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# Parental Dietary Fat Intake Alters Offspring Microbiome and Immunity

**Ian A. Myles,* Natalia M. Fontecilla,*,[1] Brian M. Janelsins,*,[1] Paul J. Vithayathil,* Julia A. Segre,† and Sandip K. Datta***

**Mechanisms underlying modern increases in prevalence of human inflammatory diseases remain unclear. The hygiene hypothesis postulates that decreased microbial exposure has, in part, driven this immune dysregulation. However, dietary fatty acids also influence immunity, partially through modulation of responses to microbes. Prior reports have described the direct effects of high-fat diets on the gut microbiome and inflammation, and some have additionally shown metabolic consequences for offspring. Our study sought to expand on these previous observations to identify the effects of parental diet on offspring immunity using mouse models to provide insights into challenging aspects of human health. To test the hypothesis that parental dietary fat consumption during gestation and lactation influences offspring immunity, we compared pups of mice fed either a Western diet (WD) fatty acid profile or a standard low-fat diet. All pups were weaned onto the control diet to specifically test the effects of early developmental fat exposure on immune development. Pups from WD breeders were not obese or diabetic, but still had worse outcomes in models of infection, autoimmunity, and allergic sensitization. They had heightened colonic inflammatory responses, with increased circulating bacterial LPS and muted systemic LPS responsiveness. These deleterious impacts of the WD were associated with alterations of the offspring gut microbiome. These results indicate that parental fat consumption can leave a "lard legacy" impacting offspring immunity and suggest inheritable microbiota may contribute to the modern patterns of human health and disease.** ***The Journal of Immunology*****, 2013, 191: 000–000.**

The prevalence of multiple immune-mediated diseases continues to increase in Western societies (1, 2). The hygiene hypothesis, originally proposed as a reason for the inverse correlation between family size and atopy (3), has expanded to become a potential explanation for the increasing prevalence of inflammatory disorders. It postulates that modern decreases in microbial exposure affect immune development and promote dysregulated immunity (1, 4, 5). Animal studies of bacterial LPS, the prototypical surrogate of infectious exposure, and its mammalian receptor, TLR4, have led to modern iterations of the hygiene hypothesis that propose environmental exposure to LPS early in life protects against the development of immune dysfunction (4). This is further supported by epidemiological studies that correlate a decreased risk for asthma and allergy in children from homes with high LPS levels (6) and a higher risk for multiple sclerosis among affluent populations (1).

The modern increase in caloric intake and dietary fat in the "Western diet" (WD) has also been correlated with both metabolic and immune-mediated diseases (7). Fatty acids have been shown to promote inflammatory responses through multiple mechanisms that include direct action on immune cells, conversion into inflammatory lipid mediators, and alteration of cell membrane characteristics (8). Dietary fats can increase colonic permeability to gut microbial products such as LPS (9), triggering colonic and systemic inflammation that may proceed to immune dysregulation. An additional potential link between dietary fats and the hygiene hypothesis is the ability of free fatty acids to stimulate TLR4, mimicking the saturated fatty acids that compose the bioactive lipid A moiety of LPS (10–12). Multiple studies have correlated fatty acid intake with changes in both LPS response and the rates of "Western" diseases (13, 14) based primarily on epidemiological data or on short-term responses to high-fat meals. Because the rates of immune-mediated diseases have dramatically increased in the population born since the late 1980s (15, 16), whose parents were among the first with robustly excessive dietary saturated fat calories (15), we hypothesized that alteration in dietary fat exposure during gestation and early perinatal development could impact the immune response later in life.

## Materials and Methods

### *Dietary exposure*

We placed breeding mice on customized specialty diets with fatty acid content derived from natural oils (Table I). The diets were derived from

*Bacterial Pathogenesis Unit, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and †Epithelial Biology Section, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892

[1]N.M.F. and B.M.J. contributed equally to this work.

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I.A.M. designed, conducted, and analyzed the experiments, and wrote the manuscript; N.M.F. conducted or assisted on all experiments; B.M.J. performed all experiments involving colonic tissue and contributed to writing the manuscript; P.J.V. assisted with chromatin immunoprecipitation; J.A.S. conducted microbiome sequencing and analysis; and S.K.D. oversaw design and analysis of the experiments, wrote the manuscript, and had primary responsibility for the final content. All authors critically read the manuscript.

The sequences presented in this article have been submitted to National Center for Biotechnology Information Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?study=SRP026657) under accession number SRP026657.

Address correspondence and reprint requests to Dr. Sandip K. Datta, Bacterial Pathogenesis Unit, Laboratory of Clinical Infectious Disease, National Institute of Allergy and Infectious Disease, National Institutes of Health, 9000 Rockville Pike, Building 33, Room 2W10A, Bethesda, MD 20892. E-mail address: dattas@niaid.nih.gov

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Abbreviations used in this article: EAE, experimental autoimmune encephalitis; LBP, LPS binding protein; LF, low fat; MRSA, methicillin-resistant *Staphylococcus aureus*; NIAID, National Institute of Allergy and Infectious Diseases; Treg, regulatory T cell; WD, Western diet.



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a master mix of proteins and micronutrients before the carbohydrates and dietary fats were added to ensure differences between diets were primarily in fatty acid content and the fat/carbohydrate ratio. All diet pellets were purchased from Research Diets (New Brunswick, NJ) with independent mass spectrometry content verification (Covance, Princeton, NJ). Mass spectrometry of samples from two areas of each chow bag upon arrival and 6 mo after storage at −80°C confirmed the reported fatty acid content. All samples were within 5% of the reported content and had the expected ratios of fatty acids. There was <7% breakdown of the fatty acids during storage, with maintenance of the fatty acid ratios.

The breeders used in the study were all littermates and were placed on the special diets 1 d before being placed in the breeding cages. Their pups were thus exposed to these diets in utero until birth and for an additional 3 wk via breast milk. At 3 wk of age, the pups were weaned to new cages and all pups were placed on our low-fat (LF) control diet. After 2–3 wk on the LF diet, the mice were placed into the challenge models described later and were maintained on the LF diet for the duration of each experiment. Therefore, at time of challenge, the only difference between the mice tested was the dietary fat and carbohydrate consumed by their parents during gestation and nursing. For investigation of the effects of actively being on the WD, we placed mice on WD chow for 2 wk after weaning from breeders on a standard diet.

### *Mice*

BALB/c and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) to set up breeders. Littermates were used as the breeders that were exposed to the experimental diets. Two to three breeder pairs per dietary group were maintained active at all times for approximately 5 mo each. The breeders were renewed as a unit on two separate occasions during the study; all breeder cages were stopped and replaced with new breeding pairs. For cohousing experiments, mice from at least two breeders were used so that a cage with two pups from a given diet group would come from two different breeding pairs. Mice were given autoclaved, acidified water (pH 2.7–3.1). The bedding provided was Maple Sani Chip (Harlan Laboratories, Indianapolis, IN). All animal experiments were done in compliance with the guidelines of the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Animal Care and Use Committee in specific pathogen-free National Institutes of Health animal care facilities that were documented to be free of Norovirus and *Helicobacter*.

### *Pulmonary fat content*

Lungs from mice were harvested 1 wk after weaning and stored in PBS at −80°C. Mass spectrometry was performed at Covance (Madison, WI).

### Escherichia coli *sepsis*

Mice were infected i.p. with $10^4$ CFUs *E. coli* K1018 (gift from M. Lu, NIAID) and followed for 2 wk for evidence of moribundity.

