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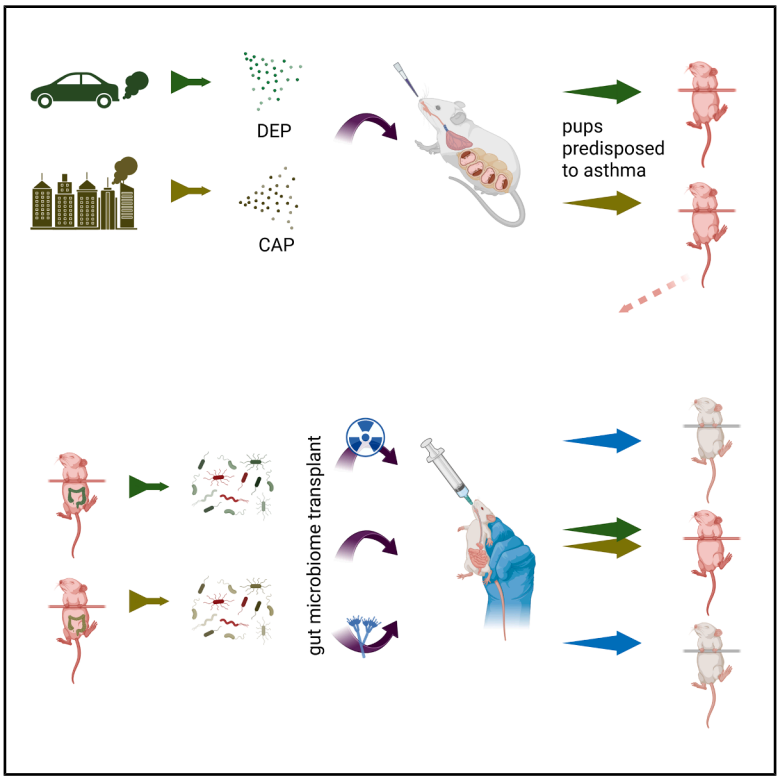
Live bacteria in gut microbiome dictate asthma onset triggered by environmental particles via modulation of DNA methylation in dendritic cells

Graphical abstract



Highlights

- The model uses intra-airway exposure of pregnant dams to environmental particles
- Such exposure predisposes neonates to asthma, mimicking human observations
- Gut microbiome transplantation confers asthma predisposition to naive recipients
- Gamma sterilization or antibacterials abrogate the transplant effect

Authors

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In brief

Ramar et al. demonstrate that gut microbiome transplantation confers asthma predisposition elicited by maternal intra-airway exposure to environmental particles. Radiation sterilization or antibacterials ablated the effect, suggesting that viable bacteria are required. Transplant recipients show DNA methylation alterations in dendritic cells, potentially explaining how microbiome-host crosstalk leads to asthma onset.

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Live bacteria in gut microbiome dictate asthma onset triggered by environmental particles via modulation of DNA methylation in dendritic cells

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SUMMARY

Despite broad knowledge of the pathogenesis, our understanding of the origin of allergy and asthma remains poor, preventing etiotropic treatments. The gut microbiome is seen to be altered in asthmatics; however, proof of causality of the microbiome alterations is lacking. We report on gut microbiome transplantation (GMT) from mice predisposed to asthma by maternal exposure to pro-allergy environmental particles into naive recipients. This GMT confers asthma predisposition, and the effect is abrogated by gamma sterilization of the transplant material or by co-administration of antibacterials, indicating that viable bacteria are mediating the effect. Metagenomics identifies key changes in the "pro-asthma" microbiome, and metabolomics links the identified species to altered production of butyrate known to act on immune cells and epigenetic mechanisms. We further show that transplant recipients develop DNA methylation alterations in dendritic cells. Finally, dendritic cells with an altered methylome present allergen to T cells, and this effect is abrogated by an epigenetically acting drug *in vitro*.

INTRODUCTION

Allergy and allergic asthma are major health problems linked to significant morbidity and mortality[1,2] and commonly have onset in childhood.[3] Despite extensive knowledge of the pathophysiologic mechanisms of allergy and asthma, the onset of the disease and its etiology remain in large part poorly understood, which prevents etiotropic curative strategies. Although contributing genetic factors have been identified, inheritance alone cannot explain the high prevalence of asthma and allergies observed in the human population and the rapid increases registered over the past several decades.[1–5] Attention has thus shifted to environmental exposure, epigenetic mechanisms, or microbiome effects,[6,7] providing an optimistic premise that these controllable, variable factors can inform preventive and curative approaches.

It is recognized that environmental airborne factors, especially particulate matter (PM), can trigger asthma onset.[8–10] Environmental particles promote allergic sensitization and co-trigger or exacerbate asthma in humans and mice[8–10]; this is true for diesel exhaust particles (DEPs) and concentrated urban air particles (CAPs), among other particulates.[11–19] However, there is an intriguing dichotomy of "cleaner air but more asthma"; as the prevalence and severity of asthma in adults and children have been increasing over the past decades and/or remain steadily high,[1,2] the particle levels in the environment have been declining since the 1980s, particularly noticeable in the US and Europe.[20,21] This can be explained mechanistically, we postulate, by heritable transmission of a pro-asthma signal across generations, which results in high asthma incidence in progeny despite lower exposure.

Maternal (more so than paternal) exposure and maternal asthma particularly predispose the offspring to asthma onset.[22,23] Acting through poorly known mechanisms, maternal exposure causes the neonatal immune system to start recognizing and processing normally innocuous proteins as allergens (Ag).[11,24–28] Deciphering these mechanisms may illuminate our understanding of allergy and asthma causality.

Our maternal asthma model is perfectly suited to address this challenge.[11,24,26] In prior work, environmental particles used as a relevant pro-asthma but Ag-independent trigger affected dendritic cells (DCs) so that they present an otherwise innocuous protein as Ag to T cells, inciting asthma.[26] This altered decision-making process in antigen-presenting cells, particularly in

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DCs, is a key culprit in the onset of allergic sensitization.[6,7,29–31] However, it remained elusive how the PM, including relatively "inert" particles, can trigger such immune deregulation, especially when the exposure is given to the ancestor, not the subject. Here, we hypothesized that PM exposure modulates the innate immune responses toward allergy by affecting the microbiome and, subsequently, the DCs.

Since the emergence of the "microflora hypothesis" of allergic diseases,[32] microbiomes in the gut and the lung are being increasingly linked to asthma and allergy.[33,34] Despite increasing number of studies descriptively reporting changes in gut flora in asthmatics, there are a few limitations, First, the microbiome profiling results are poorly reproducible across studies,[35–39] and second, profiling alone does not allow conclusions regarding causality of the microbiome changes, as they can be secondary to allergy/asthma or to another factor.

The causality can be best addressed using a modern version of the classical Koch postulates by transplanting the hypothesized disease-causing agent (in this case, the microbiome) to healthy animals. An obstacle that may limit interpretation is that Ag-specific factors (Ag or its haptens, antibodies, cytokines, or polarized immune cells) may co-transfer with the microbiome if the transplant is performed from an allergic/asthmatic donor. Here, we avoid this issue by using a non-Ag trigger—environmental CAP and DEP particles—and by exposing the mother of the donor, not the donor directly.

Disease-conferring gut microbiome transplantation (GMT) has been used in a few studies aiming to establish a causative role of the microbiome in other disease.[40] Typically, germ-free mice are used as recipients in such GMT experiments; however, these mice pseudo-spontaneously respond to Ag challenge[41,42] and show altered immune cell populations in the lungs,[41,43] making them unsuitable for asthma and allergy GMT. These problems, and other downsides of gnotobiotic studies,[44] have informed our choice of conventionally colonized rather than gnotobiotic mice; moreover, we also point out that the human condition we aim to model develops in regular, not gnotobiotic, circumstances; thus, we opted for wild-type mice as a more relevant model.

To date, there is a lack of studies demonstrating asthma transfer via microbiome transplantation. Of interest, a recent study showed that GMT from human asthmatics planted into mice conferred an increase in lung oxidative stress and Th17 responses but failed to elicit asthma onset on its own.[45]

In parallel, emerging evidence suggests that GMT from healthy donors can be therapeutic in asthma,[46] food allergy,[39,47] and other allergic diseases.[48] The positive effect of probiotics[49] and isolated cultures[50–52] also supports the notion. However, these data alone do not allow causality conclusions because of the possibility that it is the microbial products that redirect immune signaling therapeutically; hence, these experiments do not establish that altered flora has triggered the disease.

Here, we detected asthma "predisposition" in an intentionally low-dose Ag protocol that remains innocuous in normal pups but elicits asthma in pups born to particle-exposed mothers.[11] We aimed to test how the neonatal microbiome is altered and hypothesized that, if such alterations are asthma causative, then they will confer asthma predisposition to naive recipients via GMT, thus proving the causative "role" of the gut flora. We further sought to narrow down the causative components of the GMT by gamma sterilization or by co-administration of antibacterials.

Finally, as a way to determine how the altered microbiome could be modulating the host's immune system toward allergy, we studied how GMT affects the epigenome of DCs. We hypothesized that microbiome alterations conferred either by CAP exposure or via GMT from CAP-exposed donors can induce epigenetic DC deregulation, focusing in this case on DNA methylation as a key epigenetic mechanism. Ultimately, we used Ag-stimulated *in vitro* DC-T cell co-culture to demonstrate that epigenetic alterations in DCs lead to a functional pro-allergy/asthma effect.

