum were isolated from Il10⁻/⁻ Jax mice that were or were not cohoused with Il10 mice from our colony, as described (35). The isolated tissues were homogenized, bacterial DNA was isolated using MO BIO Power Analyzer kit (MO BIO Laboratories, Carlsbad, CA), and PCR was used with unique bar-coded primers to amplify the V4 region of the 16S rRNA gene to create an amplicon library from individual samples (36, 37). The 5’ primer was 5’-AATGATACGCAGCCAGGATATGTCTCTATCTATGGTATTGTGTAAGCAGCAGGACCGTCAAT-3’ and the 3’ primer was 5’-CAAGAGAAGAGACGCAGGTCAACCTACTATCCGACGAGGCAGCTCAATAA-3’. The individual PCR products were set up as follows: 10 μL 5X reaction buffer, 1.5 μL dNTPs, 2 μL 5’ primer, 2 μL 3’ primer, 1.5 μL Long Amp enzyme kit from New England Biolabs (Ipswich, MA), 30 μL DNA with the concentration of DNA at 2–5 ng/μl, and 3 μl H2O, as described (37). The amplification cycle was as follows: denature at 94°C for 1 min, followed by 32 cycles of 94°C for 30 s, 50°C for 1 min, 65°C for 1 min, with a final extension of 65°C for 5 min. The PCR products were sequenced using NextGen sequencing Illumina GAIIx platform (36). The samples were first quantitated using Pico Green, adjusted to a concentration of 4 nM, and then used for sequencing (37). Fastq conversion of the raw data files was performed following demultiplexing. Overall reads quality was assessed using the program FASTQC, and quality filtering of fastq files was performed using the FASTX toolkit.

**Bioinformatics**

A total of 11,321,588 sequencing reads of 101-bp length was generated from 12 animals (36 samples total) using Illumina GAIIx sequencing technology. Quality filtering of raw data was performed in two steps, as follows: 1) due to low quality of bases toward the 3’ end of the reads, 11 bases were trimmed from the 3’ end for all reads and 2) only high-quality reads having >90% bases with a Q Score >30 were retained. The quality filtering step retained 78% of total reads (8,928,469) with average of 248±92 reads per sample (range: 127,940–484,143 reads per sample). We clustered those sequences using AbundantOTU* v.0.93b with the “abundantly” option at 97% similarity and generated 824 operational taxonomic units (OTUs) incorporating 99.38% of the total input sequences. We used USEARCH (38) (v. 6.1.357) and the Gold reference database to screen for the presence of chimeras in our OTU sequences, and a total of 135 OTUs was removed. The remaining 689 OTUs (representing 98.73% of the input sequences) were used for downstream analysis. We assigned taxonomic ranks through the Quantitative Insight into Microbial Ecology (QIIME) (39) (v. 1.8.0) using both ucclust consensus taxon assigner and the Ribosomal Database Project (40) Classifier (v. 2.2) (confidence set to 80%). OTU consensus sequences were then aligned to the GreenGenes core reference alignment (41) using PyNAST (42). We built a phylogenetic tree from those alignments using FastTree (43) (v. 2.1.3) as implemented in the QIIME pipeline (v. 1.8.0). We generated BIOM files from our OTU counts and fed those along with the phylogenetic tree to PhylosEquation (44) (v. 1.7.24). Raw OTU counts were normalized and log₁₀ transformed according to the following formula:

$$\log_{10} \left( \frac{\text{OTU raw count}}{\text{Number of sequences in sample}} \right) \times \text{Average number of sequences per sample} + 1$$

The normalized and log₁₀-transformed reads were used in Phyloseq to produce principal coordinate analysis (PCoA) plots from Bray-Curtis and UniFrac (weighted and unweighted) (45). The α diversity measures (Chao1 richness estimate and Shannon diversity index) were also calculated using Phyloseq after rarefying the raw counts to an even depth of 97,988 reads (the minimum count in all samples). We used a mixed linear model utilizing SAS (v. 9.5) software to analyze the data and accounting for possible effects that may arise from cohosting the mice in the same cage (46).

**Statistical analysis**

Data were analyzed using GraphPad prism software and were presented as mean ± SE. Statistical significance was determined using the nonparametric Mann–Whitney U test or unpaired t test. The p values <0.05 were considered significant. Our mixed linear model, in which origin and tissue are a fixed effect and cage is a random effect (46), takes the form of the following:

$$Y_{ik} = \mu + \varepsilon_{ik} + \text{OT}_i + \text{origin}_k + \varepsilon_{ijk}$$

where $Y_{ik}$ represents each PCoA axis value OTU log normalized count Shannon or Chao1 value for Origin i, Tissue j, Cage k, and replicate l. $\varepsilon_{ik}$ is the effect of the lth origin, where origin is set to either The Jackson Laboratory animals, The Jackson Laboratory animals cohoused with our animals, or our Il10⁻/⁻ animals. $T_j$ is effect from the jth tissue (cecum, small intestine, or large intestine). (OT)k is the interaction effect between treatment i and tissue j. $\varepsilon_{ijk}$ is the effect from the kth cage that is nested within the ith origin, and $\varepsilon_{ijk}$ denotes the error associated with measuring $Y_{ik}$. We controlled for the false discovery rate (FDR) by correcting the p values using Benjamini and Hochberg approach (47).

