Genes controlling the activation of natural killer lymphocytes are epigenetically remodeled in intestinal cells from germ-free mice

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ABSTRACT: Remodeling of the gut microbiota is implicated in various metabolic and inflammatory diseases of the gastrointestinal tract. We hypothesized that the gut microbiota affects the DNA methylation profile of intestinal epithelial cells (IECs) which could, in turn, alter intestinal function. In this study, we used mass spectrometry and methylated DNA capture to respectively investigate global and genome-wide DNA methylation of intestinal epithelial cells from germ-free (GF) and conventionally raised mice. In colonic IECs from GF mice, DNA was markedly hypermethylated. This was associated with a dramatic loss of ten-eleven-translocation activity, a lower DNA methyltransferase activity and lower circulating levels of the 1-carbon metabolite, folate. At the gene level, we found an enrichment for differentially methylated regions proximal to genes regulating the cytotoxicity of NK cells (false-discovery rate \( < 8.9 \times 10^{-6} \)), notably genes regulating the cross-talk between NK cells and target cells, such as members of the NK group 2 member D ligand superfamily Raet. This distinct epigenetic signature was associated with a marked decrease in Raet1 expression and a loss of CD56+/CD45+ cells in the intestine of GF mice. Thus, our results indicate that altered activity of methylation-modifying enzymes in GF mice influences the IEC epigenome and modulates the cross-talk between IECs and NK cells. Epigenetic reprogramming of IECs may modulate intestinal function in diseases associated with altered gut microbiota.—Poupeau, A., Garde, C., Sulek, K., Citirikkaya, K., Treebak, J. T., Arumugam, M., Simar, D., Olofsson, L. E., Bäckhed, F., Barrès, R. Genes controlling the activation of natural killer lymphocytes are epigenetically remodeled in intestinal cells from germ-free mice. FASEB J. 33, 2719–2731 (2019). www.fasebj.org

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Changes in the gut microbiota are associated with many diseases characterized by altered intestinal dysfunctions and chronic activation of the immune system. In addition to intestinal disorders, such as inflammatory bowel diseases (1), altered gut microbiota has also been linked to metabolic disorders, including obesity (2–4), type 2 diabetes (5–7), non-alcoholic fatty liver disease (8), and atherosclerosis (9, 10). Epigenetic information regulates gene expression in a cell-type–specific fashion during the various states of cell development and differentiation. DNA methylation, post-translational modification of histone proteins, and expression of small noncoding RNA constitute epigenetic marks that have the potential to be mitotically heritable. Methylation of DNA is a widely studied epigenetic mark, which alters the access of cis- and trans-activating DNA binding protein to the DNA and consequently modulates gene expression. Aside from a role in normal cell development,
DNA methylation is implicated in pathologic developments, such as the Beckwith-Wiedemann, Silver-Russell, and Prader-Willi syndromes (11). Alteration of the DNA methylation blueprint has been identified in many autoimmune diseases in which lifestyle and environmental agents are risk factors, such as type 1 diabetes (12) and inflammatory bowel diseases (13). Abnormal intestinal cell development in mice with disrupted DNA methyltransferases supports a role for DNA methylation in intestinal development (14).

The influence of the gut microbiota on intestinal epithelial cell (IEC) function and on the phenotype of the host could be mediated through the synthesis of specific metabolites. Notably, the gut microbiota may alter the epigenome of the intestinal cell by modulating availability of 1-carbon metabolites used for setting epigenetic marks (15). Indeed, folate-synthesizing gut bacteria, such as bifidobacteria, contribute to the homeostasis of circulating folate in the host (16–19) and may modulate the bioavailability of the methyl-donor S-adenosylmethionine (SAM) and, ultimately, lead to remodeling of the DNA methylome. Similarly, the gut microbiota influences the chromatin state of various tissues, including colonic cells, an effect likely driven by the production of short-chain fatty acids (20). Other metabolites, such as fatty acids that can be synthesized or transformed by the gut microbiota, influence the DNA methylation profile of somatic cells (21). Importantly, the epigenome of the intestine, and in particular of the IECs, is critical for proliferation and differentiation of IECs, as well as the inflammatory response and integrity of the intestinal–epithelial barrier (22, 23).

Germ-free (GF) mice are bred and housed in an isolated environment in the absence of microorganisms, and are commonly used as experimental models to study the influence of the microbiota on the host. Using the GF mouse model, a link among the metabolic, behavioral, and developmental features of the host, including the maturation of the immune system, has been clearly established. Although eutherian fosters reside in a sterile environment until birth (24), they are exposed to microbial products and metabolites from the mother’s microbiota during pregnancy (25). Thus, GF mice are neither exposed to microbes nor microbial products and metabolites from early development. Gene-specific investigation of the TLR4 gene showed lower methylation levels in IECs of the large intestine from GF, compared with conventionally raised (Conv-R) mice (19). This supports the notion that the gut microbiota controls intestinal function through epigenetic mechanisms.

Here, we investigated global and genomewide DNA methylation in GF, compared with Conv-R mice to determine the influence of the gut microbiota on the DNA methylation signature of IECs. We found that a lack of gut microbiota was associated with a different epigenetic signature of genes controlling the cytotoxicity of NK cells, such as genes regulating the crosstalk between NK and target cells. This distinct epigenetic signature was associated with a functional loss of CD56+CD45+ cells in the intestine of GF mice, supporting a role for gut microbiota in the regulation of the immune system.