### Staphylococcus aureus *infections*

A total of $10^7$ CFU USA300 strain of methicillin-resistant *Staphylococcus aureus* (MRSA; gift from F. DeLeo, Rocky Mountain Laboratories, NIAID) with Cytodex beads (Sigma, St. Louis, MO) was injected intradermally (100 μl) into the shaved back of each mouse. Resultant abscess size, bacterial burden, and skin cytokine analysis were done as previously described (17). TaqMan probes for TLR2 (Mm00439614_m1*), IL-17A (Mm00439619_m1*), DefB4 (Mm00731768_m1*), IL-1β (Mm01336189_m1*), IL-10 (Mm00442346_m1*), vitamin D receptor (Mm00437297_m1*), and CYP27b1 (Mm01165918_g1) were purchased from Life Technologies. Comparison of signal was performed using the ΔΔ–cycle threshold method.

### *Experimental autoimmune encephalitis*

Mice were injected with 200 μg MOG protein (AnaSpec, Fremont, CA) with 300 μg CFA (Difco, Franklin Lakes, NJ) s.c. on day 0. They were also injected with 500 ng pertussis toxin (List Biological Laboratories, Campbell, CA) i.p. on days 0 and 2. They were monitored and scored daily based on the following scale: 1, limp tail; 2, paralysis of one hind leg; 3, paralysis of both legs; 4, paralysis or clumsiness in either front leg; and 5, death. They were scored by animal care facility technicians who were blinded and independent of our study.

### *Anaphylaxis*

For 4 wk, mice were sensitized by weekly gavage with 1 mg peanut protein (Protein Plus, Fitzgerald, GA) and 20 μg cholera toxin (List Biological Laboratories, Campbell, CA) in 200 mcl PBS (Cellgro, Manassas VA). Thirty minutes before each sensitization, mice were gavaged with 150 mcl bicarbonate (Mallinckrodt, Phillipsburg, NJ). On the last day of sensitization, peripheral temperature transponders were injected s.c. (Bio Medic Data Systems, Seaford, DE). One day later, blood was taken to measure peanut-specific IgG and IgE. Then mice were challenged with 5 mg peanut protein i.p. Temperature was measured every 5–15 min for 60–90 min. Anaphylaxis scores were taken at time of temperature measurement and based on the following scale: 1, face scratching or swelling around the eyes; 2, heavy breathing or raised hair; 3, no spontaneous movement, other than breathing; 4, no movement when prodded or seizures; and 5, death. A similar protocol was followed to test anaphylactic response to OVA instead of peanut, using 1 mg for sensitization and 5 mg for challenge.

### *Colon stimulation*

Whole colons were excised, washed, weighed, and placed in 1 ml DMEM (Cellgro) with 10% FBS (Thermo, Dubuque, IA) for 24–72 h in the presence of 100 ng/ml LPS (List Biological Laboratories, Campbell, CA). Cytokine levels of the supernatants were measured with the Bio-Plex suspension array system on a Bio-Plex 200 (Bio-Rad, Hercules, CA).

### *Splenocyte stimulation*

Single-cell suspensions of live splenocytes (2e6/ml) were stimulated with 100 ng/ml LPS (List Biological Laboratories) or 1 μg/ml Con A (Sigma) at 37°C under 5% $CO_2$ in MEM (Mediatech, Manassas, VA) supplemented with 10% newborn calf serum (HyClone, from Thermo Scientific, Dubuque, IA), and 100 U/ml penicillin and 100 g/ml streptomycin (MEM/newborn calf serum) for 24–72 h. Cytokines were measured with the Bio-Plex suspension array system on a Bio-Plex 200 (Bio-Rad).

### *Regulatory T cell detection*

Colons were removed and flushed with cold 10% FBS/HBSS solution to clear fecal material. Next, colons were opened via a longitudinal incision, then cut laterally into three pieces. Samples were then incubated in a 10% FBS/HBSS solution supplemented with 0.05M EDTA for 15 min at 37°C with 125 rpm shaking. After incubation, the epithelial layer was gently scraped off and colons were minced, exposing the lamina propria. Processed samples were then incubated in a 20% FBS/HBSS solution supplemented with 2% collagenase, 2% Dispase, and 0.1 mg DNAse for 1 h at 37°C with 185 rpm shaking. Digested tissue and solution were then passed through 100- and 40-μm filters to obtain single-cell suspensions. After thorough washing with cold 1× PBS, colonic lamina propria cells were labeled with LIVE/DEAD fixable violet dead stain (Invitrogen, Grand Island, NY), anti-mouse CD45 (FITC), anti-mouse CD25 (allophycocyanin or PE-CY7), anti-mouse CD4 (PE-CY7 or allophycocyanin), and anti-mouse Foxp3 (PE), using Foxp3 staining buffer reagents (eBioscience, San Diego, CA). Flow cytometric data were acquired on an LSRFortessa (BD Bioscience, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR). Splenic regulatory T cells (Tregs) were detected in a similar manner after obtaining single-cell suspensions (2e6/ml).

### *Macrophage recruitment and processing*

Mice were injected with 1 ml sterile Brewer thioglycolate broth (Difco Laboratories, Detroit, MI), and peritoneal cells were harvested 5 d later as previously described (18). Cells from 3–5 mice were pooled. RNA was extracted using the RNeasy Kit (Qiagen) per the manufacturer's instructions and evaluated by RT-PCR identically to skin mRNA processing. TaqMan probes for TLR4 (Mm00445273_m1*) and LPS binding protein (LBP; Mm00493139_m1*) were from Life Technologies.

### *Liver LPS content*

Livers were harvested from mice and homogenized using TissueLyser (Qiagen, San Diego, CA) in a 2-ml tube (Eppendorf, Hauppauge, NY) with a steel ball bearing (Qiagen). The homogenized liquid was assayed for LPS content using a commercial kit (Hycult, Plymouth Meeting, PA).

### *Chromatin immunoprecipitation*

Immunoprecipitation of sheared chromatin from splenocytes was done according to the Millipore Magna-Chromatin Immunoprecipitation protocol as previously described (19), using Protein G beads (Millipore, Temecula, CA) and anti-H3K9Me3 or isotype control IgG (Millipore). QIAquick PCR purification kit (QIAGEN) was then used to purify DNA. Quantitative PCR was performed using SYBR green–labeled primers (Applied Biosystems, Foster City, CA). Primers (forward; reverse) were as follows: Gapdh (5′-GTCATCATCTCCGCCCCTTCTGC-3′; 5′-GATGCCTGCTTCACCACCTTCTTG-3′), LBP (5′-GGACAGCAACTCCCTAACTTACCC-3′; 5′-GCAGAAATGGAACCCAGGCTAC-3′), CD14 (5′-CAGAGAACACCACCGCTGTA-

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AAG-3′; 5′-AGATTCCTCAGGTTGGCTCCAG-3′), and TLR4 (5′-TTCAGGGCTTTCTGTGGGAAC-3′; 5′-AGATTTTCATCAGGCTTGGCAG-3′).

### *Microbiome analysis*

DNA was extracted from sterilely excised cecal stool pellets using QIAamp DNA stool mini kit (Qiagen). Female BALB/c mice were used. Quantitative analysis of 16S rDNA was performed as previously described on 1000–3000 sequences/sample using the established primer sequences (20). All microbiome sequencing data were uploaded to the National Center for Biotechnology Information Sequence Read Archive database under accession number SRP026657; this can be accessed at http://www.ncbi.nlm.gov/Traces/sra/sra.cgi?study=SRP026657.