**Results**

**MZ B cells and CD11b⁺ myeloid cells and the T-independent immune response are significantly increased in the spleens of Il10⁻/⁻ mice**

To determine whether the gut microbiota alters distal immune cell homeostasis in mice susceptible to colitis, we quantitated immune cell populations in Il10⁻/⁻ mice. For this analysis, we used Il10⁻/⁻ mice acquired from The Jackson Laboratory (Jax) in the year 2000 that were crossed to the B10.PL background (H-2u) and mice acquired from The Jackson Laboratory, animals, or our Il10⁻/⁻ animals. Il10⁻/⁻ mice acquired from The Jackson Laboratory mice are specific pathogen-free conditions. Because our Il10⁻/⁻ mice are susceptible to spontaneous enterocolitis, we used mice at 6–8 wk of age prior to enterocolitis onset and evidence of T cell activation in the gut (data not shown). First, we compared the total cellularity of Il10⁻/⁻ (WT) and Il10⁻/⁻ (WT) littermates and found no difference in the spleen (Fig. 1A), lymph node, thymus, Peyer’s patch, or BM (Supplemental Fig. 1A). We next performed a comprehensive immune phenotyping to determine whether the homeostasis of specific immune cell populations was altered in Il10⁻/⁻ mice. In the spleen (Fig. 1B) and lymph node (Supplemental Fig. 1B), we found that percentage of $\gamma\delta$ T cells was...
FIGURE 1. Increased MZ B and CD11b+ cells in the spleen of Il10<sup>−/−</sup> mice. (A) The absolute number of cells in the spleen of WT and Il10<sup>−/−</sup> mice was determined. Data are shown as the mean ± SE of three experiments (n = 8). (B) The percentage (left panel) and absolute number (right panel) of splenic TCR<sub>B</sub><sup>+</sup> T cells in WT and Il10<sup>−/−</sup> mice were determined by flow cytometry. Data are shown as the mean ± SE of three experiments (n = 8). (C) The percentage of TCR<sub>B</sub>CD4<sup>+</sup> (left panel) and TCR<sub>B</sub>CD8<sup>+</sup> (right panel) T cells in the spleen of WT and Il10<sup>−/−</sup> mice was determined by flow cytometry. Data are shown as the mean ± SE of two experiments (n = 6). (D) The percentage (left panel) and absolute number (right panel) of splenic TCR<sub>B</sub> B220<sup>+</sup> B cells in WT and Il10<sup>−/−</sup> mice were determined by flow cytometry. Data are shown as the mean ± SE of three experiments (n = 8). (E) The percentage (left panel) and absolute number (right panel) of splenic T1, T2, Fo, T2-MZP, and MZ B cell subsets from WT and Il10<sup>−/−</sup> mice were determined by flow cytometry. Data are shown as the mean ± SE of three experiments (n = 8). (F) Flow cytometric analysis showing the percentage (left panel) and absolute number (right panel) of splenic CD11b<sup>+</sup> cells from WT and Il10<sup>−/−</sup> mice. Each data point represents a single mouse from three independent experiments. (G) Immuno-
significantly reduced, whereas no difference was observed in the Peyer’s patch or peripheral blood (Supplemental Fig. 1B). We also observed a significant reduction in the absolute number of αβ T cells in the spleen (Fig. 1B). Overall, we did not observe a difference in the ratio of CD4/CD8 T cells in the spleen (Fig. 1C) or in the lymph node, thymus, or peripheral blood (Supplemental Fig. 1C, 1D). For B cells, whereas the percentage of total B cells was significantly decreased in the spleen of Il10−/− mice, the reduction in absolute number did not reach significance (Fig. 1D). Interestingly, the percentage of B cells was significantly increased in the lymph node and decreased in the peripheral blood, although no change was observed in the Peyer’s patch or BM (Supplemental Fig. 1E).

We then asked whether the homeostasis of specific B cell subsets was altered in Il10−/− mice. Using a gating strategy to identify peritoneal cavity B cell subsets, there was no difference in the absolute number of B2, B1, B1a, or B1b cells (Supplemental Fig. 2A). When we gated on splenic B cell subsets (26) (Supplemental Fig. 2B), we found that both the percentage and absolute number of the transitional 1 (T1) and transitional 2 (T2) precursors and the mature follicular (Fo) populations were significantly decreased in Il10−/− mice (Fig. 1E). There was no difference in the percentage or absolute number of T2–MZ precursors, the immediate precursor to MZ B cells (Fig. 1E). However, there was a significant increase in MZ B cells (Fig. 1E), which was confirmed using an alternative gating strategy (Supplemental Fig. 2C) (48, 49). Because MZ B cell retention is regulated in part by macrophages and perhaps neutrophils (50, 51), we quantitated splenic CD11b+ myeloid cells and found that both their percentage and absolute number were significantly increased in Il10−/− mice (Fig. 1F). We next used immunofluorescence to determine whether IL-10 deficiency altered the splenic architecture. By detecting B220+ B cells and MOMA-1+ macrophages to demarcate the MZ, we found an evident increase in the size of the MZ in Il10−/− mice, but no obvious difference in the splenic architecture (Fig. 1G). In measuring CD11b+ myeloid cells at other anatomical sites, we found a similar increase in their percentage in the peripheral blood and BM (Fig. 1H), but not the lymph node (data not shown). These data indicate that the loss of IL-10 has organ-specific effects on immune cell homeostasis.

We next determined whether the increase in myeloid cells and MZ B cells was associated with signs of inflammation. To measure local inflammation in the gut, we quantitated the protein expression level of two chemokines and six proinflammatory cytokines known to be associated with intestinal inflammation in the colon. As compared with WT mice, Il10−/− mice had significantly increased levels of the chemokines CCL2 and CXCL10 and the cytokines TNF-α, IL-1β, IL-6, and IL-17, but not IL-23 or IFN-γ (Fig. 1I). These data indicate that local inflammation is occurring in Il10−/− mice in our animal colony, which is consistent with their eventual development of spontaneous enterocolitis. To determine whether inflammation was detectable systemically, we measured the same panel of chemokines and cytokines in the serum and found no difference between the two cohorts of mice (Table I). These data suggest that the increase in MZ B cells and myeloid cells in Il10−/− mice is not due to a systemic immune response, but stems from inflammation in the colon.