RESULTS

GMT confers asthma predisposition

Prenatal exposure to CAPs or DEPs predisposes the offspring to asthma, reproducing our earlier findings.[11] Specifically, after a low-dose Ag protocol, the pups born to CAP- or DEP-exposed mothers respond with airway eosinophilia; elevated interleukin-4 (IL-4), IL-5, and IL-13 levels in lavage fluid; and lung tissue infiltration (Figures 1A–1E) as well as previously reported airway hyperresponsiveness to methacholine,[11,24,28] which does not occur in control pups after the same Ag exposure.

To test whether the gut microbiome can causatively mediate this asthma predisposition, we performed GMT from pups born to particle-exposed vs. vehicle-exposed mothers to age-matched recipients. The pups serving as donors did not receive Ag and were naive aside of their mothers' exposure (Figure 1F).

Intact recipients of stool material from CAP- or DEP-exposed donors have developed lavage eosinophilia (Figure 1G), elevated cytokine levels (Figure 1I), and lung tissue infiltration (Figures 1J and 1K) upon low-dose Ag, indicating that GMT has conferred asthma onset predisposition. The robustness of the GMT effect on recipients was similar to the "direct" maternal effect in the "donors" shown in Figures 1A–1E. Control pups that received stool material from vehicle dam donors did not show asthma predisposition (Figures 1G–1K). The effects of CAPs and DEPs were similar with slight variability; we show bronchoalveolar lavage (BAL) eosinophilia in 3 separate experiments (Figure 1H) to illustrate this point.

These results strongly suggest that particle-driven changes in the gut microbiome are causative for asthma predisposition early in life.

A viable microbiome is required for asthma predisposition

To test whether live microbes are required for the GMT effect, we performed radiation (gamma) sterilization of the donor material prior to GMT (Figure 2). Fecal matter was irradiated using the standard "medical" sterilization dose of 50 kGy over 24 h on ice and then transplanted into naive pups as in Figure 1. Gamma sterilization ablated the effect of the transplant (Figures 2A1–2A3): recipients of the sterilized GMT no longer responded to Ag with the asthma phenotype compared to recipients of an

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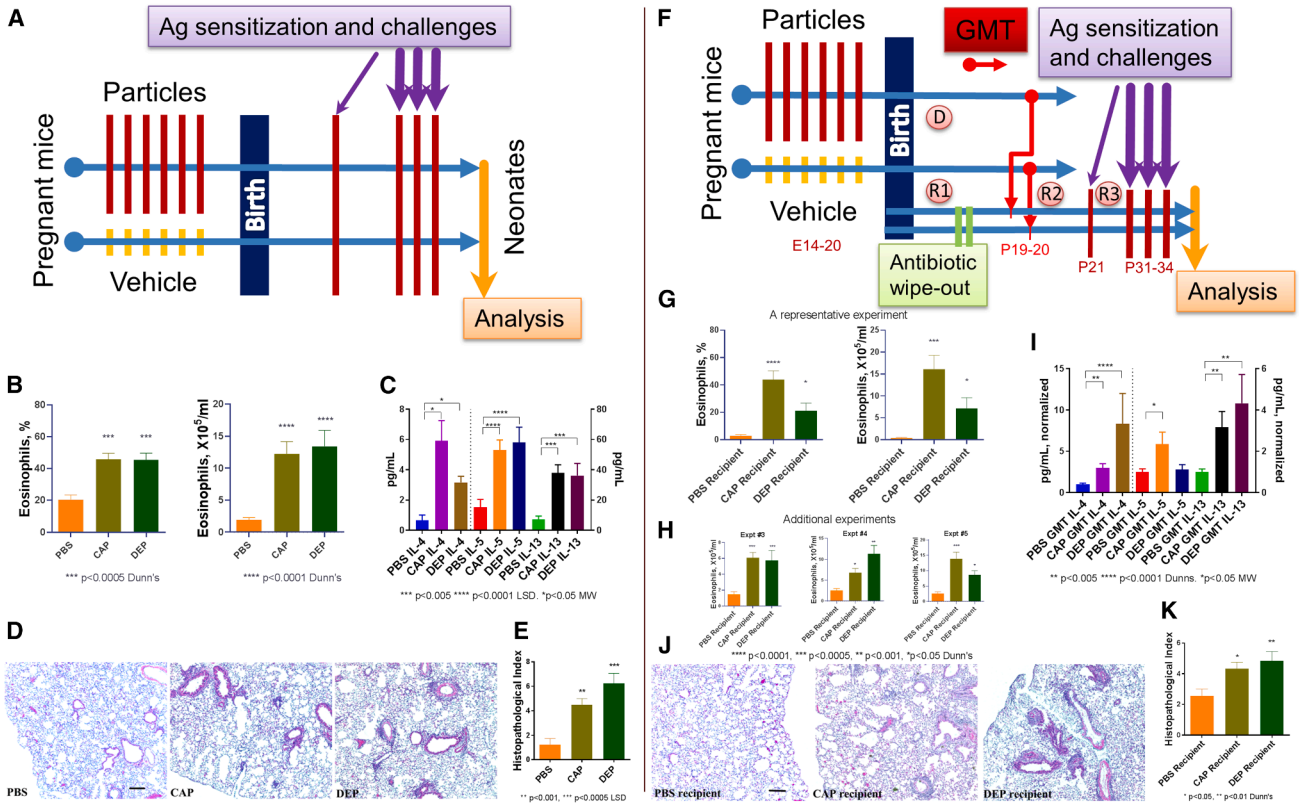


Figure 1. Direct and GMT-conferred asthma predisposition effect

(A–E) Direct effects. A maternal model was used to study asthma predisposition after environmental particle exposure (A). Dams are exposed at embryonic day 14 (E14)–E20 days of gestation to particles or vehicle (PBS). Neonates are tested in the "low-dose allergen (Ag, OVA) protocol" with a single intraperitoneal (i.p.) sensitization and 3 daily aerosol challenges (A), which results in lavage eosinophilia (B), cytokine increases (C), and lung tissue infiltration (D and E) (H&E staining, ×100) in neonates of mothers exposed to particulates but not the control. n = 36/experiment (1E n = 12).

(F–J) GMT-conferred effects.

(F) GMT model. Naive recipients after an antibiotic wipe-out receive GMT (post-natal day 19 [P19] and P20) from the offspring of dams exposed to CAPs, DEPs, or vehicle and are then tested in the low-dose Ag protocol with a single i.p. sensitization (P23) and 3 daily Ag aerosols (P31–P34). D, R1, R2, and R3: collection of microbiome samples for sequencing.

(G–K) BAL eosinophils in a representative experiment (G) and the same in 3 additional experiments (H), showing fluctuations per animal cohort, (I) BAL cytokines (pooled data from 3 experiments), (J) representative lung infiltration (H&E staining, ×100; scale bar, 100 μm), and scoring (K).

Data are represented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$, and ****$p \leq 0.0001$ (ANOVA with Dunn's or LSD, as detailed). n = 30–36/experiment.

unsterilized GMT. Due to the kinetics of the gamma sterilization procedure, we included both a "fresh" control GMT performed immediately after harvest and an additional control were donor samples were stored on ice overnight but not sterilized ("control"). The results suggest that microbial viability is important for asthma predisposition.

Moreover, to control for radiation as a method of sterilization, we performed co-administration of the antibacterials ciprofloxacin and metronidazole (Cip/Met) with the GMT, which also abrogated the effect of the transplant (Figures 2B1–2B3). Notably, Cip/Met treatment had no effect on asthma when given directly to the "donor" neonates of CAP- or DEP-exposed mothers (Figure S1), indicating that admixture of antibiotics in transplantation has no effect on the host's allergic susceptibility. In combination, these data suggest that the transfer of asthma predisposition involves host changes driven by bacteria that are viable and active during the process of colonization.

Metagenomic sequencing detects partial microbiome remodeling in pro-asthma gut microbiome transplant material and during its establishment after transplantation

We assessed the microbial abundance and diversity of the gut microbiome present in the fecal samples. Prior to evaluation of the donor and recipient stool samples, we determined the most abundant species in the mothers' gut microbiome to assess whether particle exposure directly led to gut microbiome remodeling. As detailed in Figure 3A, both types of particles induced a change in the gut flora composition profile, with some shared and some particle-specific changes among the top 15 most abundant species.

We then looked at the donor gut microbiome from the offspring of the aforementioned exposed and control dams. The most abundant flora was mainly composed of bacteria from *Alistipes*, *Bacteroides*, Lachnospiraceae, *Cropoplasma*, *Gallimonas*, MGBC139354, and uncultured bacteria (Figure S2A). Donors