### *Ab measurements*

Serum was drawn from orally sensitized mice 1 d before anaphylaxis challenge. Total IgE was detected by ELISA using commercial kits per manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). For peanut-specific Abs, serum was incubated in immunoplates (Thermo Scientific, Dubuque, IA) coated with whole peanut protein (Protein Plus, Fitzgerald, GA). After 1 h nonspecific protein blockade with 10% FBS (Thermo Scientific), the plate was washed with PBS and 0.05% Tween (Acros, Pittsburgh, PA). ELISA assay for IgG was performed using commercial kits (Bethyl Laboratories). For peanut-specific IgE, serum was added to ELISA plates coated with anti-IgE (Bethyl Laboratories). Whole peanut protein, biotinylated with a commercial kit (Anaspec, Fremont, CA), was added. Colorimetric detection using streptavidin-HRP and TMB reagents (Sigma) was done on a Beckman Coulter DTX880.

### *Vitamin D assay*

Vitamin D3 levels were evaluated by ELISA per the manufacturer's instructions (ALPCO, Windam, NH).

### *Weight and glucose monitoring*

Pups from each litter were weighed weekly after weaning. For fasting blood glucose measurements, food was removed at 3 pm. At 9 am the following morning, a small incision was made in the tail vein and glucose was measured on the second drop of blood using the Freestyle Lite (Abott Diabetes Care, Alameda, CA).

### *Statistics*

Means were compared using either two-tailed unpaired *t* test or ANOVA with Bonferroni's posttest correction for multiple-group comparison with Prism software (GraphPad, San Diego, CA). The *p* values are designated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, or $****p < 0.0001$. NS, Not significant.

### *Declaration of approval for animal studies*

All animal experiments were done in compliance with the guidelines of the NIAID Institutional Animal Care and Use Committee.

## Results

### *Dietary exposure*

The modern WD departs from the recommended diet in 3 major ways: high percentage of calories from fat (40% rather than 30%) (21), increased ratio of ω6/ω3 fatty acids (8–15:1 rather than 2:1) (21), and twice the recommended simple carbohydrates (22). To isolate the effects of dietary fatty acids, we formulated experimental diets that did not contain excess sugars. Our WD formulation reflected the average American diet in both fat percentage and source (saturated fat predominantly from palm oil and ω6 from soy) (22) but did not provide excess simple carbohydrates. The LF control diet was essentially identical to standard mouse chow. All diets had equivalent micronutrient composition and caloric density, with the differences in fatty acid content being accounted for by changes in carbohydrate content (Table I). Breeders were placed on the diets 1 d before placement in breeding cages and remained on the diets throughout the study. Pups were exposed to these diets in utero and during nursing. All pups from all breeders were weaned to new cages and placed on the LF control diet for 2–3 wk before immunologic challenge. Thus, the primary difference in dietary exposure between the mice tested was the fatty acid composition and the fat/carbohydrate ratio consumed by their parents during gestation and lactation. Fig. 1 provides an overview of the study design. Lung fatty acid composition has been shown to reflect dietary fat intake (23), and analysis of lung tissue by mass spectrometry confirmed differential saturated fat exposure in the pups from WD and LF breeders (Supplemental Fig. 1A, 1B). Although the WD and LF chow had different ω3 and ω6 fatty acid composition, there was no difference in the ω3 or ω6 content in the lung (Supplemental Fig. 1B). This may be because the smaller differences, compared with saturated fat exposure, were below the assay detection capability or because migration of unsaturated fatty acids across the placenta may be regulated differently than saturated fats. Importantly, there were no differences between WD and LF pups in fasting blood glucose or weight (Supplemental Fig. 1C–E), indicating any observed differences between these mice would not be confounded by diabetes or obesity. Of note, both groups ate identical LF chow postweaning, and the lack of weight differences correlated with our finding that the amount of food consumed per mouse per week did not differ between the LF or WD pups (data not shown).

Table I. Fatty acid content and source for the diets studied

| Dietary Components | Human RD | ω6 | Western | LF |
|---|---|---|---|---|
| Protein (% kCal) | 20 | 20 | 20 | 20 |
| Carb (% kCal) | 50 | 40 | 40 | 70 |
| Fat (% kCal) | <30 | 40 | 40 | 10 |
| % Fat saturated | 10 | 10 | 40 | 10 |
| % Fat PUFA | | 77 | 21 | 72 |
| % Fat MUFA | | 14 | 39 | 18 |
| Saturated fat source | | — | Palm oil | — |
| ω6 source | | Safflower | Soy | Soy/Safflower |
| ω3 source | | – | Menhaden | Flaxseed |
| ω6/ω3 ratio | 2:1 | 63:1 | 8:1 | 2:1 |

Breakdown of dietary components in the diets studied are shown, including protein, carbohydrates (carb), fat, and % of fat that was saturated, polyunsaturated fatty acids (PUFA), or monounsaturated fatty acids (MUFA). The dietary source for each fatty acid is shown. All diets were made from natural oils. Human recommended diet (RD) reflects the guidelines of the U.S. Department of Agriculture.

### *Pups from WD breeders had altered disease susceptibility*

Because direct fatty acid exposure has been reported to affect LPS responses (9–12), and immune responses to Gram-negative bacteria directly involve LPS-triggered innate immunity, we first evaluated the impact of parental fatty acid intake on pup susceptibility to a model of Gram-negative bacterial sepsis. Pups from WD breeders injected i.p. with the clinical isolate *E. coli* K1018 had significantly greater mortality than pups from LF breeders (Fig. 2A). To test whether the effects of WD exposure extended to infectious agents that do not contain LPS and thus do not bind to TLR4, we next used a skin infection model with the Gram-positive bacterium MRSA. Suggesting that WD exposure resulted in immune modulation beyond direct effects on TLR4, WD pups developed larger abscesses with greater bacterial burdens compared with LF pups (Fig. 2B, 2C). Saturated fats have also been reported to influence signaling by TLR2 (10), the receptor for microbial lipoteichoic acid and peptidoglycan, both of which are produced by *S. aureus*, induce an inflammatory cascade, and have been implicated in the hygiene hypothesis (4). In the abscess tissue of WD pups, we found a significant reduction in transcript levels of central mediators of the cutaneous anti-MRSA response: TLR2, IL-1β, IL-17A, and β-defensin 4 (24), as well as the regulatory cytokine IL-10 (Fig. 2D). WD pups also showed reduced expression of the vitamin D receptor and the vitamin D activating enzyme, CYP27b1 (Fig. 2D), both of which are induced by TLR2

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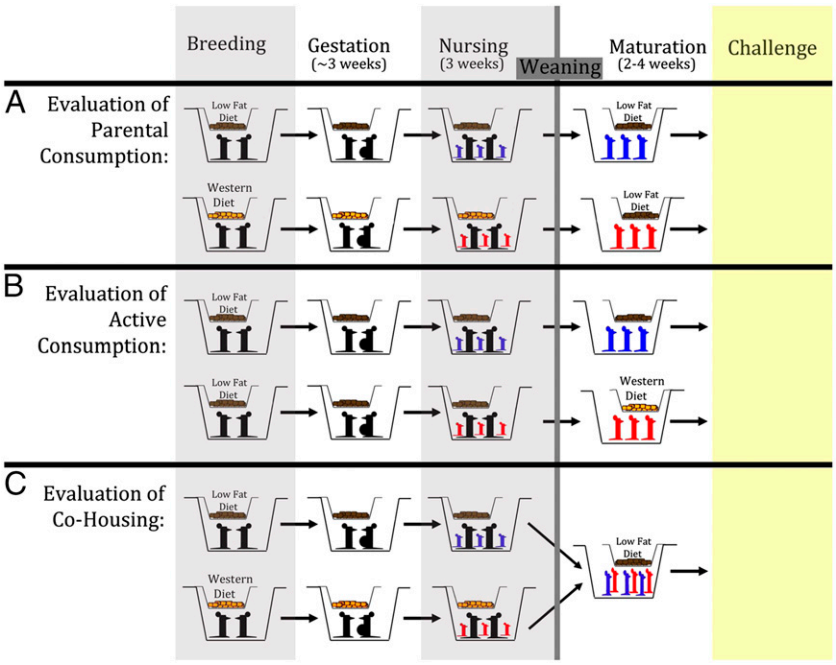