We next examined the immunological consequence of an increase in MZ B cells and found that resting levels of IgM, but not IgG3, were significantly elevated in Il10−/− mice (Fig. 1J). To determine whether the increase in MZ B cells impacted the amplitude of the humoral immune response, we immunized with the T-independent Ag NP-Ficoll. On day 0, there was a significant increase in NP-specific IgM in Il10−/− mice that was retained on day 7 (Fig. 1J). NP-specific IgG3 was not detectable on day 0 (data not shown), and there was no difference in its generation on day 14 in WT versus Il10−/− mice (Fig. 1J). The increase in NP-specific IgM was not accompanied by an increase in the percentage of plasma cells in the spleen (Supplemental Fig. 2D). The increase in IgM was not likely due to B1 cells (52) because we found no difference in the absolute number of peritoneal cavity B cell subsets (Supplemental Fig. 2A). Thus, the increase in resting levels of total IgM in Il10−/− mice is most likely due to increased numbers of MZ B cells contributing to the IgM pool. Following immunization, the increase in NP-specific IgM is consistent with an increase in the absolute number of plasma cells.

Defects in MZ B cell and myeloid cell homeostasis are dependent upon the housing environment of the mice

To determine whether the alterations in B cell and myeloid homeostasis in Il10−/− mice were unique to our colony, we performed the same splenic phenotypic analysis on mice purchased directly from Jax and found no significant difference in the absolute number of T cells, B cell subsets, and myeloid cells between WT Jax and Il10−/− Jax mice (data not shown). The contrast between mice housed long-term in our colony and mice acquired directly from Jax supports the hypothesis that the microbiome acquired after housing in isolated animal colonies can specifically alter immune cell homeostasis. To evaluate this hypothesis, we determined whether Il10−/− Jax mice cohoused with Il10−/− mice from our colony would acquire the immune homeostasis defect. We found that 4 wk of cohousing was sufficient to induce an increase in both MZ B cells and CD11b+ myeloid cells, but not Fo B cells (Fig. 2A). Kinetically, the increase in the percentage of MZ B cells and CD11b+ myeloid cells was evident as early as 1 wk following cohousing, which peaked at 4 wk (Fig. 2B). In contrast, the percentage of Fo B cells within the total B cell population did not change (data not shown). To further demonstrate that the acquisition of an imbalance in immune homeostasis was driven by the microbiome, we included a treatment group whereby Il10−/− Jax cohoused mice were provided a combination of nonabsorbable broad-spectrum antibiotics in the drinking water (32). The inclusion of antibiotics from the outset of the experiment prevented the increase in the percentage and absolute number of MZ B cells and CD11b+ myeloid cells (Fig. 2C).

Both neutrophil and monocyte/macrophage numbers are specifically increased in Il10−/− mice following cohousing

To determine the phenotype of the expanded CD11b+ myeloid population in Il10−/− mice, we subdivided CD11b+ cells by Ly6C and Ly6G expression. Specifically, we quantitated Ly6C−Ly6Ghigh fluorescence analysis of spleen sections from WT and Il10−/− mice stained with B220-PE (red) and MOMA-1-FITC (green) was performed. Representative images from one of three mice are shown (original magnification ×200). (H) The percentage of CD11b+ cells in the peripheral blood (left panel) and BM (right panel) in WT and Il10−/− mice was determined by flow cytometry. Data are shown as the mean ± SE (n = 3). (I) Cytokines and chemokines in the colon of naïve WT and Il10−/− mice at 7–8 wk of age are shown (n = 4). (J) Resting serum IgM and IgG3 levels in naïve WT and Il10−/− mice were measured by ELISA (n = 6–7) (left panels). NP-specific IgM (days 0 and 7) (middle panels) and IgG3 (day 14) (right panel) serum titers in NP-Ficoll–immunized WT and Il10−/− mice were determined by ELISA. Data are shown as the mean ± SE (n = 3–4). *p < 0.05, **p < 0.01, ***p < 0.001.
neutrophils, Ly6C<sup>hi</sup>Ly6G<sup>-</sup> inflammatory and Ly6C<sup>lo</sup>Ly6G<sup>-</sup> resident monocytes/macrophages, and Ly6C<sup>-</sup>Ly6G<sup>-</sup> cells that are most likely a combination of resident monocytes/macrophages, dendritic cells, and NK cells (Fig. 3A, Supplemental Fig. 2E) (53, 54). When we measured the absolute number of each CD11b<sup>+</sup> myeloid cell population in II10<sup>−/−</sup> Jax mice following cohousing with II10<sup>−/−</sup> mice from our colony, we found a significant increase in the Ly6C<sup>lo</sup>Ly6G<sup>-</sup> neutrophil and in the Ly6C<sup>hi</sup>Ly6G<sup>-</sup> and Ly6C<sup>lo</sup>Ly6G<sup>-</sup> monocyte/macrophage populations as compared with both WT mice and noncohoused II10<sup>−/−</sup> Jax mice (Fig. 3B). No change in the Ly6C<sup>-</sup>Ly6G<sup>-</sup> population was observed (Fig. 3B). Antibiotic treatment during the cohousing period prevented the increase in the absolute number of neutrophils and monocytes/macrophages (Fig. 3B). When we measured the relative distribution of each cell type within the CD11b<sup>+</sup> population, we observed a significant increase in the percentage of neutrophils and monocytes/macrophages with cohousing (Fig. 3C). We then examined the activation status of the macrophages and found that their absolute number increased upon cohousing (Fig. 3F). Interestingly, antibiotic treatment did not prevent the increase in MARCO<sup>+</sup> macrophages (Fig. 3F), suggesting that their increase alone is not responsible for the increase in MZ B cells.