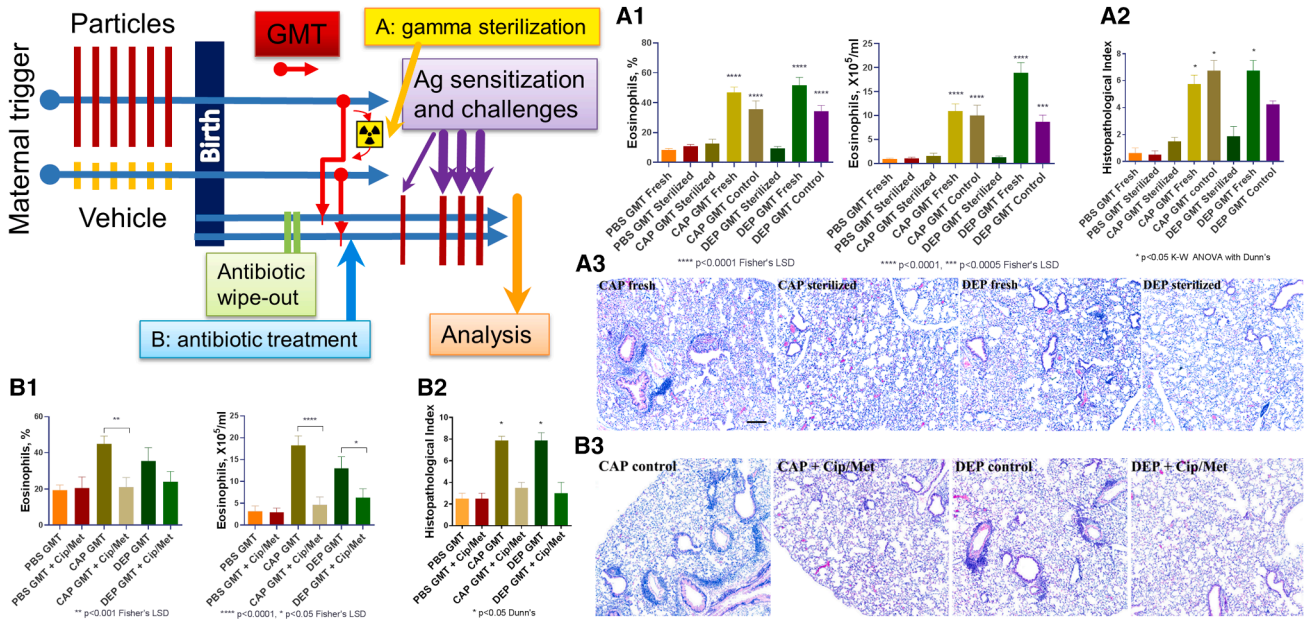


Figure 2. "Live" vs. "dead" GMT effects: sterilization by gamma-radiation or antibacterials

(A) BAL eosinophilia (A1), lung histopathology scoring (A2), and lung tissue micrographs (H&E staining, ×100; scale bar, 100 μm) (A3) in recipients of fresh gut microbiome transplant material or control gut microbiome transplant material (stored overnight on ice) vs. gamma-sterilized gut microbiome transplant material from offspring of CAP- or DEP-exposed (or PBS vehicle control) mothers. (B) Co-administration of the antibiotics Cip/Met with the gut microbiome transplant: BAL eosinophilia (B1), lung histopathology scoring (B2), and lung tissue micrographs (H&E staining, ×100) (B3).

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$, and ****$p \leq 0.0001$ (ANOVA with Dunn's or LSD, as detailed). $n = 20–30$/experiment. Data are represented as mean ± SEM. See also Figure S1.

born to CAP- and DEP-exposed dams showed reduced overall diversity compared to the material from vehicle-exposed mice (Figure S2B). After GMT at time point R2, there was an observed colonization period where all groups showed similar diversity (Figure S2C). Finally, at the later time point R3, both CAP and DEP fecal samples showed higher microbial diversity compared to that of the vehicle group (Figure S2D).

Bacterial species that were more abundant in the vehicle group compared to the CAP and DEP groups were *Alistipes timonensis*, *RACS-045*, *CAG-115 sp910577365*, *Ventrimonas sp003611875*, *Enterenecus sp910585265*, and *Lachnospiraceae bacterium UBA3282 sp009774585* (Figure 3B). At multiple time points, we observed that *A. timonensis*, *RACS-045*, and *Lachnospiraceae bacterium UBA3282 sp009774585* had an over 1% decrease in relative abundance compared to the vehicle controls (Figure 3B). Of importance, these bacteria are known to metabolize polysaccharides and produce short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate.[53–60]

Species that increased in the CAP and DEP groups compared to the control were *Acetatifactor sp910578215*, *Butyribacter sp910579085*, *CAG-95 sp910577445*, *CAG-873 sp002490635*, *Coproplasma sp910578765*, *Eubacterium F sp910589665*, *Kineothrix sp910588855*, *Sporofaciens sp011960625*, and *V9D3004* (Figure 3C). These species, overabundant in both the donors and the recipients of the "asthma risk" microbiome, contain the causative agents of the phenotype. While over a quarter are unculturable bacteria (Figure S2E), suggesting space for future discovery within these major bacterial effectors in asthma predisposition, the "suspects" *Acetatifactor*, *Butyribacter*, *Kineothrix*, *Coproplasma* and other Lachnospiraceae bacteria are also linked to production of SCFAs: acetate, butyrate, and propionate.[61–65] These results provided a potential mechanistic lead into the following metabolomics studies.

Particle-driven changes in microbial genes are associated with altered metabolic pathways and lead to a reduction of butyric acid levels

A major way for microbiome changes to alter immune regulation is through functional changes that modify release of metabolites.[66] To assess this, we employed Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology and pathway analysis of microbial genes. The donor material showed shared metabolism changes in both the CAP and DEP groups (Figure S3). Two specific genes were found to be changed in a similar manner in both particle groups, the beta-exotoxin I transport system permease protein was found to be upregulated, and the adenine-specific DNA methyltransferase (K07318) was found to be downregulated (Figure S3A). For the R2 time point, several genes were found to be downregulated in both the CAP and DEP groups; specifically, the 4-hydroxycutyryl coenzyme A (CoA) dehydratase (abfD), la (rph), rifampicin phosphotransferase (rph), the zinc carboxypeptisa, the phage terminase small subunit (xtmA), and a putative restriction endonuclease (K07454) (Figure S3B). Of note, abfD facilitates the dehydration of 4-hydroxybutyryl-CoA to form crotonyl-CoA, an essential intermediate in the biosynthesis of butyric acid. For the last time point

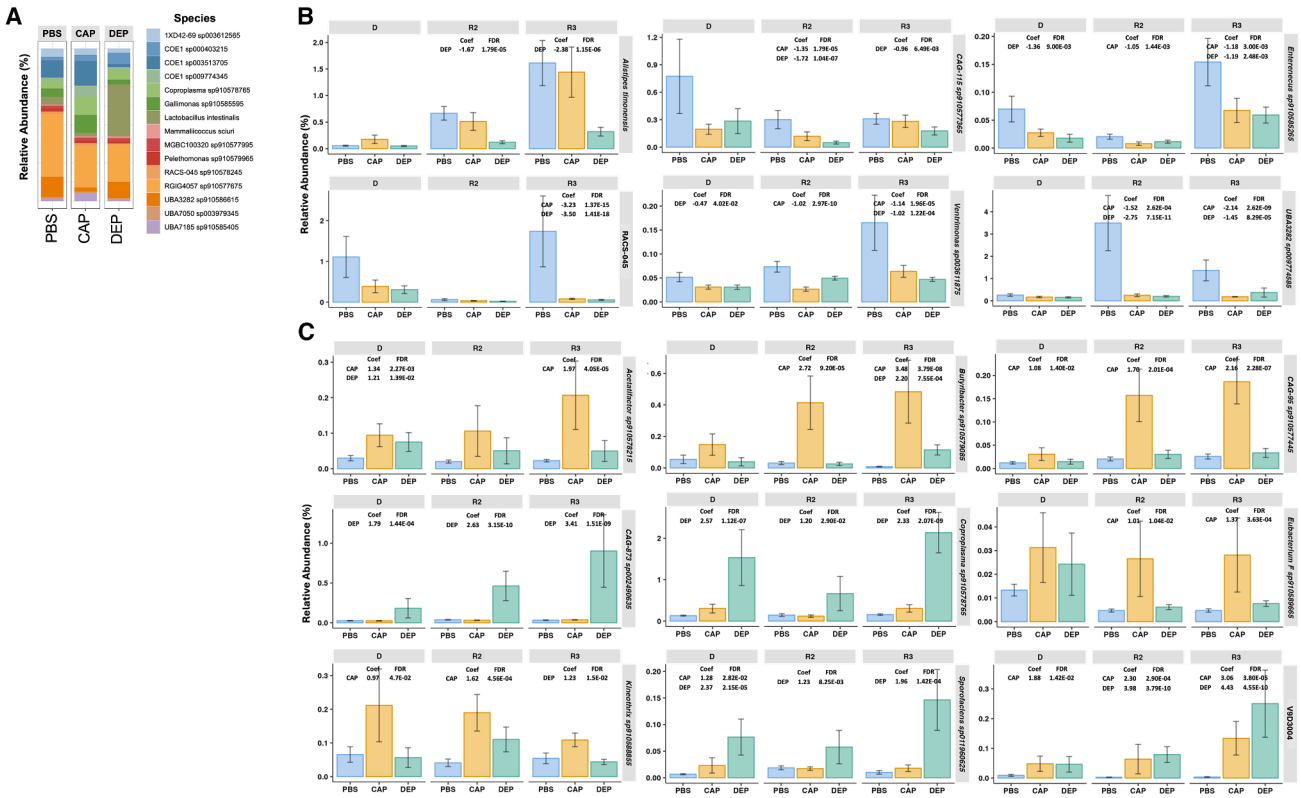


Figure 3. Pro-asthma GMT material leads to partial gut microbiome remodeling in mice

(A) Maternal microbiome: relative abundance of the top 15 species in the stool of pregnant dams exposed to PBS, CAPs, or DEPs.

(B) Offspring microbiome: significantly changed bacterial species that show higher relative abundance in PBS group compared to the CAP and DEP groups.

(C) Offspring microbiome: significantly changed species of bacteria that show higher relative abundance in the CAP and DEP groups compared to the PBS group. "D" samples are from donor neonates born to CAP- or DEP-exposed and control mothers, and "R2" and "R3" samples are from recipients after the transplantation (see Figure 1F). Also related to Figure S2.