**FIGURE 1.** Diagrammatic presentation of study design. (**A**) For experiments evaluating the effects of parental diet, littermate mice were placed on either LF or WD formulations 1 d before being placed in breeding cages. Breeder mice were maintained on the different diets throughout gestation and nursing. When the pups were 3 wk postpartum, they were weaned to new cages. All pups were weaned onto the LF control diet. Two to 4 wk after weaning, the mice were evaluated in the described models. (**B**) For evaluation of the effects of active diet consumption, the converse experiment was performed. Pups from breeders on the LF control diet were weaned into new cages and placed on either the WD or LF control diet. (**C**) For experiments involving cohousing of mice, pups from both LF and WD breeders were weaned into the same cage and both placed on the LF control diet.

stimulation and mediate antistaphylococcal immune activity (25). There were no differences in serum vitamin D levels (data not shown), suggesting the observed effects on vitamin D metabolism were not systemic but occurred within the context of the anti-MRSA immune response.

LPS responsiveness has also been implicated in autoimmune and allergic disease (26, 27). To test the effect of fat exposure on these disease states, we examined these pups in established models of experimental autoimmune encephalitis (EAE) and oral peanut sensitization. In BALB/c mice, which are relatively resistant to EAE (28), WD pups were more likely to develop signs of EAE (Fig. 2E), but the severity of disease was similar to the LF pups (data not shown). In the more susceptible C57BL/6 mouse strain (28), WD pups showed both a higher incidence and more severe manifestations of EAE compared with LF pups (Fig. 2F). WD pups sensitized orally to peanut protein had more pronounced temperature declines in response to challenge, the most sensitive measure of murine anaphylaxis (29) (Fig. 2G). Clinical anaphylaxis scores mirrored the temperature findings, but differences did not reach statistical significance (Fig. 2H). There was no difference between groups in the induced levels of total IgE, peanut-specific IgE, or peanut-specific IgG (Supplemental Fig. 2A–C). Mice were sensitized to OVA in an identical manner to test the effect of dietary fat on anaphylactic response against another Ag. There were no differences between WD and LF groups in temperature change during OVA challenge (Supplemental Fig. 2D). Peanut sensitization of the less susceptible C57BL/6 mice (30) also revealed that exposure to WD had no effect on anaphylaxis compared with LF controls (Supplemental Fig. 2D). Given the lack of effect in the OVA model and the requirement for genetic predisposition, it seems that the WD had a stronger effect on sepsis and autoimmunity than on allergic sensitization. Taken together, these data suggest that WD fat exposure during gestation and early life increased susceptibility to a range of infectious and immune-mediated diseases.

### *WD pups had hyperinflammatory colonic responses but decreased systemic responses to LPS*

To explore the immunological basis for the effect of WD exposure on disease susceptibility, we first compared colonic immune responses in WD and LF pups. Other studies have shown that a high-fat diet can produce a low-grade inflammatory response in the colon (9). We postulated that this might drive altered systemic immunity because the gut is a major site for immunological education. In response to ex vivo LPS stimulation, WD pup colons produced enhanced levels of IL-6, IL-1β, and IL-17A (Fig. 3A–C), suggesting a hyperinflammatory milieu. WD pups had reduced frequency of colonic Tregs (Fig. 3D, 3E), further indicating dysregulated gut immunity. In contrast, splenic LPS responses in WD pups suggested a muted systemic LPS response, with reduced production of TNF-α and IL-6, but no differences in IL-17A (Fig. 3F–H) or IL-1β (data not shown). Similar to the colonic Treg findings, WD pups had reduced frequency of splenic Tregs (Fig. 3I, 3J). WD and LF pups did not significantly differ in splenocyte production of IL-4, IL-5, IL-13, IL-17A, or IFN-γ in response to Con A (data not shown), suggesting no baseline skewing of effector T cell polarization in WD pups. The colonic inflammatory response to a high-fat diet has been shown to increase LPS leakage from the colon into the portal circulation (9). Consistent with this finding, we found the LPS content in liver tissue was higher in WD pups (Fig. 3K). Macrophage TLR4 and LBP mRNA expression was suppressed in WD offspring (Fig. 3L), suggesting a downregulated capacity for LPS signaling after increased LPS exposure.

These effects of the WD could stem from the increased saturated fats, the skewed ω6/ω3 ratio, or both. To isolate the contribution of high dietary ω6, we evaluated offspring from breeders fed a high-fat diet with an overrepresented ω6/ω3 ratio but low saturated fat content (Fig. 1A, Table I). Compared with pups from LF breeders, pups from the ω6 diet breeders had mild increases in only a subset of colonic inflammatory markers (Supplemental Fig. 3A–C). They showed trends toward enhanced susceptibility to infection and EAE (Supplemental Fig. 3D–G), but these did not achieve the statistically significant differences seen in the WD pups. In further contrast with WD pups, ω6 pups were protected against allergic sensitization (Supplemental Fig. 3H–L). Taken together, these data suggest that the high saturated fat content of the WD was required to induce colonic inflammation, resulting in increased systemic LPS exposure and reduced LPS responsiveness that may have contributed to immune dysregulation and disease susceptibility in WD pups.