### The increase in MZ B cells in II10<sup>−/−</sup> mice is due to enhanced MZ B cell development

Next, we investigated the mechanism driving the immune homeostasis defect. First, we examined proliferation and found that total B cells from II10<sup>−/−</sup> mice exhibited a slight proliferative advantage over WT when stimulated with LPS, CpG, or anti-CD40, but not anti-IgM (data not shown). However, this did not translate into increased steady state proliferation of II10<sup>−/−</sup> MZ B cells in vivo as measured by BrdU incorporation (Fig. 4A), nor did cohousing increase the number of Ki-67<sup>+</sup> MZ B cells (Fig. 4B). Interestingly, MZ B cells were proliferating less in the II10<sup>−/−</sup> mice (Fig. 4A, 4B). These findings are consistent with similar expression of the activation markers CD80, CD86, MHC class II, and CD40 by WT and II10<sup>−/−</sup> MZ B cells (data not shown) (57). To determine whether II10<sup>−/−</sup> MZ B cells have a survival advantage, we repeated the in vitro culture using FACSpurified MZ B cells and found that in the absence of stimulation II10<sup>−/−</sup> MZ B cells had a higher level of survival after 3 d in culture (Fig. 4C). Following stimulation with LPS or CpG, both the WT and II10<sup>−/−</sup> MZ B cells expanded with a similar dose-dependent kinetics that was proportional to the percentage of surviving cells without stimulation, which resulted in a significant increase in the percentage of surviving II10<sup>−/−</sup> MZ B cells (Fig. 4C). Although anti-CD40 induced expansion of both WT and II10<sup>−/−</sup> MZ B cells, there was no significant difference in their survival (Fig. 4C). Anti-IgM only led to a marginal expansion of both populations (Fig. 4C). The survival advantage was marginally evident following a BrdU pulse-chase assay after 30 and 45 d (Fig. 4D). This finding is consistent with the similar expression levels of the cell survival proteins Bcl-2 and Bcl-xL in MZ B cells from WT and II10<sup>−/−</sup> mice (data not shown). In addition, II10<sup>−/−</sup> MZ B cells were not undergoing reduced levels of apoptosis (data now shown). These cumulative data indicate that the increased numbers of MZ B cells in II10<sup>−/−</sup> mice are not due to increased cell survival or proliferation. Thus, we examined whether B cell differentiation is altered in II10<sup>−/−</sup> mice. In the BM, we found no alteration in the proportion of pre/pro and immature B cells in II10<sup>−/−</sup> mice, indicating that IL-10 is not required for early B cell differentiation (Fig. 4E). To specifically examine whether enhanced MZ lineage differentiation is occurring in the spleen, we measured Notch-2 expression in the T1 subset

### Table I. Chemokine and cytokine levels in serum of naive WT and II10<sup>−/−</sup> mice

<table>
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<tr>
<th></th>
<th>IL-23 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>CCL2 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IP-10 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-17 (pg/ml)</th>
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<td>Mean ± SE</td>
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<td>9.2 ± 2.5</td>
<td>94.2 ± 67.4</td>
<td>na</td>
<td>10.6 ± 2.4</td>
<td>222.4 ± 149.9</td>
<td>15.3 ± 7.2</td>
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<td>330.4</td>
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<td>Mean ± SE</td>
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<td>34.2 ± 7.5</td>
<td>225.7 ± 57.4</td>
<td>34.9 ± 9.5</td>
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</table>

Mice aged 7–8 wk.
IP-10, IFN-γ-inducible protein-10; na, not applicable; nd, not detected.
because it is required to drive precursor maturation into the MZ B cell lineage (58, 59). Using flow cytometry, we found that Notch-2 expression was significantly increased on Il10−/− T1 B cells (Fig. 4F). However, Notch-2 expression was not altered in the mature Fo and MZ subsets (data not shown). We then determined whether the increase in Notch-2 expression led to increased signaling by measuring the transcript levels of its downstream effector molecules (60, 61). Although we saw no difference in mRNA levels of Rbpj and Hes1, we found that Deltex 1 (Dtx1) transcripts were significantly increased in Il10−/− T1 B cells (Fig. 4G). In addition, the cell surface expression of CD21, another Notch-2–regulated protein (59), was significantly increased on T1 B cells in Il10−/− mice (Fig. 4H). Finally, we asked whether the expression of the ligand for Notch-2, Delta-like 1, was increased on CD11b+ myeloid cells in Il10−/− mice. Although we did not detect a difference in the expression level of Delta-like 1 on myeloid cells (Fig. 4I) or in the percentage of CD11b+ cells that express Delta-like 1 (data not shown), there was a ∼15-fold increase in the absolute number of CD11b+Delta-like 1+ cells in Il10−/− mice (Fig. 4J). When we examined the proliferation of Il10−/− CD11b+ myeloid cells in the spleen in the steady state, we found that in Il10−/− mice they underwent a significantly higher rate of proliferation as compared with WT Jax mice (Fig. 4K). Identical results were obtained in WT and Il10−/− mice from the home colony (Fig. 4L). These cumulative data suggest that the increase in MZ B cells in Il10−/− mice is due to increased differentiation of the T1 subset into the MZ lineage via upregulation of Notch-2 and increased availability of its ligand Delta-like 1, whereas the increase in myeloid cells is due to increased proliferation.