(R3), we observed upregulation in both particle groups of the arginine utilization regulatory protein rocR, the n-acyl-D-amino acid deacylase (E3.5.1.81), the dimethyl arginase ddaH, and an uncharacterized protein (K09703). Downregulated in both particle exposure groups were the pyrimidine-nucleoside phosphorylase pdp, the antimicrobial peptide resistance transport system ATP-binding protein yknS, and the recombination protein RecT (recT) (Figure S3C). Downregulation of recT, a major recombination protein used by certain bacteriophages to facilitate homologous recombination,[67] suggests a reduction in the propagation of phages and the possible completion of microbial abundance changes. Taken together, the changes in the KEGG orthology suggest that asthma-at-risk status is linked to specific changes to the functional state of the gut microbiome that may modulate SCFA production and bacterial abundance through phage activity.

To test whether the functional changes observed in the microbiome led to alteration of key immunomodulatory metabolites, we used a metabolomics panel to define the abundance of SCFAs in the donor material and in the stool of recipients at two time points post GMT (Figure 4). The tested SCFAs were butyric acid, acetic acid, iso-butyric acid, isocaproic acid, heptanoic acid, and propanoic acid. Only butyric acid was significantly changed between the control and particle samples at all time points (Figure 4). The reduction in butyric acid may be linked to the aforementioned functional changes in the microbiome (Figure S3). Taken together, these results show that *in utero* particle exposure causing asthma predisposition leads to changes in the composition and metabolic state of the gut microbiome of newborns, including reduced butyric acid levels.

Of importance, negative changes in butyric acid during early years have a strong correlation with the development of allergy and asthma in humans,[68–71] and butyrate effects have been linked to epigenetic changes in host immune cells.[72–75]

The epigenome of the recipient host's DCs is altered by GMT similar to maternal exposure

Earlier, we demonstrated a key role of DCs in the origin of asthma predisposition conferred by maternal allergy or particle exposure[26] and detailed epigenetic changes in the DCs,[27] some of which are inherited transgenerationally.[28] Here, we tested to what extent GMT confers the DNA methylation alterations to recipients' DCs to test the premise that microbiome-based epigenetic deregulation may occur besides the direct epigenetic inheritance, and may explain how the altered gut flora leads to altered responses to Ag.

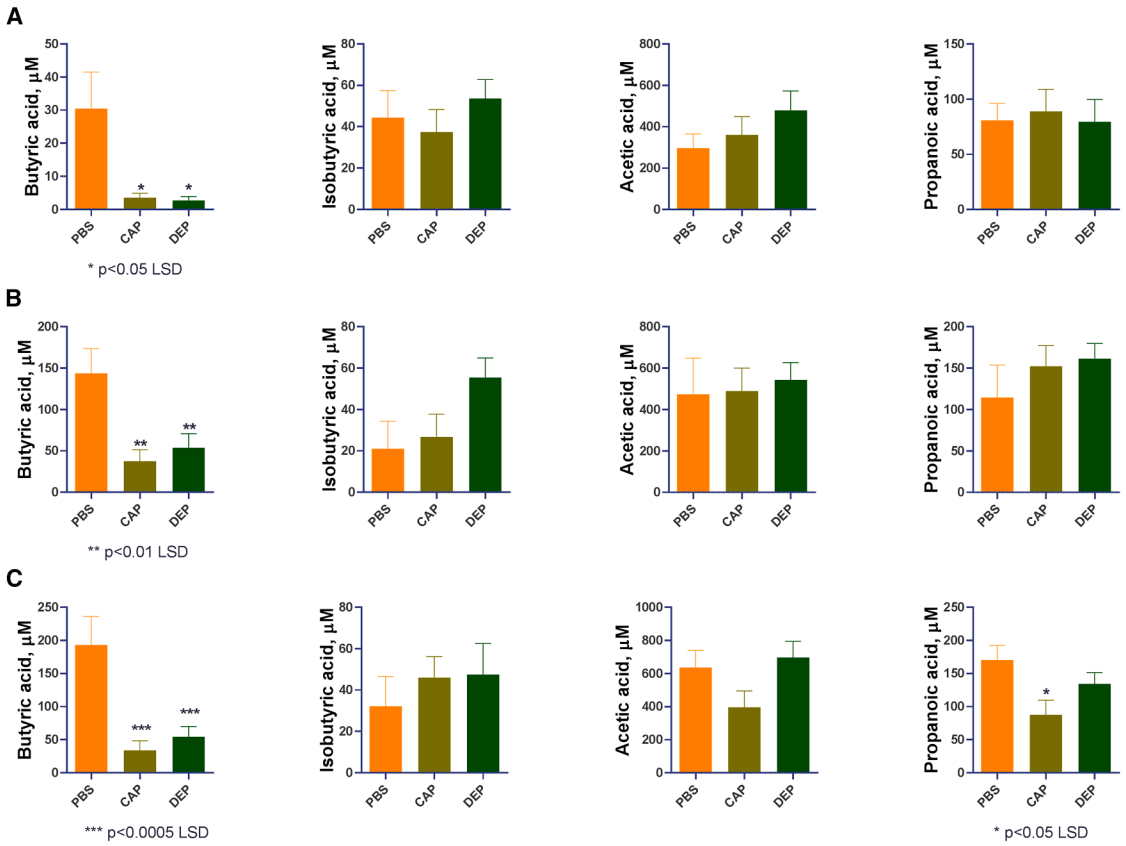


Figure 4. Targeted metabolomic analysis of short chain fatty acids in stool samples of donors and recipients

(A–C) The metabolites tested were butyric acid, isobutyric acid, acetic acid, propanoic acid, isocaproic acid (below detection), and heptanoic acid (below detection).

(A) Donor material.

(B) R2 recipients.

(C) R3 recipients.

Butyric acid is depleted in pro-asthma gut microbiome transplant material and in the post-GMT established microbiome. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.0005$, and (ANOVA with LSD). $n = 30$. Data are represented as mean ± SEM.

We compared DNA methylation profiles of the DCs from pups born to CAP-exposed mothers (the donors) and the recipients of gut microbiome transplants from these pups. An additional control was the recipients of sterilized gut microbiome transplants. All pups were Ag naive. Our bioinformatics strategy aimed to identify loci where methylation is altered by maternal CAP exposure vs. PBS control *and* by transplantation of gut microbiome from pups of CAP-exposed mothers but *not* by the sterilized transplant (i.e., the effect of CAP-altered gut microbiome on the DC epigenome).

Methylation values from the 4 groups of samples (in this order: "PBS," "CAP," "CAP fresh GMT recipients," and "CAP sterilized GMT recipients") were subjected to Pavlidis template matching analysis with the template 0_1_1_0 (meaning low in PBS, high in CAP, high in CAP GMT recipients, and low in CAP sterilized GMT recipients), which identified 484 differentially methylated loci (DMLs) with a p value of 0.01 (Figure S4A). The opposite template, 1_0_0_1, identified 2,479 DMLs with a p of 0.01 (Figure S4B).

ANOVA with post-tests reported (Figure 5A) that PBS vs. CAP comparison at $p = 0.05$ produces 8,369 DMLs. Of these, 1,598 DMLs overlap with (are also present in) the comparison PBS vs. CAPfresh. These DMLs therefore are shared by CAP and CAPfresh GMT effects vs. the PBS control; in other words, they are induced by either prenatal CAP exposure and by GMT from prenatally CAP-exposed donors. In this list, 935 loci are not present in the PBS vs. CAPsterilized comparison, indicating that they occur as a result of maternal CAP exposure and are conferred by GMT but disappear when the GMT is gamma sterilized (Figures 5A and 5B). This 935 DML list is the key output of our epigenomic analysis and informs the network and pathway analysis below.

Network and pathway analysis of this list has shown that key allergy- and asthma-relevant pathways are affected. A direct interactions algorithm (Figure 5C) has linked the majority of the factors/genes in the 935 list (806 objects recognized), indicating a likely co-involvement of these genes in biological processes and supporting biological feasibility of the bioinformatics result. Compare the number of connections (each representing manually curated evidence [e.g., a published paper] of an interaction between two factors) in Figure 5C with connections in an identically sized (935) list of random gene names (Figure 5D).