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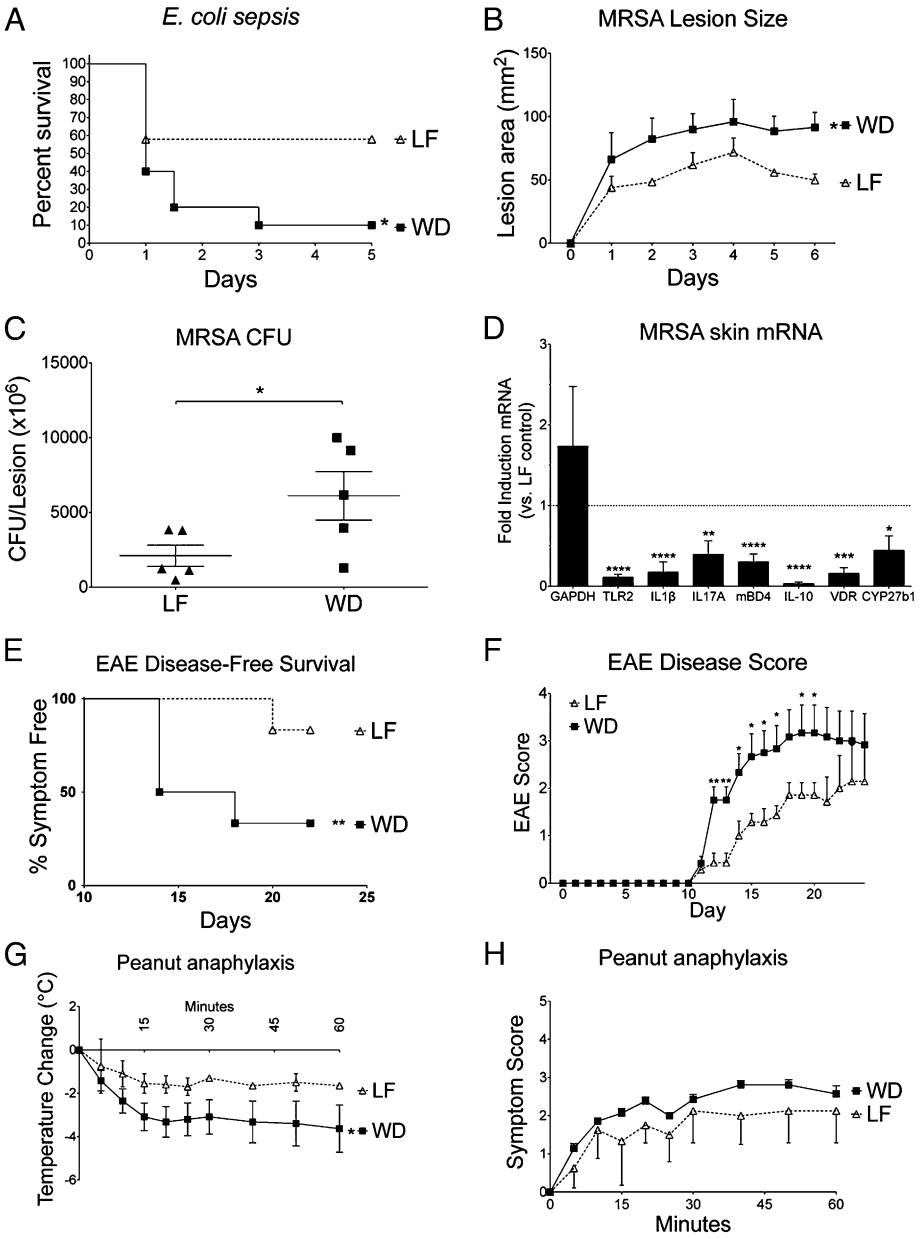


**FIGURE 2.** Pups from WD breeders had altered disease susceptibility. (**A**) Survival postinfection with *E. coli* K1018 in BALB/c mice ($n$ = 10–19). (**B–D**) *Staphylococcus aureus* (MRSA USA300) skin infection in male BALB/c mice. Lesion sizes (B), day 6 bacterial counts from homogenized skin (C), and mRNA expression in skin abscess tissue normalized against LF controls (D, dotted line) ($n$ = 5–6). (**E**) Disease-free survival after induction of EAE in female BALB/c mice ($n$ = 6). (**F**) EAE scores in C57BL/6 mice ($n$ = 7–12). (**G** and **H**) Weaned male BALB/c pups were gavaged with peanut protein and cholera toxin weekly for 4–8 wk before challenge. Temperature decline (G) and symptom scores (H) after challenge ($n$ = 5). Results are representative of three or more (B, E–H) or combined from two to four (A, C, D) independent experiments and displayed as mean + SEM. Significance determined by $t$ test (A–C, E–H) or ANOVA with Bonferroni's correction (D). All experiments were repeated with similar results in both sexes. Sex is indicated when representative experiments are shown; otherwise, data reflect both male and female mice with matched ratios within experiments. Each symbol designates one mouse unless otherwise specified. $n$ designates mouse number per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

*Active consumption of WD did not fully recapitulate the phenotype of mice exposed during early development*

The immune phenotype of WD pups conceivably represented a direct and residual effect of saturated fat consumption during the 3 wk of gut exposure through breast milk. In addition, newborn mice may sample the food eaten by their parents, indicating a window wherein direct consumption could be the cause of our observed phenotypes. To test whether such direct exposure could account for the observed immune modulation, we performed the converse of the previous experiments, placing female mice on WD chow after weaning from breeders fed a standard diet (Fig. 1B). Consistent with previous reports (31), active WD consumption decreased the survival rate from sepsis (Fig. 4A). However, active consumption did not affect MRSA-induced skin lesion size (Fig. 4B) or transcript levels of anti-MRSA cytokines (Fig. 4C). Active intake of the WD also did not impact susceptibility to EAE (Fig. 4D) or peanut anaphylaxis (Fig. 4E, 4F). Active ingestion increased colonic IL-6 production in response to LPS (Fig. 4G), but did not affect other tested cytokines or colonic Treg frequency (Fig. 4H–J). Similar to mice exposed only early in development, active WD consumption increased liver LPS concentrations and reduced splenocyte IL-6 and TNF-α responses (Fig. 4L–N). However, splenic Treg frequency was not altered (Fig. 4O). Thus, exposure to the WD after weaning appeared to partially alter responses directly related to inflammatory effects on the colon but could not fully recapitulate the immune dysregulation seen in mice exposed during prenatal and perinatal development. It is possible that prolonged adult exposure to the WD would further modulate immune responses, but these results suggest that the observed phenotype of WD pups required either prenatal or perinatal parental WD exposure and could not be explained solely by the direct postnatal WD exposure during nursing.

*Inheritance of the WD immune phenotype was dependent on altered gut microbiota*

Beyond direct exposure, potential explanations for the altered immune phenotype in WD pups include paternal germline epigenetic changes and/or an altered maternal microbiome, both of which could be transmitted and influence pup immune responses. Previous studies have linked high-fat diets to epigenetic alterations

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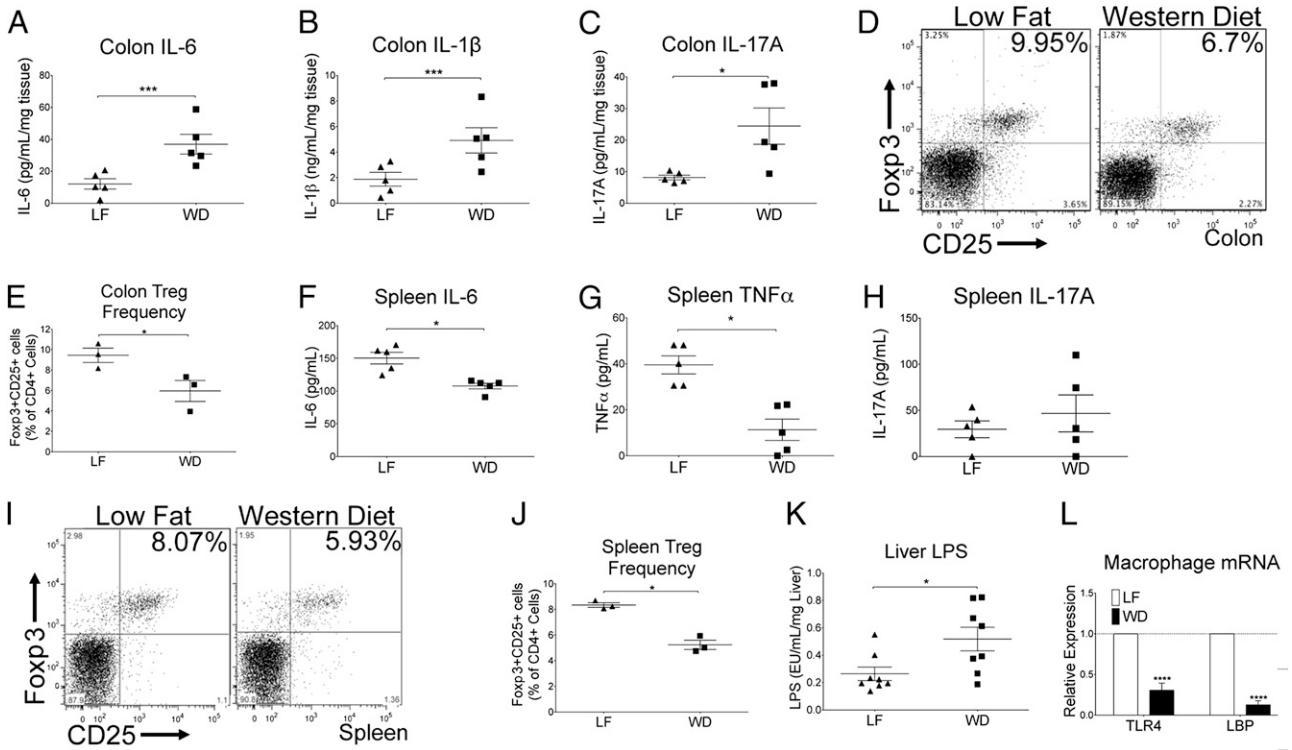