**FIGURE 2.** Microbiota-mediated increase in splenic MZ B and CD11b+ cells in Il10−/− mice. (A) The percentage of MZ B cells (gated on B220) (left panel), CD11b+ myeloid cells (middle panel), and Fo B cells (gated on B220) (right panel) was quantitated in Il10−/− Jax housed alone or cohoused with Il10−/− mice from our colony for 4 wk. Each data point represents a single mouse. (B) WT and Il10−/− Jax mice were cohoused with Il10−/− mice from our colony, and the percentage of MZ B cells (gated on B220) and CD11b+ myeloid cells in the spleen was determined on days 7, 21, 28, and 35. Data are shown as the mean ± SE (n = 3). (C) The percentage (top panels) and absolute number (bottom panels) of splenic MZ B cells (gated on B220) (left panels) and CD11b+ myeloid cells (right panels) in WT Jax cohoused and Il10−/− Jax and Il10−/− Jax cohoused mice (4 wk) with and without antibiotic treatment were determined by flow cytometry. Data are shown as the mean ± SE of two experiments (n = 6–7). *p < 0.05, **p < 0.01.
FIGURE 3. The increase in splenic CD11b+ myeloid cells in Il10<sup>−/−</sup> mice is due to increased numbers of monocytes/macrophages and neutrophils. (A) Representative dot plots showing Ly6C and Ly6G expression by splenic CD11b+ myeloid cells from WT Jax cohoused, Il10<sup>−/−</sup> Jax, Il10<sup>−/−</sup> Jax cohoused (4 wk), and antibiotic-treated (4 wk) Il10<sup>−/−</sup> Jax cohoused mice. (B) Using the indicated mice in (A), the absolute number of CD11b+Ly6C<sup>+</sup>Ly6G<sup>+</sup>, CD11b<sup>+</sup>Ly6C<sup>h</sup>Ly6G<sup>−</sup>, and CD11b<sup>+</sup>Ly6C<sup>−</sup>Ly6G<sup>+</sup> cells was determined by flow cytometry. Data are shown as the mean ± SE of two experiments (n = 6–7). (C) The percentage of the indicated cell populations in (A) was determined by flow cytometry. (D) Immunofluorescence analysis of spleen sections from WT and Il10<sup>−/−</sup> mice stained with B220-Alexa Fluor 594 (blue), CD11b-Alexa Fluor 488 (green), and Ly6G-Alexa Fluor 647 (red) was performed. Representative images from one of two mice are shown (×100). (E) The expression of MHCII and CD86 on splenic CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> (top panels) and CD11b<sup>+</sup>Ly6C<sup>h</sup>Ly6G<sup>−</sup> (bottom panels) cells from the mice indicated in (A) was measured by flow cytometry, and the mean fluorescence intensity (MFI) is shown. Data are shown as the mean ± SE (n = 6). (F) The absolute number of splenic MARCO<sup>+</sup> cells (CD11b gated) was determined in the indicated mice in (A). Data are shown as the mean ± SE (n = 3–4). *p < 0.05, **p < 0.01, ***p < 0.001.
The increase in MZ B and myeloid cells in Il10<sup>−/−</sup> mice is macrophage, but not B cell, intrinsic

We next determined whether the increase in MZ B cells and myeloid cells was due to a loss of IL-10 signaling in one or both cell types. To examine the necessity for IL-10 signaling in B cells, we used IL-10<sup>−/−</sup>CD19Cre<sup>+</sup> mice and found that upon cohousing these mice exhibited no alteration in the percentage of total (Fig. 5A) or Fo B cells (Fig. 5B) as compared with IL-10<sup>−/−</sup>LysMCre<sup>−/−</sup> control mice. Interestingly, the percentage of MZ B cells was significantly decreased (Fig. 5C). In addition, the percentage of...
CD11b⁺ cells was not altered (Fig. 5D). To render monocytes/macrophages and other myeloid subsets deficient in IL-10R signaling, we used IL-10Rα(fl/fl)LysMCre⁺ mice and found that upon cohousing these mice had an increase in both the percentage and absolute number (data not shown) of both MZ B cells and CD11b⁺ cells at a level similar to Il10⁻²⁄⁻ mice from our colony for 4 wk. Data are shown as the mean ± SE of two experiments (n = 6). (E) The percentage of splenic MZ B cells (left panel) and CD11b⁺ myeloid cells (right panel) from Il10⁻²⁄⁻, IL-10Rα(fl/fl)LysMCre⁺, IL-10Rα(fl/fl)LysMCre⁺, and IL-10Rα(fl/fl)LysMCre⁺ cohoused (4 wk) mice was determined by flow cytometry. Each data point represents a single mouse from three experiments. **p < 0.01.

FIGURE 5. The increase in CD11b⁺ myeloid cells in Il10⁻²⁄⁻ mice is due to enhanced proliferation in a cell-intrinsic manner. The percentage of total splenic B cells (B220⁺) (A) and Fo (B) and MZ (C) B cells within the B220 gate and CD11b⁺ (D) cells was determined by flow cytometry from IL-10Rα(fl/fl)CD19Cre⁺ and IL-10Rα(fl/fl)CD19Cre⁻ mice cohoused with mice from our colony for 4 wk. Data are shown as the mean ± SE of two experiments (n = 6). (E) The percentage of splenic MZ B cells (left panel) and CD11b⁺ myeloid cells (right panel) from Il10⁻²⁄⁻, IL-10Rα(fl/fl)LysMCre⁺, IL-10Rα(fl/fl)LysMCre⁺, and IL-10Rα(fl/fl)LysMCre⁺ cohoused (4 wk) mice was determined by flow cytometry. Each data point represents a single mouse from three experiments. **p < 0.01.

FIGURE 6. Distinct and significant clusters are formed between Il10⁻²⁄⁻ Jax mice as compared with Il10⁻²⁄⁻ Jax cohoused and Il10⁻²⁄⁻ from our colony. (A) Bray-Curtis PCoA plots generated from the OTU colored by origin (A) were generated comparing Il10⁻²⁄⁻ Jax mice analyzed immediately upon receipt from Jax (n = 4) to Il10⁻²⁄⁻ mice in our facility (n = 4) and to Il10⁻²⁄⁻ Jax mice cohoused with our mice for 4 wk (n = 4). (B) PCoA was conducted as for (A) colored by tissue source. (C) PCoA was conducted as for (A) colored by cage. α diversity analysis using Chao1 richness estimate (D) and Shannon diversity index (E) between the three groups is shown.
our colony (Fig. 5E). WT levels of both populations were observed in the IL10R$^{−/−}$/LysMCre$^{−}$ and IL10R$^{−/−}$/LysMCre$^{+/−}$ non-cohoused controls (Fig. 5E). These cumulative data indicate that IL-10R signaling in monocytes/macrophages, but not B cells, is required to regulate the steady state homeostasis of MZ B cells and CD11b$^{+}$ myeloid cells in the spleen.

**II10$^{−/−}$ Jax mice acquire a distinct microbial community after cohousing**

To identify the microbial community associated with the transition from The Jackson Laboratory, we performed Illumina sequencing targeting the V4 region of the 16S rRNA gene, after quality filtering 8,928,469 sequences of length = 90 from 12 animals (four in each group sampled from three different tissues). Those sequences were then clustered into OTUs, groups of sequences with an average percent identity of 97%. A PCoA analysis of these OTUs revealed that II10$^{−/−}$ Jax mice upon immediate receipt from Jax showed distinct clustering from II10$^{−/−}$ Jax mice cohoused with our animals (Fig. 6A). The FDR was $p < 0.05$ for II10$^{−/−}$ Jax mice compared with II10$^{−/−}$ mice in our facility and cohoused II10$^{−/−}$ Jax mice.