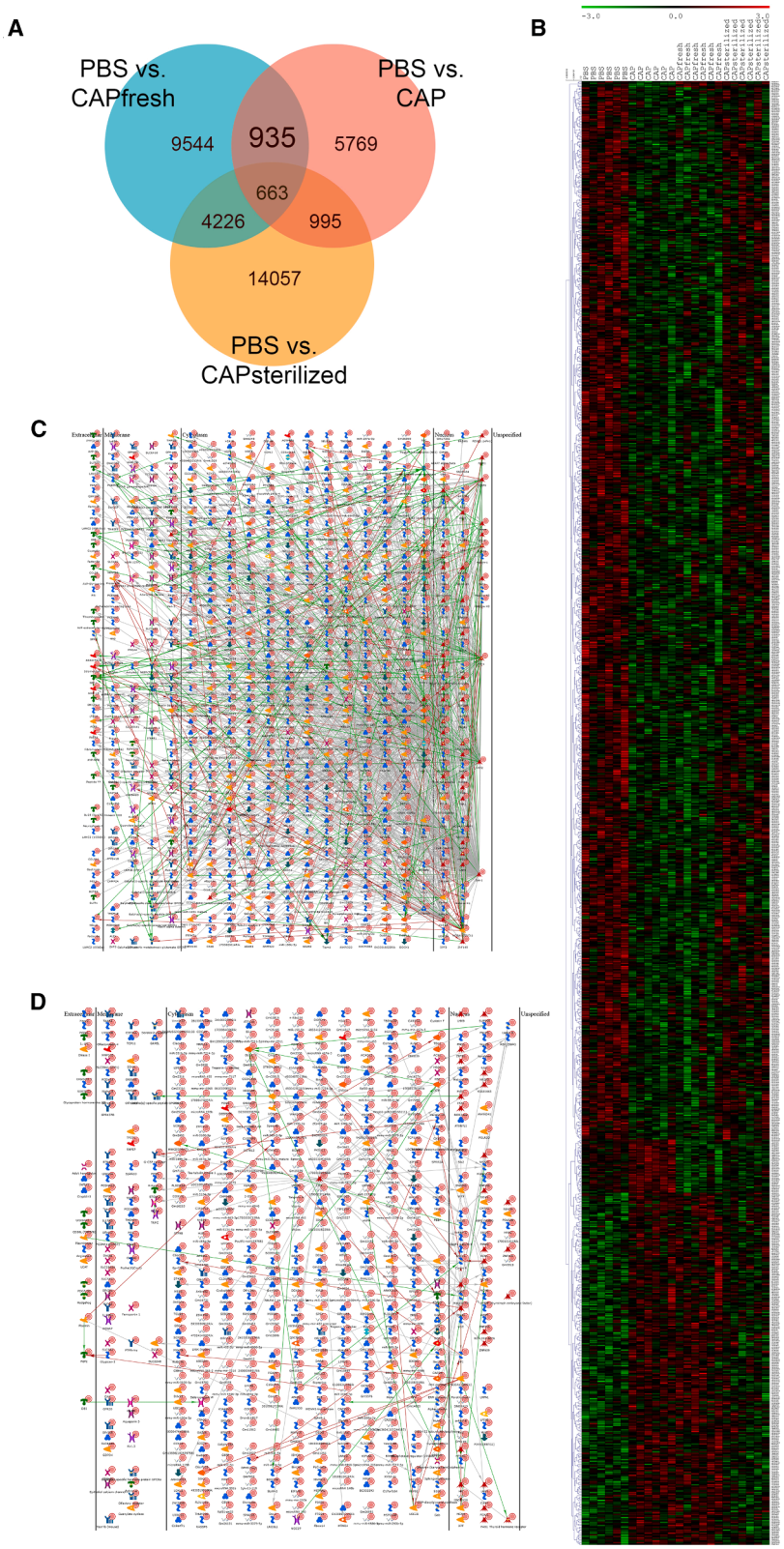


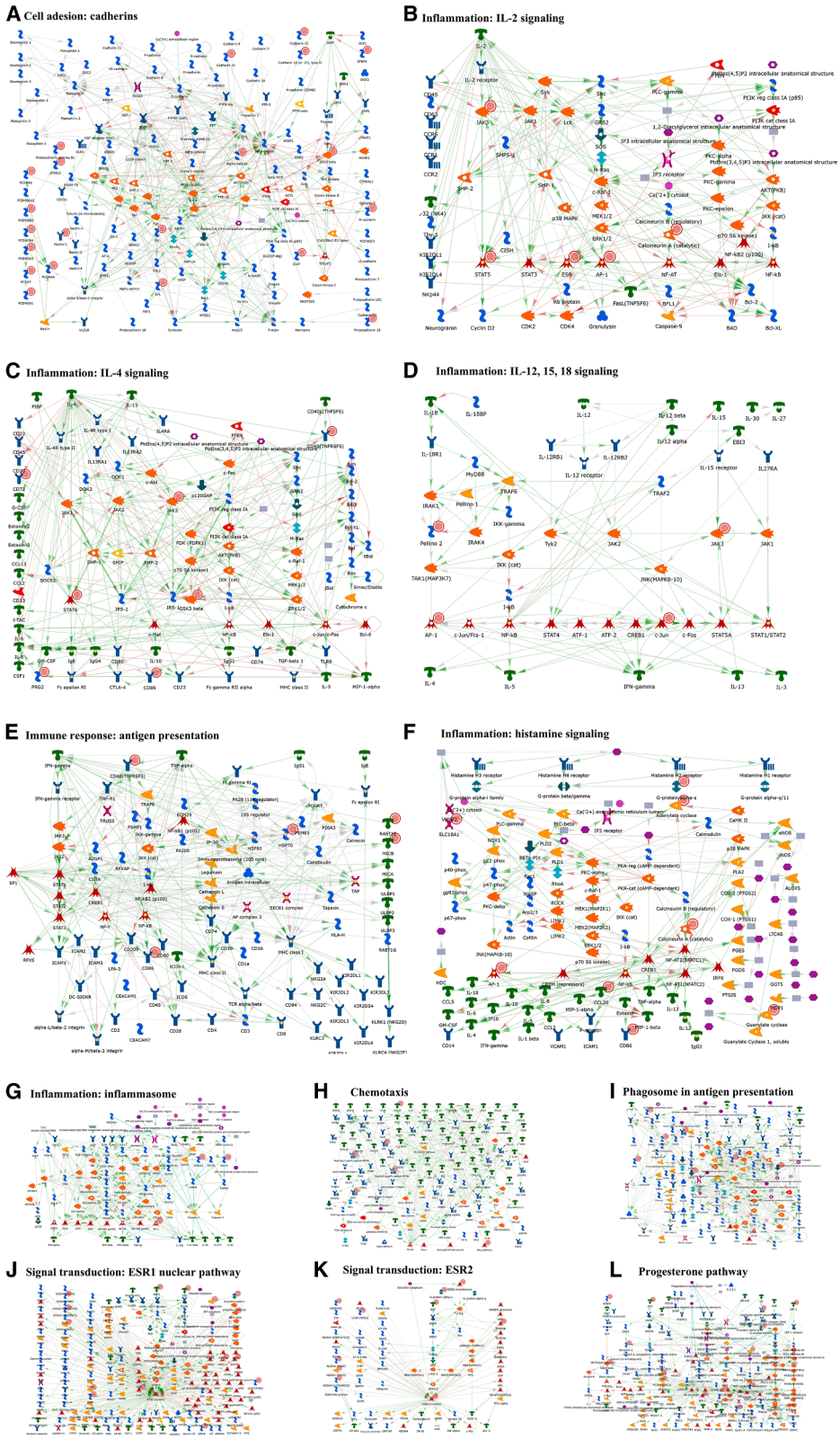
Figure 5. Effect of GMT on the epigenome of host's DCs

Study groups included offspring born to CAP-exposed mothers (CAP) or PBS control mothers (PBS) and recipients of either a fresh gut microbiome transplant (CAPfresh) or gamma-sterilized gut microbiome transplant (CAPsterilized) from the offspring of CAP-exposed mothers. All mice were age matched and Ag naive.

(A) Venn diagram detailing post-ANOVA contrasts.

(B) Hierarchical clustering (Pearson, single linkage) of 935 DMLs significant after intersection ANOVA, where PBS vs. CAP *and* PBS vs. CAPfresh *not* PBS vs. CAPsterilized criteria were met.

(C and D) Network analysis via Metacore, a direct interactions network. Shown are the actual gene list (C) and the control list of 935 random gene names (D). $n = 24$.



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Process networks analysis indicated that the following highly relevant processes were affected: apoptosis (several pathways); cell adhesion and cell adhesion/cell-matrix interactions; chemotaxis; inflammation: histamine signaling, immunoglobulin E (IgE) signaling; IL-2, IL-4, IL-5, IL-12, IL-15, IL-18, and interferon signaling; inflammasome; innate inflammatory response; immune response: antigen presentation; immune response: phagocytosis and phagosome in antigen presentation; proliferation: positive and negative regulation of cell proliferation and cell cycle regulation; signal transduction: NOTCH and WNT signaling; transforming growth factor β, GDF, and Activin signaling; estrogen receptor ESR1 and ESR2 pathways; and progesterone signaling pathways. All significant process networks (top 100) are summarized in Table S1. The involvement typically included 5–10 factors in each process with a maximum of 21. We selected 12 key processes/pathways (Figure 6) to illustrate where the altered genes (marked with red circles) are located on their maps. This result is highly robust in showing that the epigenetic alterations in DCs after GMT occur to genes directly participating in Ag capture and presentation activities of DCs and their inflammatory signaling.

The epigenetically altered DCs are pro-allergy in co-culture with T cells

To test the premise that DNA methylation changes in DCs of asthma-predisposed pups of asthmatic dams are mechanistically responsible for eliciting the predisposition, we performed functional testing of DCs *in vitro*. We co-cultured them with $CD4^+$ T helper responders from DO11.10 mice that are transgenic for the ovalbumin (OVA)-specific T cell receptor (TCR). These T helpers respond with proliferation when OVA is presented to them by antigen-presenting cells and are broadly used to test Ag presentation.[76] DCs from asthma-predisposed pups produced increased proliferation (vs. normal naive DCs) of OVA-TCR T helpers *in vitro* in OVA-stimulated co-culture. However, when treated with zebularine or decitabine—DNA methyltransferase (DNMT) inhibitors that non-selectively demethylate DNA and "reshuffle" the epigenome—this difference was no longer seen, indicating that DNA methylation was a factor of increased Ag presentation (Figure 7). A control drug, cytarabine, of similar structure, chemistry, and toxicity but without epigenetic action, did not have such an effect.

DISCUSSION

Whether the gut microbiota alterations seen in asthmatics are causative of this disease (and, more broadly, of allergy) has been a growing question. Despite bioinformatic effort to derive causality from microbiome profiles[77] and effects of "therapeutic" GMT and probiotics in asthmatics,[49–52] it remains unclear whether microbiome composition altered by, e.g., an environmental exposure can elicit asthma. Troubled with the aforementioned limitations of germ-free mice for allergy microbiome studies, the poor overlap between human profiling studies, and by Ag-specific effects in commonly used asthma models, the issue has been lacking direct experimental causality confirmation.