**FIGURE 3.** Pups from WD breeders had increased colonic and decreased systemic LPS responses. (**A**–**C**) Cytokine production from female excised colons stimulated with LPS for 24–72 h. (**D** and **E**) Representative plots of Foxp3 and CD25 expression of pooled female colonic CD45$^+$, CD4$^+$ cells analyzed by flow cytometry (D), and values from three replicate experiments (E) ($n$ = 3–5). (**F**–**H**) Cytokine production from male splenocytes stimulated with LPS for 24–72 h. (**I** and **J**) Representative plots of Foxp3 and CD25 expression of pooled female splenic CD4$^+$ cells analyzed by flow cytometry (I) and values from three replicate experiments (J) ($n$ = 3–5). (**K**) Liver LPS content in homogenized livers. (**L**) mRNA expression for TLR4 and LBP from thioglycolate-elicited macrophages ($n$ = 3–6). Results are representative of three or more (A–D, F–I) or combined from (E, J–L) two to three independent experiments in BALB/c mice and displayed as mean + SEM. Significance determined by $t$ test. All experiments were repeated with similar results in both sexes. Sex is indicated when representative experiments are shown; otherwise, data reflect both male and female mice with matched ratios within experiments. Each symbol designates one mouse unless otherwise specified. $n$ designates number of mice per experiment. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

of inhibitory histone markers such as H3K9Me3 (32, 33). Focusing on genes related to LPS response, we found WD male breeders had significantly greater H3K9Me3 histone modifications associated with the TLR4 and LBP loci compared with their LF counterparts (Fig. 5A). The pups of the WD breeders had the identical epigenetic modifications at the LBP locus (Fig. 5B), indicating potential germline inheritance of the altered LPS response. However, despite a difference in TLR4 transcript expression (Fig. 3K), we did not find any significant differences in H3KMe3 histone modification at the TLR4 or CD14 loci in WD pups (Fig. 5B), suggesting other mechanisms of regulation.

The gut microbiota has been recognized as a key mediator of immunologic development and control of colonic inflammation, and is an inheritable characteristic passed from mother to child at time of birth (7). Compared with LF controls, 16S rRNA gene analysis of stool from WD pups showed an increased ratio of Firmicutes to Bacteroidetes (LF, 2.2:1; WD, 4.3:1; Fig. 5C, Supplemental Table I). Across all sequences, other bacterial phyla were not represented at >0.2% of the population. WD pups had significantly greater representation of the genera *Lachnospiraceae* and *Clostridiales* compared with the LF pups (Supplemental Table I). Overall diversity, as measured by the Shannon index, was significantly lower in the WD pups compared with the LF pups (Supplemental Table I). The ω6 diet generated different, but equally pronounced, alterations on the gut microbiome (Supplemental Fig. 3P) without imparting the same pathologic immune manifestations (Supplemental Fig. 3A–O). We cohoused WD and LF pups at time of weaning to equilibrate their microbiomes, which occurs within 2–4 wk of cohousing (34, 35). After 4 wk, the cohoused pups had no significant differences in gut microbial content or diversity (Fig. 5C, Supplemental Table I). With Yue and Clayton as well as Jaccard analyses, the cohoused mice segregated together in a group distinct from both noncohoused LF and WD littermates (data not shown). Unexpectedly, there was an emergence of *Bacteroides* and a mixture of minor genera including Akkermansia from the phyla Verrucomicrobia ("Other" in Supplemental Table I) in the cohoused mice compared with the noncohoused groups (Fig. 5C, Supplemental Table I); an intriguing finding likely because of cage variations but unlikely to explain the observed differences in phenotype between WD and LF pups given the lack of difference in frequency of these organisms in either of the non-cohoused groups. Cohousing abrogated the differences in colonic cytokine production (Fig. 5D–F), liver LPS content (Fig. 5G), colonic Tregs (Fig. 5H), splenic cytokines (Fig. 5I–K), splenic Tregs (Fig. 5L), and expression of TLR4 and LBP (Fig. 5M). To evaluate whether the normalization of immune responses after cohousing translated to normalization of disease susceptibility, we exposed cohoused WD and LF pups to *E. coli* sepsis, the disease model that appeared to be most robustly and rapidly affected by WD exposure (Figs. 2, 4). Cohousing rescued the susceptibility of WD pups to this infection (Fig. 5N). These findings suggest that the immunologic differences between LF and WD pups were dependent on the altered microbiome.

## Discussion

We show that parental dietary fat intake during gestation and nursing can negatively alter the subsequent immune responses and disease

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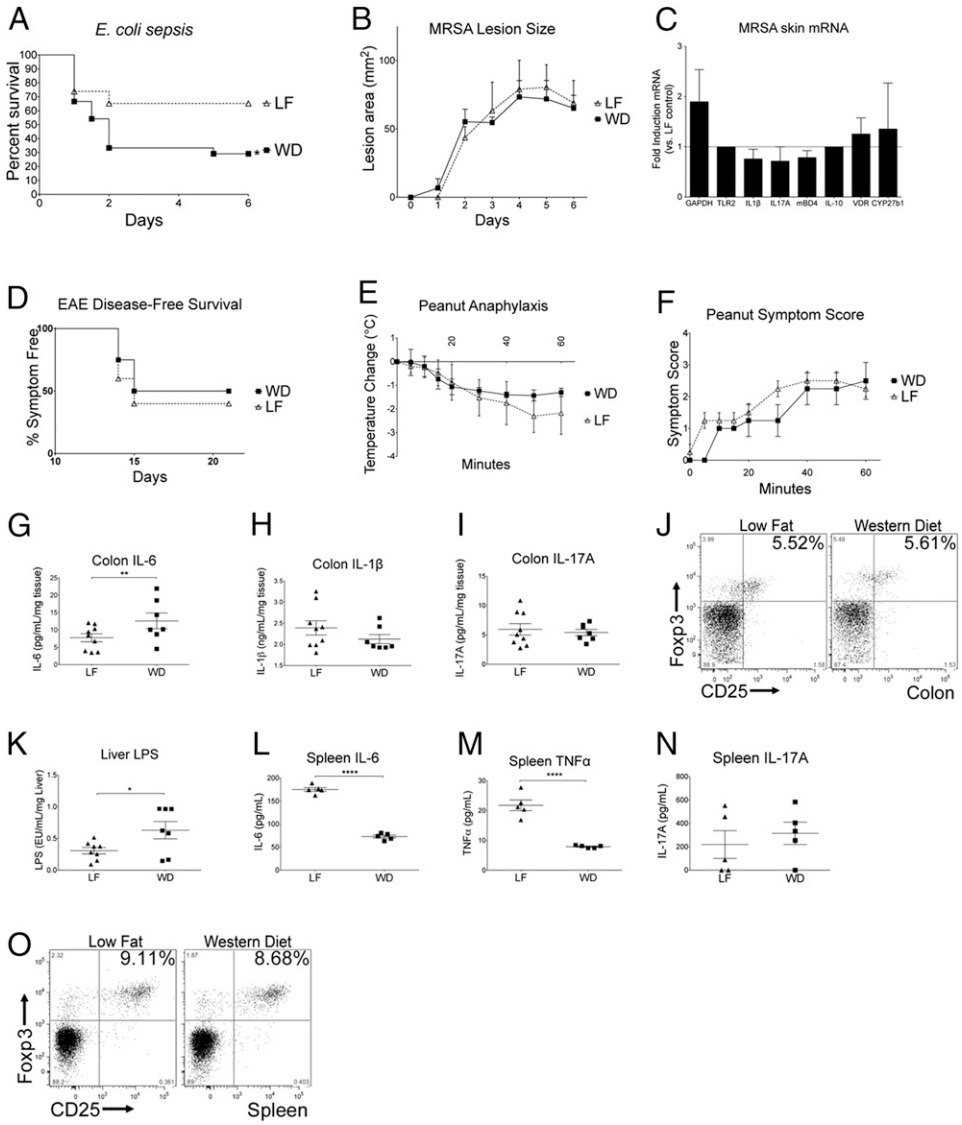