To separate the effect of the Jax microbiota from the tissue type from which we sampled (Fig. 6B) and the cage (Fig. 6C) in which the animals were housed in, we used a mixed linear model, as described previously (46), with the following terms: origin, indicating II10$^{−/−}$ Jax mice, II10$^{−/−}$ Jax mice cohoused with our mice or our II10$^{−/−}$ mice; tissue, indicating the source tissue of the sample (cecum, small intestine, or large intestine); and cage, indicating the cage at which the animals were housed. The results from this analysis have been deposited at http://metagenomics.anl.gov/linkin.cgi?project=14383. Among the first five PCoA axes, we observed many highly significant effects for tissue, tissue $\times$ origin, and origin. These results are consistent with the II10$^{−/−}$ Jax mice having a distinct microbiome that shifts over time and with cohousing in our animal facility. It is interesting to note that the differences between the Jax mice gut microbiome and our facility’s mice gut microbiome (Fig. 6A) appear to be larger than the differences that are attributable to the tissue that is sampled (Fig. 6B). This emphasizes the profound influence of the animal facility environment over the gut microbiome. Analysis of these data utilizing alternative algorithms (for example, utilizing UNIFRAC distance as opposed to Bray-Curtis or assigning OTUs through the open-reference algorithm in QIME) yielded nearly identical patterns of clustering (Supplemental Fig. 3A–I). Our conclusion of a distinct microbial community in the II10$^{−/−}$ Jax mice is therefore unlikely to be the result of particular analysis choice that we made in our pipeline. However, $\alpha$ diversity analysis (Fig. 6D, 6E) showed no significant differences in terms of richness or diversity between the three groups.

**MZ B cell and myeloid cell homeostasis is dysregulated by introduction of a single bacterial strain, H. hepaticus**

To determine the biological impact of differences between Jax mice and mice in our facility, we focused on *Helicobacter* because of its known association with colitis (11, 18). In addition, our II10$^{−/−}$ mice harbor *Helicobacter* and succumb to spontaneous colitis, whereas the II10$^{−/−}$ Jax mice do not. We found that Consensus3 classified to genus level as *Helicobacter* by both Ribosomal Database Project Classifier (confidence score $> 0.8$) and uclust was significantly lower in II10$^{−/−}$ Jax mice (Fig. 7A).

To determine whether the presence of *Helicobacter* was responsible for the immune homeostasis defect in II10$^{−/−}$ mice, we inoculated II10$^{−/−}$ Jax mice once with *H. hepaticus* by gavage. We found that II10$^{−/−}$ Jax mice gavaged with *H. hepaticus* had a significant increase in both MZ B cells and CD11b$^{+}$ myeloid cells as compared with II10$^{−/−}$ mice that were gavaged broth (Fig. 7B). WT Jax mice gavaged with *H. hepaticus* did not exhibit

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**FIGURE 7.** II10$^{−/−}$ Jax mice as compared with II10$^{−/−}$ Jax cohoused and II10$^{−/−}$ Jax from our colony had a decreased abundance of *Helicobacter* that when introduced to II10$^{−/−}$ Jax mice was sufficient to drive an increase in MZ B and CD11b$^{+}$ cells. (A) A box and whisker plot shows the minimum, first quartile, median, third quartile, and maximum relative abundance of Consensus3 (*Helicobacter spp.*) in II10$^{−/−}$ Jax with and without cohousing and II10$^{−/−}$ from our colony ($n=4$). (B) The percentage of MZ B (left panel) and CD11b$^{+}$ (right panel) splenic cells in *H. hepaticus* or control broth-gavaged WT Jax and II10$^{−/−}$ Jax mice was determined by flow cytometry. Data are shown as the mean ± SE from one representative experiment of two ($n=4$). (C) The percentage of splenic MZ B cells (left panel) and CD11b$^{+}$ cells (right panel) from WT, II10$^{−/−}$, and antibiotic-treated (4 wk) II10$^{−/−}$ mice was determined by flow cytometry. Data are shown as the mean ± SE of two independent experiments ($n=5–6$). *$p < 0.05$, **$p < 0.01$. **
an increase in either MZ B cells or CD11b+ myeloid cells (Fig. 7B). We next asked whether the immune homeostasis defect was permanent and found that antibiotic treatment of our Il10−/− mice reversed the increase in MZ B and CD11b+ myeloid cells (Fig. 7C). Together, these results indicate that a single bacterial strain upon introduction to the intestinal track can modulate immune cell homeostasis at distal sites in addition to contributing to a disease state in immunocompromised individuals.

Discussion

We have demonstrated that differences in the microbiota between genetically similar mice can vary profoundly depending on the animal husbandry history, leading to an imbalance in immune cell development and homeostasis at distal sites outside of the intestinal track. Specifically, we showed that MZ B and CD11b+ myeloid cell numbers were significantly increased in the spleen of Il10−/− mice from our colony as compared with WT mice from our colony and Il10−/− Jax mice. Sequencing of the microbiome revealed that our mice were colonized with a distinct bacterial microbiota, which was transferred to Il10−/− Jax mice upon cohousing with our mice in conjunction with the acquisition of the homeostasis defect in MZ B and myeloid cells. We also found that the transfer of a single bacterial species was sufficient to drive the imbalance in immune homeostasis seen in our colony. These results have profound implications for the reproducibility of experimental data when animals with deficiencies in immune regulatory mediators are used and emphasize the importance of controlling the composition of the microbial community.