Here, we used a model of gestational exposure to particles—CAPs or DEPs—that trigger predisposition to asthma in the offspring[11] to determine whether the predisposition is due to a particle-altered gut microbiome passed from the mother to the pup that can also be passed to other, naive control recipient pups. Using a GMT approach, we tested the causality of particle-induced microbiome changes and demonstrated that GMT confers asthma predisposition to naive recipients. We further discerned the input of live microbes versus bacterial metabolites or other solutes by gamma-sterilizing the GMT material or by antibiotic co-administration and showed that either method ablates the effect of the transplant. These data lend support to our hypothesis that viable gut bacteria are required for GMT to confer asthma predisposition triggered by environmental particles.

Our study is consistent with the findings that environmental exposures of the mother, especially during gestation, are associated with an altered gut microbiome of the newborn,[78,79] which has been linked to a number of diseases,[80] and that airway exposure to PM specifically can modify the gut microbiome with a link to airway disease.[81,82] Because the maternal gut microbiota is essential for healthy development of the neonate's immune system, the link to allergy and asthma is reasonable.[83] However, direct evidence showing that airway exposure alters gut microbiota composition so that it is causative of asthma has been lacking.

Here, we provide such evidence and link it to a plausible mechanistic explanation. Previous studies have revealed that microbiome-derived SCFAs play significant roles in the offspring's immune system.[84] These solutes form what has been called microbiome-metabolome interactions and are increasingly seen as potential mechanistic and therapeutic targets in asthma.[85–87] While some forms of SCFAs have been shown to contribute to ozone-induced airway hyperresponsiveness,[88] this is debated by other findings suggesting that SCFAs ameliorate microbiome-driven allergic lung inflammation.[89] Acetate, butyrate, and propionate, the three end products of microbial fermentation of macronutrients,[90] are substantially decreased in the stool of asthmatics, including children.[70,91,92] It appears that not all SCFAs act synchronously, but they have shown a strong anti-inflammatory effect on DCs,[93] which is important for our study. Specifically, butyrate and propionate inhibit the production of cytokines in DCs[93,94]; butyrate induces tolerogenic human DCs[95] and has other prominent effects on DC maturation and function,[96–98] implicating counterinflammatory outcomes,[99–101] including downstream interactions with T cells.[102] Hence, SCFAs, possibly in combination with other factors,[103–106] directly modulate DC function and gene expression, mediating what is becoming known as "gut microbiota-to-innate immunity crosstalk."[106–108]

Figure 6. Process network enrichment illustrations for the most relevant of the 100 top significant processes that involve gene loci from the 935 DML list

(A–L) Each process network is pre-designed in Metacore; genes from the input list that fall onto a network are labeled with a red circle, which illustrates to what extent each pathway was affected.

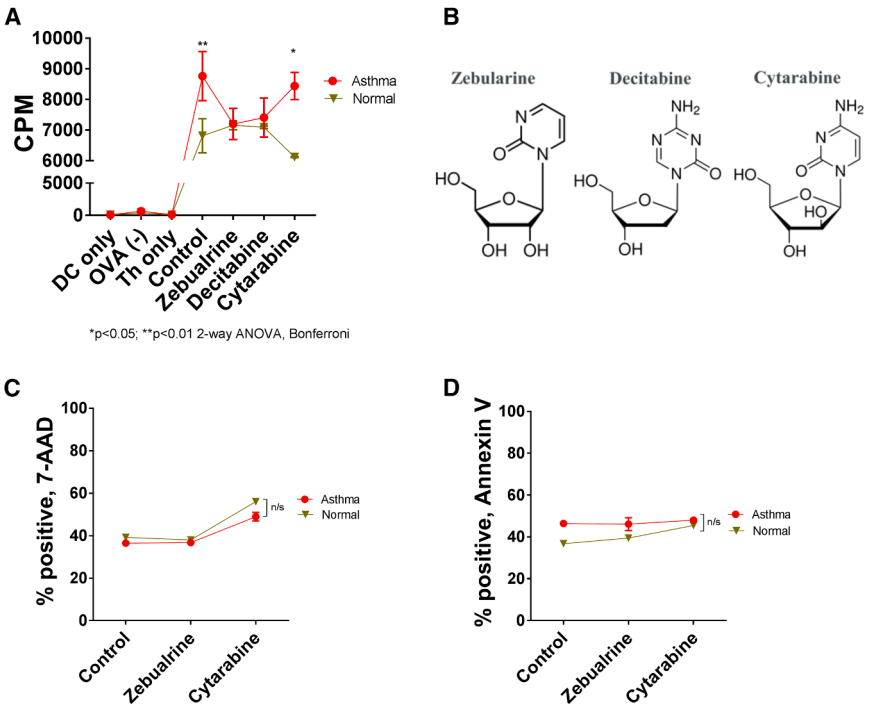

Figure 7. Allergen presentation *in vitro* assay

DCs isolated from asthma-predisposed pups were co-cultured with OVA-TCR transgenic CD4$^+$ T helper responders cells.

(A) Proliferation of the T helpers was registered in OVA-stimulated co-culture using the radioactive tritium incorporation method and expressed as counts per minute (CPM). DCs from asthma-predisposed pups present OVA as antigen, eliciting increased proliferation of T-helpers, which is not seen with normal control DCs. *In vitro* pre-treatment with the epigenetically-acting DNMT inhibitors zebularine or decitabine abrogates this effect.

(B) A control drug, cytarabine, that is structurally similar but does not have epigenetic action did not have an effect.

(C and D) Zebularine (or decitabine, data not shown) did not affect DC viability, whereas cytarabine had a mild effect on both asthma-predisposed and normal DCs.

$^*p \leq 0.05$ - $^{**}p \leq 0.01$, ANOVA with Bonferroni. $n = 56$. Data are represented as mean ± SEM.

Our metagenomics sequencing revealed key microbial differences in the neonates born to PM-exposed vs. control mothers. The species shown in Figure 3 are shared between the CAP and DEP study groups and by both the donors and the recipients of the gut microbiome transplant, suggesting that this profile is the "signature" of asthma predisposition. The reduced bacteria in the exposed groups are known to metabolize polysaccharides and produce the SCFAs. Driven by these data, we performed SCFA profiling (Figure 4), which revealed a substantial decrease of stool butyrate in these study groups, which is consistent with the aforementioned data in asthmatics.[70,91,92] Of interest, those of the SCFA-producing bacterial species that were observed to be increased in the CAP and DEP groups mostly did not surpass the 1% relative abundance; thus, it is possible that their outgrowth is an attempt to compensate for the butyrate-producing flora. It is also possible that this transplanted flora suppresses the local butyrate-producing flora, leading to an overall drop in butyrate levels. Another plausible explanation is that the butyrate permeates into the circulation through gut leakage induced by the transplanted altered flora and is then excessively consumed by DCs.

Our focus on DC epigenetics as a potential mechanism was based on the key role of these cells in allergy and asthma origin[29–31] and was fueled in part by literature indicating epigenetic effects of the microbiome in general[109–111] and the epigenetic action of SCFAs in particular,[112–115] including specifically the epigenetic effects of butyrate on DCs.[95,100]

Previously, we described in this model that maternal particle exposure does induce epigenomic changes in neonatal DCs and that a DNA demethylation treatment has abrogated asthma risk transmission to progeny.[28] Here, we performed epigenome-wide DNA methylation profiling not only of pups born to a particle (CAP)-exposed mother but also of those receiving a gut microbiome transplant from such pups and have found that the same altered DNA methylation patterns are seen in either group and are not seen after receiving a sterilized gut microbiome transplant (Figure 5). Hence, the live, but not dead, microbiome confers this alteration.

Pathway and network analyses (Figure 6) show that the affected genes belong in pathways highly relevant to asthma and DC antigen presentation functions, which points toward the biological significance of our finding as well as indirectly confirms the relevance of our bioinformatics strategy. Further gain- and loss-of-function assays will be necessary to test the premise that SCFAs could potentially be mediating the epigenetic effects.

Finally, our DC:T-cell co-culture provides an endpoint linking the epigenetically altered DCs to a functional allergy test (Figure 7). While these experiments could not be done in recipients of a gut microbiome transplant in our CAP model because DO11.10 T-helper responses entail OVA-specific effects, they serve to support the critical importance of altered DNA methylation in DCs for the onset of allergic sensitization.

Limitations of the study

A few potential limitations of our work merit discussion. We note that we did not determine to what extent the DNA methylation effects are linked to expression of the corresponding genes, and we note the relatively small ($n = 24$) in this particular profiling. We did not seek direct evidence that it is the SCFA butyrate that exclusively mediates the DC effects. Our metagenomics

data did not detect the decreases in lacto- and bifidoflora that have been reported in some profiles of human asthmatics.

While we chose gamma radiation as a method that is maximally effective in eliminating the broadest spectrum of live microbes (in contrast to, e.g., UV transillumination) and at the same time minimally affects the solutes (in contrast to, e.g., thermal processing/boiling), it is not without side effects. For example, we observed partial degradation and loss of proteins in the gamma-sterilized gut microbiome transplant sample, likely indicative of protein radiolysis. However, our treatment with Cip/Met serves as an alternative method of eliminating live bacteria during GMT; hence, in combination, our data support the conclusion that live bacteria are required for the gut microbiome transplant effect.