**FIGURE 4.** Postweaning exposure to WD did not recapitulate the phenotype of mice exposed during development. Pups from breeders on a standard diet were placed at 3 wk of age on LF or WD for 2 wk before challenge. (**A**) Survival after injection with *E. coli* K1018. (**B**) Lesion size in male mice and (**C**) mRNA expression in skin abscess tissue normalized against LF controls (dotted line) after injection with MRSA. (**D**) Disease-free survival in female mice after induction of EAE. (**E** and **F**) Temperature change and symptom scores for orally sensitized male mice after challenge with peanut protein. (**G**–**I**) Colonic cytokine induction by LPS in female mice. (**J**) Representative plots of Foxp3 and CD25 expression of pooled female colonic $CD45^+$, $CD4^+$ cells analyzed by flow cytometry ($n$ = 5). (**K**) Liver LPS content. (**L**–**N**) Splenic cytokine induction by LPS in male mice. (**O**) Representative plots of Foxp3 and CD25 expression of female splenic $CD4^+$ cells analyzed by flow cytometry ($n$ = 5). Significance determined by $t$ test. Results are representative of 2–3 independent experiments, 5–10 sex- and age-matched BALB/c mice/group unless designated as individual symbol, and displayed as mean + SEM.*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

susceptibility of offspring mice. The inheritance of this immune phenotype is associated with an altered gut microbiota. Prior reports have described the direct effects of high-fat diets on the gut microbiome and inflammation, and some have additionally shown metabolic consequences for offspring (31–39). Our study has expanded on these previous observations to identify the effects of parental diet on offspring immunity. Seeding of microbiota occurs from the mother during parturition and further diversifies during early life (40). Fatty acid exposure causes rapid changes in the microbiotic composition (41), implying that diet-induced changes in the maternal microbiota were passed on to offspring in our studies. Studies on the durability of these inherited alterations in microbiota would provide additional information on disease susceptibility as would direct comparison of maternal versus offspring microbiota. Because of the coprophagic (stool-consuming) habits of mice, cohousing has been shown to be an effective means of transferring microbiota between mice, generating similar microbial shifts and immunological effects compared with direct fecal or microbial transfer (34, 42, 43). The results from our cohousing experiments thus implicate altered microbiota as the most likely driver of the observed immunological phenotypes. We also found limited inheritance of paternal epigenetic changes consistent with prior observations of epigenetic influences on metabolic, developmental, and cardiovascular dysregulations (44–46). However, microbiome alteration by cohousing superseded potential contributions of these epigenetic changes in our studies. Future identification and targeting of species-level changes in the microbiota promises the possibility of reversing or preventing harmful dietary effects through isolation and transfer of specific gut organisms.

High-fat diet effects have been characterized by increased Gram-negative bacteria and an increased Firmicutes/Bacteroidetes ratio in the gut microbiome (47), increased colonic inflammation and permeability (9), and decreased Treg frequency (48). We find that similar effects are inherited by progeny of mice fed a WD and are reversed by subsequent microbiota alteration, suggesting that the changes in microbiota are a primary effector of the diet-induced immune effects. Multiple mechanisms have been proposed to drive the influences of the microbiota on host immunity, including gut nutrient utilization, microbial metabolic products such as short-chain fatty acids, and differential triggering of gut immune responses (47). Similarly, dietary fats likely alter gut microbiota composition through multiple mechanisms, including altered microbial nutrient availability and host inflammatory effects. The reported ability of saturated fats to directly trigger inflammatory TLR4 signaling (9–12), and the increased levels of LPS in the circulation after dietary fat exposure in our study and others (9), raises the intriguing possibility that modern diets alter our exposure to TLR4 signaling, potentially resulting in a systemic hyporesponsiveness to LPS that paradoxically mimics the low LPS exposure postulated by the

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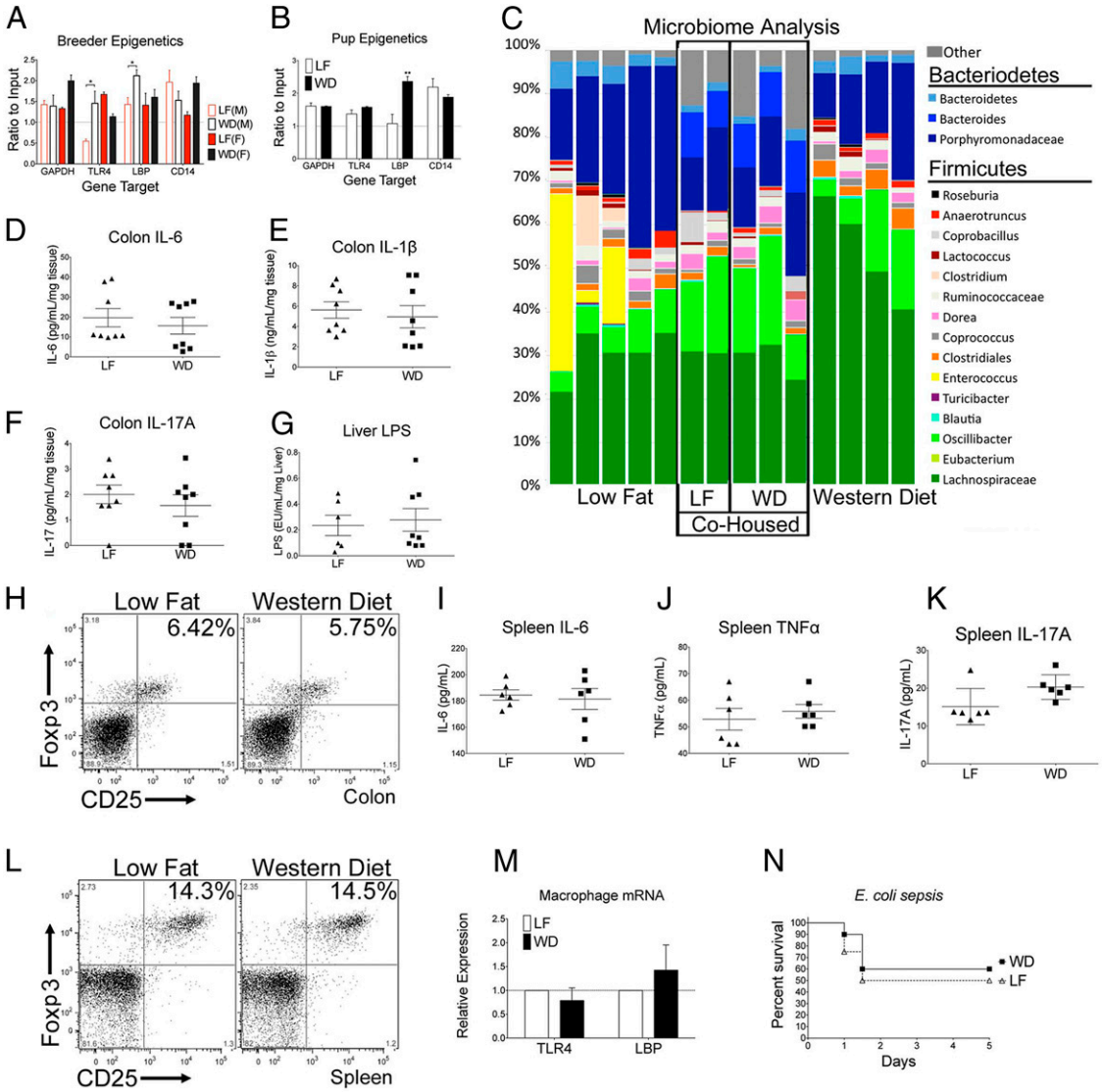