When genetically altered mice are phenotyped for deficiencies in immune cell populations, it is very uncommon for specific B cell subsets to be examined. Thus, even profound differences in the relatively rare population of MZ B cells (4–8% of splenic B cells) can be missed. This is clearly demonstrated in our study, in which we found that total B cells were decreased in Il10−/− mice from our colony (Fig. 1D). This reduction was largely due to a decrease in Fo B cells (Fig. 1E), and, because Fo B cells are the major B cell subset in the spleen, their decrease was able to offset the increase in MZ B cells in the total B cell pool (Fig. 1E). We found that in Il10−/− mice from our colony, there were increased levels of resting IgM and Ag-specific IgM following immunization with a T-independent Ag (Fig. 1J). Although we attribute this finding to an increase in MZ B cells, if the increase in MZ B cells had not been observed, an alternate explanation would be that IL-10 directly regulates IgM production. If the same experiment had been performed with Il10−/− Jax mice, no alteration in IgM levels would have been observed, which would be interpreted as IL-10 playing no role in IgM production. Thus, two laboratories doing the same experiment using the same mouse strain would come to very different conclusions. In support of the importance of phenotyping B cell subsets, in previous studies in mice deficient in the cannabinoid receptor 2, we found that whereas the percentage of Fo B cells was not altered in the spleen, the percentage of MZ B cells was significantly reduced (28). These data indicate that assuming B cell development and homeostasis is normal based on using global B cell markers such as B220 and CD19 is not sufficient when phenotyping genetically modified mice.

Using two different gating strategies (26, 48), we were able to definitively demonstrate that MZ B cell numbers are increased in the spleen of Il10−/− Jax mice as early as 7 d after cohousing with mice from our colony (Fig. 2B). Using a variety of approaches, we determined that the increase in MZ B cells in Il10−/− mice was not due to increased levels of proliferation or enhanced cell survival (Fig. 4). However, we cannot rule out a role for either increased expression of B cell survival factors or increased MZ B cell responsiveness to survival factors such as BAFF in Il10−/− mice. We do not favor the increased survival factor hypothesis because it was previously shown that serum levels of the B cell survival factor BAFF were not increased in Il10−/− mice (62). Given the requirement for Notch-2 signaling for the differentiation of the MZ lineage (59), our data demonstrating an increase in Notch-2 expression by the T1 precursors (Fig. 4F) suggest that the increase in MZ B cells is due to increased lineage commitment to the MZ lineage. This would mean that fewer progenitors would be available to commit to the Fo lineage, which is what we observed (Fig. 1E). The increase in the mRNA expression of the Notch-2 target gene Dtx1 (Fig. 4G) and surface expression of CD21 (Fig. 4H) indicates that the increase in Notch-2 protein has a functional outcome most likely due to enhanced signaling. Little is known regarding how Notch-2 expression is regulated in MZ B cells, but its function is dependent upon binding to its ligand Delta-like 1 (20, 58). Delta-like 1 expression by splenic non-hematopoietic cells, most likely a stromal cell, is sufficient to drive MZ B cell differentiation (63). The site of MZ commitment is unknown, but it most likely occurs outside of the MZ. Our finding that Delta-like 1 is expressed by monocytes/macrophages in the spleen (Fig. 4I) is consistent with the literature (64). We found that neither the mean fluorescence intensity of Delta-like 1 on CD11b+ cells (Fig. 4I) nor their percentages expressing Delta-like 1 (data not shown) were altered in Il10−/− mice. However, the increase in total myeloid cells in the Il10−/− spleen resulted in an increase in the total number of Delta-like 1+ cells by ~15-fold (Fig. 4J). Thus, although in the steady state hematopoietic cells do not promote MZ B cell differentiation, we propose that during inflammation the increase in CD11b+Delta-like 1+ cells in the spleen increases Notch-2 ligand availability, transiently increasing the number of MZ B cells most likely by promoting both their differentiation and retention. This speculative hypothesis is supported by our finding that antibiotic treatment of Il10−/− mice, presumably dampening the inflammatory response, was sufficient to reduce the number of MZ B cells and CD11b+ myeloid cells in the spleen (Fig. 7C). Further support of this hypothesis is the finding that intradermal injection of Borrelia burgdorferi, the causative agent of Lyme disease, led to a transient increase in MZ B cells in mice that correlated with the first appearance of the spirochete in other tissues (65).

Although we favor the hypothesis that the increase in MZ B cells in Il10−/− mice is due to enhanced differentiation, we cannot rule out the possibility that MZ B cells are being retained within the spleen. Using a Delta-like 1 blocking Ab, it was shown that Notch-2 engagement with its ligand was required for retention of the pre-established long-lived MZ B cell pool within the spleen in adult mice (64). In addition, MARCO+ macrophages within the MZ are also thought to contribute to MZ B cell retention (56). However, the increase in MARCO+ macrophages in cohoused Il10−/− mice with and without antibiotics suggests that retention is not likely the major mechanism that drives the increase in MZ B cells (Fig. 3F). An unanswered question is the myeloid cell type that potentially drives MZ B cell differentiation. By flow cytometry phenotyping, we found that both neutrophils and monocytes/macrophages are significantly increased in Il10−/− Jax mice upon cohousing with mice from our colony (Fig. 3B, 3C). Based on the composition of the myeloid cells, we favor the hypothesis that the Ly6C+Ly6G− monocytes/macrophages are the required cell. Cohousing of Il10−/− Jax mice significantly increased the absolute number of neutrophils (Ly6C+Ly6G−), inflammatory macrophages (Ly6C+Ly6G−), and the Ly6C+Ly6G+ monocytes/macrophages, the expansion of which was prevented by the inclusion of antibiotics (Fig. 3B). However, when the composition...
of the myeloid cell population was determined, the percentage of neutrophils within the CD11b\(^+\) population was identical in the cohoused with and without antibiotic groups (Fig. 3A, 3C). The most dramatic change in composition was in the Ly6C\(^+\)/Ly6G\(^-\) population, which was increased by >50% after cohousing. We cannot rule out a role for neutrophils given that Puga et al. (51) recently demonstrated that they are located peri-MZ in the spleen. That elegant study largely conducted in humans further demonstrated that neutrophils provide a helper function to MZ B cells by producing factors promoting isotype class switching, somatic hypermutation, and Ig production (51). Interestingly, a broad group of neutropenic patients was shown to have reduced numbers of circulating MZ B cells with a poorly developed MZ (51). However, the study was not designed to determine whether neutrophils are required for development of the MZ and/or lineage commitment of MZ B cells.