Notably, antibiotics had no effect on the recipient host allergy/asthma status (Figure S1); thus, admixture of a small amount of antibiotics with the gut microbiome transplant has no significance. This lack of a direct antibiotic effect is consistent with human data showing that antibiotics are not a treatment for allergic asthma. We interpret this to mean that oral Cip/Met with the gut microbiome transplant has precluded the transplanted bacteria from homing but was not sufficient to affect the already homed flora.

Here, within one study, we provide direct experimental support for the premise that a maternally altered microbiome can confer asthma origination and that environmental airway exposure triggers the causative changes in the gut flora, narrowed to viable bacteria as the key component. Metagenomic sequencing results inform new species-specific perspectives, including the potential for understudied unculturable species. Of note, we have reported previously that asthma predisposition transmits through 3 generations of intact progeny after a single ancestral exposure,[28] but we have not yet set out to determine whether microbiome changes accompany this transmission, which can be a subject of future research. Future experiments could also be aimed at DC-targeted modifications to strengthen evidence of their mechanistic involvement in microbiome-mediated asthma onset. Finally, we did not seek direct evidence that SCFAs (e.g., butyrate) mediate the effects of the altered flora on DCs, which could be done via gain- and loss-of-function approaches.

Conclusions

Prenatal exposure to CAPs or DEPs changes the gut microbiome of newborns, making it causative of increased asthma predisposition upon transplantation to healthy recipients. Live bacteria in the microbiome transplant are required to confer the effect, as radiation or antibacterials abrogate the effect of the transfer. Metagenomic sequencing identifies specific bacterial species; many of these bacteria are linked to SCFA production. The transplanted microbiome interacts with host immunity, leading to an altered epigenome of DCs—a mechanism that can lead to aberrant Ag presentation as a launch mechanism of the disease.

RESOURCE AVAILABILITY

Lead contact

Requests for further information, resources, and reagents should be directed to and will be fulfilled by the lead contact, Alexey V. Fedulov (alexey@brown.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Metagenomics data have been deposited at NCBI SRA, and epigenomic data have been deposited at NCBI GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

M.R. and N.Y. conducted mouse experiments. R.W.-R. conducted microbiome DNA isolation and participated in data analysis. H.S. performed metagenomics and metabolomics data analyses. E.L. and M.S. performed radiation sterilization experiments. N.G.-J. supervised microbiome sequencing and metabolomics and metatranscriptomics analyses and participated in omics data analyses. A.V.F. supervised mouse studies, including particle and allergen exposure and GMT experiments, and performed epigenomics analyses. M.R., R.W.-R., N.G.-J., and A.V.F. co-authored the manuscript. All authors participated in study design and planning, interpretation of results, and manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD11c MicroBeads, mouse	Miltenyi Biotech	130-125-835
CD11c Antibody, anti-mouse	Miltenyi Biotech	130-122-939
MHC Class II Antibody, anti-mouse	Miltenyi Biotech	130-102-896
CD4 (L3T4) MicroBeads, mouse	Miltenyi Biotech	130-117-043
Chemicals, peptides, and recombinant proteins		
Concentrated urban air particles (CAP)	Harvard Ambient Particle Concentrator (Demokritou et al.[116]; Lawrence et al.[117]; Savage et al.[118]; Petros et al.[119])	N/A
Diesel exhaust particles (DEP)	provided by Dr. Ian Gilmour at the U.S. Environmental Protection Agency (Fedulov et al.[11]; Gregory et al.[28]; Ramar et al.[120])	CAS Number 1333-86-4
Ovalbumin; grade V	Sigma-Aldrich	A5503
Critical commercial assays		
ELISA kits for IL-4, IL-5, IL-13	R&D Systems	N/A
DNeasy PowerSoil Pro Kit	Qiagen	47016
DNeasy kit	Qiagen	69504
NEBNext Ultra II FS DNA Library Prep Kit	New England Biolabs	E7805L
NEBNext Multiplex Oligos for Illumina	New England Biolabs	E6448S
Ampure XP beads	Beckman Coulter	A63881
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher	Q33231
Deposited data		
Metagnomics data	NCBI SRA	BioProject PRJNA1182340
Epigenomics data	NCBI GEO	record GSE247470
Experimental models: Organisms/strains		
Mouse: BALB/c	Charles River Laboratories	BALB/c
Mouse: DO11.10	Jackson Laboratories	DO11.10
Software and algorithms		
GraphPad Prism	GraphPad Prism	Graphpad.com
Metagenomic data analysis pipeline using VEBA, SPAdes, MaxBin2, Metabat2, and CONCOCT using GTDB-Tk and CheckM and Bowtie2, as detailed in text (Espinoza and Dupont[121]; Nurk et al.[122]; Camargo et al.[123]; Alneberg et al.[124]; Parks et al.[125]; Langmead and Salzberg[126])		
Illumina Genome Studio V2011.1 with Methylation Module 1.9.0	Illumina	N/A
TIGR MeV 4.9	TIGR MeV Team	https://mev.tm4.org
Partek Genomics Suite 7.20	Partek	Partek.org
GeneGo Metacore	Clarivate	https://portal.genego.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

Time-pregnant (E13) BALB/c mice from Charles River Laboratories were used in this study. The mice were maintained individually at the specific pathogen –free (SPF) barrier facility of Rhode Island Hospital and maintained at 22°C–24°C temperature with a 12-h dark,

12-h light cycle. All mice were provided with commercial pelleted diet & water *ad libitum*. Animal studies were performed in compliance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the IACUC of Rhode Island Hospital (504718). We adhered to the ARRIVE Guidelines in our work.

Particles

The concentrated urban air particles (CAP) were obtained from Boston city air using Harvard Ambient Particle Concentrator[116,117] and are well characterized.[28,118,119] Diesel exhaust particles (DEP), CAS Number 1333-86-4, were generously provided by Dr. Ian Gilmour at the U.S. Environmental Protection Agency and used in earlier studies.[11,28,120] All particles were of comparable, although not identical, ''fine'' size of the PM2.5 class with mean particle diameter of ~1 μm (micrographs can be found in[127]). Particle samples had been baked at 165°C for 3 h to eliminate endotoxin, aliquoted and stored frozen at – 80°C. Before instillation the particles were freshly sonicated on ice to break up clumps and assure a homogeneous suspension using Qsonica Q55 probe sonicator.

Exposures were performed via intranasal instillation of particle suspensions. After light isoflurane anesthesia, a droplet (25 μL) was placed on the nares and inspired by the mouse, followed by another 25 μL volume. The exposures were performed at E14-E20 days of gestation at 8.3 μg/mouse/day. Dams were allowed to give birth; their newborns received a single intraperitoneal (i.p.) sensitization with 50 μg ovalbumin (OVA; grade V, Sigma-Aldrich)) + aluminum hydroxide (Baker) adjuvant at postnatal day P3 and a set of 3 daily 7-min 1% OVA challenges in phosphate-buffered saline (PBS) 2 weeks later (the ''low-dose OVA protocol''). The aerosol exposure was performed within individual compartments of a mouse pie chamber (Braintree Scientific) using a Pari IS2 nebulizer (Sun Medical Supply) connected to an air compressor (PulmoAID; DeVilbiss). This allergen protocol remains innocuous in control pups (which contrasts with a 2X i.p. protocol commonly used in other studies) but elicits asthma-like phenotype in the offspring from particle-exposed mothers and serves to test how exposure to particulates predisposes to asthma. Littermates were randomly assigned to experimental groups.

METHOD DETAILS

Pathophysiologic analysis

24 h after the last allergen aerosol, the mice were euthanized with sodium pentobarbital. Bronchoalveolar lavage (BAL) followed a standard procedure of 5 times X 300 μL washes with PBS.[11] After centrifugation at 1200 RPM (300 G) for 10 min the fluid was stored for cytokine assays. The pellet was resuspended in 100 μL PBS; BAL differential cell counts were performed on cytocentrifuge slides (Cytospin 2; Shandon, Pittsburgh, PA). After lavage, the lungs were extracted and fixed with 10% buffered formalin. After paraffin embedding, sections for microscopy were stained with hematoxylin and eosin (H&E). Slides were scored by a blind observer for 'severity' (score of 1 for 1–3 cells thick, 2 for 4–10 cells thick, and 3 for >10-cells thick) and 'extent' (score of 1 for <25%, 2 for <50% and 3 for >50% coverage) of inflammatory infiltration, and the 'Inflammatory Index' was calculated as 'severity' multiplied by 'extent'.[11,28,120]

Cytokine detection

Levels of cytokines in BAL fluid were measured via ELISA (R&D Systems, Minneapolis, MN). The sensitivity of the IL-4, IL-5 and IL-13 kits varied between 0.3 and 20 pg/mL depending on the cytokine. All the samples were tested in duplicates.

Gut microbiome samples and DNA extraction

Fresh stool pellets (5–6 pellets (~50 mg) from each individual donor mouse) were collected and homogenized in sterile LPS-free PBS without Ca or Mg stirring gently (until the pellets broke up), and the suspension was centrifuged at 1200 rpm (300G) for 3 min. The clear supernatant was transferred into another tube and placed on ice until transplant.

DNA was extracted according to protocol using QIAGEN DNeasy PowerSoil Pro Kit (Cat# 47016, QIAGEN, Hilden, Germany) with mechanically lysed using QIAGEN PowerLyzer 24 Homogenizer (110/220 V) (Cat# 13155, QIAGEN, Hilden, Germany).

Gut microbiome transplant (GMT)

P15-16 (postnatal days 15–16) recipients were pre-treated with antibiotics to wipe-out their host flora: 100 μL of a mixture of ciprofloxacin 5 mg/mL and metronidazole 10 mg/mL were given by oral gavage. The GMT followed at days P19 and P20: the transplant used 100 μL of the homogenized pellet material via oral gavage at two consecutive days (P19 -20). Every recipient was given material from only one donor. After 2 days to recover and for the transplant to settle the recipients were exposed to our low-dose allergen protocol followed by pathophysiologic analysis as described above.