**FIGURE 5.** Cohabitation rescued WD pups from immune alterations. (**A** and **B**) Quantitative PCR of selected genes after anti-H3K9Me3 immunoprecipitation of splenic DNA from breeders and both male and female offspring, displayed as the ratio of input DNA and normalized to isotype Ab control. (**C**) 16S rRNA genes in cecal stool samples of female mice. Pups from indicated breeder diets were all weaned to LF diet. Female pups were placed in cages with their littermates or cohoused for 4 wk with pups from breeders fed opposing diet. Each bar represents one mouse; further breakdown of composition within each phylum can be found in Supplemental Table I. (**D**–**F**) Colonic cytokine induction by LPS in cohoused mice. (**G**) LPS content in homogenized liver tissue from cohoused mice. (**H**) Representative plots of Foxp3 and CD25 expression of pooled colonic $CD45^+$, $CD4^+$ cells from cohoused mice ($n$ = 3–5) analyzed by flow cytometry. (**I**–**K**) LPS induction of cytokines from splenocytes in cohoused mice. (**L**) Representative plots of Foxp3 and CD25 expression of splenic $CD4^+$ cells from cohoused mice analyzed by flow cytometry ($n$ = 3–5). (**M**) mRNA levels of TLR4 and LBP from thioglycolate-elicited macrophages in cohoused mice relative to LF control ($n$ = 3–4). (**N**) Survival postinfection with *E. coli* K1018 ($n$ = 10). Significance determined by $t$ test (D–N) or ANOVA with Bonferroni's correction (A, B). Results are representative of two to three independent experiments in BALB/c mice and displayed as mean + SEM. Each symbol designates one mouse. $n$ designates number of mice per experiment. $*p < 0.05$, $**p < 0.01$.

hygiene hypothesis to partially drive immune dysregulation. Although previous work has established TLR4-dependent effects of dietary fat on gut inflammation (49), the altered intrinsic susceptibility of TLR4-deficient mice to the tested models of infection, autoimmunity, and allergy may complicate future evaluation of the role of TLR4 in dietary fat-induced development of disease. Furthermore, the immune dysregulation we observed extended beyond direct effects on TLR4 signaling, as evidenced by alteration of autoimmunity, vitamin D regulation, and TLR2 expression. In addition, dysregulation of Treg cells, which are protective against sepsis (50), *S. aureus* skin disease (51), autoimmunity (52), and allergic sensitization (53), appeared to correlate with the pathology generated by the altered microbiome in our studies. Trending increases in the incidence of sepsis may be explained by the aging population and invasive medical procedures, but it is interesting to note that, similar to the other diseases, sepsis is characterized by immune dysregulation that may contribute to disease susceptibility (2). It appears that broad immune dysregulation induced by altered microbiota contributed to the range of disease susceptibility observed in our studies. Direct changes in LPS responsiveness by dietary fat likely dominated the susceptibility to sepsis seen in both adult and offspring mice, whereas indirect or developmental effects on Tregs and other immune compartments may be more important for allergy and autoimmunity models that manifested in the offspring. Although direct exposure postweaning did not mimic the effects of early life saturated fat exposure, our studies do not absolutely distinguish between effects of intrauterine and breastfeeding exposure in the offspring. Cross-fostering

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studies that place WD pups with adopted mothers immediately after birth may further discriminate between these windows for deleterious exposure, although the reported inability to introduce new microbiota into mice preweaning (54) may complicate such experiments. Regardless, the relevant translational implications and public health strategies to decrease early-life exposure would be similar for both scenarios.

Previous reports have documented intermittent prenatal LPS exposure as a negative risk factor for the development of allergic disease (55). This apparent contradiction with our results may be partially explained by the chronic nature of LPS signaling after dietary fat exposure, as well as additional inflammatory effects of dietary fat. In addition, we did not find pronounced effects of high ω6 intake on the tested disease models despite reports that dietary ω6 inhibits TLR4 activation (11) and has proinflammatory properties (56). A plausible explanation for our findings is that the ω6 diet generated changes in the microbiome that were different from the WD, perhaps because of differences in the nature of the inflammatory response triggered by these fatty acids and the interaction of these fatty acids with TLR4 (11). Our study did not delineate whether immune dysfunction in the WD pups was solely due to increased saturated fats or whether the addition of the skewed ω6/ω3 ratio was required. However, a skewed ratio in the absence of high saturated fat intake did not fully recapitulate the immunologic changes. Importantly, because of their lack of obesity or hyperglycemia, our WD pups provide a model to study the immune and microbiome effects of dietary fat exposure without confounding by the metabolic dysfunction seen in most other studies using a directly fed WD with excessive sugars.

Human gut microbiomes are more dynamic than mice raised in controlled, specific pathogen-free cages (36). Similar to mice, our microbiota can be influenced by dietary exposure (41). Human studies have associated altered microbiota with inflammatory bowel disease, enteric infections, liver inflammation, and gastrointestinal cancers (57). Moreover, gut bacteria alter the energy-absorbing potential of the mucosa (36), indicating influence over metabolism that could confer additional immune impacts. Modern increases in fat consumption have been accompanied by altered infectious exposures, reduced nutrient intake, and an ever-changing array of chemical and environmental exposures, all of which may have their own impacts on immunity. In fact, dietary intake of refined sugars enhances inflammatory microbiota (58), and a high-salt diet may enhance autoimmunity (59, 60). Considering the WD is enriched for sugar, salt, as well as fat, it may be the perfect recipe for driving multiple pathways of immunological dysfunction. Our results identify the potential impact of a proinflammatory diet on immune development and the possible contribution of inheritable microbiota to the modern patterns of health and disease.

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## Disclosures

The authors have no financial conflicts of interest.

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