In our kinetic study, upon cohousing MZ B cells and CD11b\(^+\) cells increased in parallel in the Il10\(^{-/-}\) Jax mice with a significant increase evident as early as day 7 postcohousing (Fig. 2B). From these data, it was not clear whether the expansion of the two populations is linked or are independent events. We investigated this question by conditionally knocking out the IL-10R in B cells or myeloid cells (24). Depletion in neutrophils has also been reported (24). Loss of IL-10R signaling in B cells had no impact on B cell or myeloid cell homeostasis (Fig. 5A–D) and supports our findings in global Il10-deficient mice that IL-10 has no major intrinsic role in B cell development, function, or homeostasis. In contrast, loss of IL-10 signaling in myeloid cells was sufficient to drive an increase in both MZ B cells and myeloid cells, further supporting our hypothesis that macrophages and/or neutrophils can promote MZ B cell differentiation (Fig. 5E). The expansion in splenic myeloid cells was due to increased proliferation with up to 90% of the cells proliferating in the absence of IL-10 (Fig. 4K, 4L). Because peripheral neutrophils do not divide and they make up 10% of the myeloid population in the Il10\(^{-/-}\) mice, this indicates that 100% of the monocytes/macrophages were undergoing cell division. This remarkable finding indicates that IL-10 is a master regulator of monocyte/macrophage proliferation. The increase in CD11b\(^+\) cells in the BM (Fig. 1H) indicates that IL-10 may exert this control even during myeloid cell development. In support of this hypothesis are studies in the Rag1\(^{-/-}\) CD4\(^+\)CD25\(^-\)CD45RB\(^{high}\) T cell transfer model of colitis (Fig. 5E). The expansion in splenic myeloid cells was due to increased proliferation with up to 90% of the cells proliferating in the absence of IL-10 (Fig. 4K, 4L). Because peripheral neutrophils do not divide and they make up 10% of the myeloid population in the Il10\(^{-/-}\) mice, this indicates that 100% of the monocytes/macrophages were undergoing cell division. This remarkable finding indicates that IL-10 is a master regulator of monocyte/macrophage proliferation. The increase in CD11b\(^+\) cells in the BM (Fig. 1H) indicates that IL-10 may exert this control even during myeloid cell development. In support of this hypothesis are studies in the Rag1\(^{-/-}\) CD4\(^+\)CD25\(^-\)CD45RB\(^{high}\) T cell transfer model of colitis (Fig. 5E). The expansion in splenic myeloid cells was due to increased proliferation with up to 90% of the cells proliferating in the absence of IL-10 (Fig. 4K, 4L).
partially activated phenotype (57). Thus, a specific increase in MZ B cells would facilitate faster clearance of potential pathogens that transverse the gut endothelium and enter the blood during inflammation. Future work will undoubtedly continue to define the role of gut bacteria in signaling to the immune system via breaches in the gut barrier.

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Disclosures

The authors have no financial conflicts of interest.

References

founder effects drive cage effects in microbial community assembly in a mouse model. ISME J. 7: 2116–2125.


Supplemental Figure 1. Characterization of immune cells in the inguinal lymph node, thymus, Peyer’s patch, peripheral blood and bone marrow of WT and Il10<sup>-/-</sup> mice. (A) The cellularity in the lymph node, thymus, Peyer’s patch and bone marrow were determined in WT and Il10<sup>-/-</sup> mice. Data are shown as the mean ± SE (n = 3). (B) In the same mice as (A) the percentage of TCRβ<sup>+</sup> cells was determined by flow cytometry in the inguinal lymph node, Peyer’s patch, and peripheral blood. (C) In the same mice as (A) the percentage of CD4<sup>+</sup> among TCRβ<sup>+</sup>-gated cells was determined by flow cytometry in the inguinal lymph node, thymus, and peripheral blood. (D) In the same mice as (A) the percentage of CD8<sup>+</sup> among TCRβ<sup>+</sup>-gated cells was determined by flow cytometry in the inguinal lymph node, thymus, and peripheral blood. (E) In the same mice as (A) the percentage of B220<sup>+</sup> cells was determined by flow cytometry in the inguinal lymph node, Peyer’s patch, peripheral blood and bone marrow. *p<0.05.
Supplemental Figure 2. Flow cytometry gating strategies and plasma cell quantitation. (A) A representative flow cytometry gating strategy for the analysis of peritoneal cavity B cell subsets is shown. The percentage of B2, B1, B1a and B1b peritoneal cavity B cell subsets was determined by flow cytometry in WT and Il10<sup>-/-</sup> mice. Data are shown as the mean ± SE (n = 3). (B) A representative flow cytometry gating strategy for the analysis of the T1, T2, T2-MZP, MZ and Fo splenic B cell subset is shown. (C) A representative flow cytometry alternative gating strategy for the analysis of splenic MZ and Fo B cells is shown for WT and Il10<sup>-/-</sup> mice. (D) WT and Il10<sup>-/-</sup> mice were immunized with NP-ficoll and 14 days later the percentage (left panel) and absolute number (right panel) of B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>-</sup>CD138<sup>+</sup> plasma cells was determined in the spleen. Each data point represents one mouse. (E) A representative flow cytometry gating strategy for the analysis of splenic CD11b<sup>+</sup>-gated cells expressing the Ly6C<sup>-</sup>Ly6G<sup>+</sup>, Ly6C<sup>+</sup>Ly6G<sup>hi</sup>, Ly6C<sup>hi</sup>Ly6G<sup>-</sup> or Ly6C<sup>lo</sup>Ly6G<sup>-</sup> phenotypes is shown. *p<0.05.
Supplemental Figure 3. Change in microbial community composition analyzed using the QIIME open-reference OTU picking approach. Plots representing Bray Curtis PCoA (A-C), unweighted-Unifrac PCoA (D-F) and weighted-Unifrac PCoA (G-I) are shown with each PCoA colored by either origin (A, D, G), tissue source (B, E, H), or cage (C, F, I).