To determine whether viable microbes are necessary for the effect we sterilized the GMT material by gamma radiation prior to its transfer to recipient. We used the Gammacell Cobalt-60 gamma irradiator (Department of Nuclear Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA) to sterilize GMT material using the standard 'medical' sterilization dose of 50 kGy over 24 h on ice. A control sample was shipped alongside the sterilized sample but was not sterilized (termed 'control'), and an additional control included a fresh GMT sample that has not been shipped (termed 'fresh') as effective in preceding studies. Negative controls include sterilized and unsterilized (fresh) samples from the neonates of PBS (vehicle) –exposed mothers.

To test whether viable *bacteria* mediated the effect we used ciprofloxacin 0.5 mg/mouse and metronidazole 1 mg/mouse administered by oral gavage twice with the GMT, then tested as above.

Metabolomics

A metabolomics panel of short chain fatty acids (butyric acid, acetic acid, iso-butyric acid, isocaproic acid, heptanoic acid and propanoic acid) was done by Cosmos ID (Cosmos, Germantown, MD). (n) = 6–15 mice per group.

Cell purification

Splenic DCs were prepared from sterile cell suspensions using positive selection (retaining of CD11c+ cells) via the MACS magnetic bead system (Miltenyi Biotech, Auburn, CA). Purity was monitored via flow cytometry (Miltenyi) by labeling for CD11c/MHC-II. More than 95% of the purified cells were positive for these antigens, and viability was >93% by trypan blue staining. **T-helpers** were isolated similarly as $CD4^+$ cells using Miltenyi MACS system from the spleens of DO11.10 mice and validated for purity via CD4 and KJ1-26 staining. After purification, the cells were washed two times in LPS-free sterile PBS**.**

Additional rationale: allergy is a systemic process and is not confined to the lungs. Spleen is a central organ of the immune system, and is the largest organ from which DCs can be obtained, whereas lung DC yields were prohibitive of the planned analyses. Splenic DCs have been widely used in asthma studies as they acquire the pro-allergy skew,[128–130] possibly due to their active migration and re-migration[30] including from the lung into spleen[131,132] and within spleen[133] and because allergy affects DCs systemically, so that pro-allergy polarization in splenic DCs occurs independent of the sensitization route and in various allergen models.[26,134–136]

Co-culture

Purified cells were plated in 96-well tissue culture plates (Nunc) in RPMI medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin mix using a 1:5 ratio of 0.5X10∧5/well DCs with 2.5X10∧5/well of responder $CD4^+$ KJ1-26+ OVA-specific T-helpers purified from spleens of DO11.10 transgenic mice. Antigen stimulation was achieved by adding tissue culture grade OVA (Sigma) at 100 μg/mL. For the first 24 h (epigenetic treatment) the cells were maintained at 4°C in presence of 125 μM Zebularine or 200 μM Decitabine or 20 μM Cytarabine used as a control agent of similar structure and toxicity but without epigenetic action,[137,140] then co-cultured for another 48 h at 37°C in 5% CO_2 incubator for allergen presentation and proliferation. For the last 18 h the co-cultures were pulsed with 1 μCi/well of tritiated [3H+] thymidine (Sigma). Cells were harvested onto glass fiber pads using the semi-automated PHD cell harvester (Cambridge Technology, Inc, Watertown, MA) which were dissolved in liquid scintillation fluid and counted on a scintillation counter (Beckman, Brea, CA). Negative controls included background (scintillation fluid only), unstimulated OVA-negative co-cultures and 'DC alone' or 'T-helper alone' samples stimulated with OVA. (n) = 4 per group.

DNA for DC epigenomics was isolated using Qiagen DNeasy kit (Qiagen GMBH, Hilden, Germany), in complete adherence with the instructions, and after spectrophotometric assessment of quality, submitted for epigenome-wide methylation profiling.

QUANTIFICATION AND STATISTICAL ANALYSIS

Basic statistics for pathophysiology studies (lavage cells, ELISA, histopathology)

Data were analyzed and plotted as Mean ± SEM in GraphPad Prism 7.02. Normality was first determined via D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. For entirely or largely normally distributed data we applied ANOVA with Fisher's LSD test (linear ANOVA in Prism employs Geisser-Greenhouse correction so as to not assume equal variances); for non-normally distributed data we applied Kruskal-Wallis ANOVA with Dunn's test; in select cases we employed Mann-Whitney U-test for pairwise comparisons. Differences were considered significant when $p < 0.05$. All key experiments were reproduced at least 3 times; (n) was at least 3 dams per group per experiment. Figures show one representative experiment with legends detailing the (n, number of mice) in this experiment; in some cases additional experiments are also detailed (in Figure 1H) or pooled samples from 3 experiments were analyzed (ELISA data). Data are plotted as Mean ± SEM.

Metagenomics

Metagenomic libraries were prepared using the NEBNext Ultra II FS DNA Library Prep Kit (Cat# E7805L, New England Biolabs, Ipswich, MA, USA) in combination with the NEBNext Multiplex Oligos for Illumina (Cat# E6448S, New England Biolabs, Ipswich, MA, USA), following the manufacturer's protocol. For adaptor ligation, the NEBNext Adaptor for Illumina was diluted 10-fold in 10 mM Tris-HCl (pH 7.5–8.0) with 10 mM NaCl. Samples were then cleaned using Ampure XP beads (Cat# A63881, Beckman Coulter, Pasadena, CA, USA) at a 0.9X bead-to-sample ratio. Samples were enriched by PCR and libraries were quantified by Qubit 1X dsDNA HS Assay Kit (Cat# Q33231, Thermo Fisher Scientific, Waltham, MA, USA) and the average fragment size was assessed using the Bioanalyzer High Sensitivity DNA Analysis (Cat# 5067-4626, Agilent Technologies, Santa Clara, CA, USA). Libraries were manually normalized based on DNA concentration and average fragment size, then pooled. The library was then loaded onto a NovaSeq 6000 S2, 300 cycles (2x150 bp) v1.5 system according to the manufacturer's instructions (Cat# 20028314, Illumina Inc., La Jolla, USA). (n) = 12–14 mice per group.

Sequencing data analysis

Data analysis was done as previously described by our group.[138] We employed VEBA v1.2.0 to conduct a metagenomics analysis, assembling and binning metagenomes separately for species-level clustering and orthology analysis.[121] Metagenomes were assembled using SPAdes v3.15.2q. Prokaryotic genomes were binned with MaxBin2, Metabat2, and CONCOCT, and further refined with DAS Tool, which were classified and quality-assessed using GTDB-Tk and CheckM.[122–125] Read preprocessing and mapping were handled using VEBA's preproces.py, Bowtie2, and feature Counts to assess abundance and expression across the metagenomics dataset.[126]

KEGG pathway enrichment was performed using the gseKEGG function in clusterProfile, identifying significant pathways associated with differentially abundant microbes, and metabolomics data were analyzed using a t test with an FDR threshold of 0.05. All analyses were conducted with adjusted p-values <0.05.

Metagenomics data were made publicly available via NCBI SRA, BioProject: PRJNA1182340.

Epigenome-wide methylation profiling

Epigenome-wide methylation profiling was performed via The Infinium Mouse Methylation BeadChip arrays (Illumina, San Diego, CA) at the Eurofins Clinical Enterprise Inc. (Framingham, MA, USA) – an Illumina-approved service vendor. Data analysis is detailed in part in the Results section. In other detail, after quality control via Illumina Genome Studio V2011.1 with Methylation Module 1.9.0 which determined that the performance of samples and chips including hybridization and bisulfite conversion were satisfactory, a matrix of beta-values was extracted. The beta-values informed Pavlidis Template Matching analysis and some of the ANOVA and cluster analysis in TIGR MeV 4.9. Moreover, raw data were loaded into Partek Genomics Suite 7.20 (Chesterfield, MO, USA) with extraction of beta-values and conversion into M-values which informed most of the analyses. The extraction and conversion employed normalization with Noob (normal-exponential out-of-band) background correction and dye correction; X and Y chromosome loci were excluded hence the analysis only includes autosomal loci. We then employed ANOVA with select pairwise post-ANOVA 'contrasts' to obtain lists of differentially methylated loci (DML) at particular p-value and fold-change thresholds specified in Results. Data are plotted as Venn diagrams illustrating the number of DMLs in select comparisons, and as heatmaps with hierarchical clustering. (n) = 24. Raw data have been submitted to NCBI GEO, record GSE247470.

Network analysis and pathway enrichment

Network analysis and pathway enrichment were performed via GeneGo Metacore (Clarivate, PA, USA). The CpGs in a list of DMLs were annotated by the nearest gene using Illumina annotation manifest ''MouseMethylation-12v1-0_A1_Annotation_Mus_musculus'', the resultant list of gene names was used for network and pathway analyses.

Metacore is a large curated database of known factor-to-factor interactions and allows designing networks based on these known 'interactions'.[139] The 'direct interactions' algorithm thus builds a graphical network of factors from a list (without adding intermediaries) connected by lines depicting a published interaction between the two factors. Metacore also performs Go enrichment using public Go Process and Go Molecular Functions data. Finally, Process Networks are network models created by Metacore on the basis of cellular process and pathway knowledge in their database; each Process Network reported as significantly enriched with the genes from the input list can be visualized, is interactive, and highlights each gene/factor from the list with a red circle to stand out in contrast to other factors that form the network but were not in the input list.