MATERIALS AND METHODS

Animal experiments

Experiments were approved by the Danish Animal Experiments Inspectorate (Glostrup, Denmark) and the local Animal Ethics Committee in Gothenburg, Sweden. All experiments were performed according to local guidelines. For the succinylsulfathiazole treatment, 20 Swiss Webster, 8-wk-old mice were purchased from Taconic Farms (Germantown, NY, USA) and were housed in ventilated cages. Ten mice were acclimated for 7 d then fed an additional 7 d of a control chow diet ad libitum (5020; 2.9 ppm folic acid; LabDiet, St. Louis, MO, USA) or the same diet supplemented with 1% succinylsulfathiazole. Mice were euthanized at 12 wk old. For the GF and Conv-R mice sequencing experiments, 5 Conv-R and 7 GF Swiss Webster male mice were maintained in flexible film isolators under a 12/12-h light/dark cycle and were freely fed an autoclaved chow diet (5010; LabDiet) (26) at the University of Gothenburg (Gothenburg, Sweden). All mice were euthanized at 12–13 wk old. For the folic acid supplementation, GF and Conv-R mice were divided in 2 groups with 1 group supplemented with folic acid at 2 μg/g/d in drinking water, and the other group supplemented with vehicle (NaOH) (7 Conv-R–vehicle, 7 Conv-R–folic acid, 6 GF–vehicle, 7 GF–folic acid).

IEC preparation

Mice were anesthetized with isoflurane, and blood samples were collected from the portal vein or the vena cava with a syringe coated with heparin or EDTA, respectively. Blood was centrifuged at 10,000 g at 10°C for 15 min, and plasma was snap frozen in liquid nitrogen for later analysis. Small intestines, cecum, colon, and liver were dissected. Liver was immediately snap-frozen in liquid nitrogen for later analysis. To collect epithelial cells from the intestines, we adapted a protocol previously described by Larsson et al. (26) and Nik and Carlsson (27). The small intestine was divided into 8, and the colon into 2, equal-sized segments, and IECs were collected from the first (duodenum), fifth (jejunum), and eighth (ileum) segments and from the proximal piece of the colon (Supplemental Fig. S1A). The specific intestine segments were washed with cold PBS (without Ca2+ or Mg2+), inverted inside-out, and bloned with air to expose the epithelial cell layer to a nonenzymatic buffer for 40–50 min (Cell Recovery Solution; Corning Life Sciences, Corning, NY, USA). Epithelium cell sheets were detached from the lamina propria by applying vigorous shakes. Recovered cells were divided into aliquots then centrifuged for 5 min at 5000 g and 10°C. Cell pellets were immediately snap-frozen in liquid nitrogen until further analysis. Cell integrity was assessed by optical microscopy (Supplemental Fig. S1B).

Nucleic acid purification

Genomic DNA was extracted with a DNeasy Mini Kit (Qiagen, Hilden, Germany) and the concentration was measured by fluorometry (Qubit double-stranded DNA; Thermo Fisher Scientific, Waltham, MA, USA). RNA was extracted using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. RNA concentration and integrity were measured by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and capillary electrophoresis on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) analysis was performed as follows. For the RT step, 1 μg of RNA was used as template for cDNA synthesis using the SuperScript III Reverse Transcriptase

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Proteins were extracted from both cytosolic and nuclear fractions.

Protein extraction and Western blot analysis

Blood analyses

Blood analyses were performed at the Klinik Biokemisk Afdeling (Rigshospitalet, Copenhagen). Folate and cobalamin concentrations in plasma were measured by competitive electrochemiluminescent immunoassay, with the Eлючys Folate III and Eлючys Vitamin B12 II Kits (Roche Diagnostics, Basel, Switzerland) on a Cobas 8000 instrument (Roche Diagnostics). These assays were calibrated for a concentration range corresponding to levels found in human plasma; thus, mice plasma was diluted up to 37 times before analysis.

Quantification of folate from IECs

Quantification of folate in colonocytes was performed with the Accubind ELISA Folate test system (Monobind, Lake Forest, CA, USA), according to the manufacturer’s instructions, in replicates on 3–10 µg of nuclear extract. Absorbance (optical density, 450 nm) was measured on a Hidex Sense microplate reader and reported to the negative and positive controls provided by the manufacturer. For SAM measurements, duodenal and colonic cells were lysed in 50 µl of 10% NP40 detergent solution and sonicated for 3 cycles of 30 s at 4°C using a Bioruptor Plus (Diagenode, Liege, Belgium) sonication device. Solubilized fragments were sonicated at 12,000 g at 4°C for 10 min; 100 µl of supernatant was transferred into 10-ml glass tubes to proceed with the manufacturer’s protocol. Absorbance at 450 nm on one-fifth–diluted fractions was determined with a Hitex Sense microplate reader (Hitex, Turku, Finland) and was reported to the standards provided by the manufacturer.

Protein extraction and Western blot analysis

Proteins were extracted from both cytosolic and nuclear fractions. Colonic epithelial cells were homogenized with a dounce tissue grinder and pestle (MilliporeSigma); 25 µl of 10% NP40 detergent solution was added to the cell homogenates for 15 min at 4°C, then centrifuged for 10 min at 3000 g. The cell lysate buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSE, 10 mM NaF, 5% glycerol, and Complete EDTA-Free Inhibitor Cocktail 1 time) and centrifuged for 30 min at 14,000 g at 4°C; 3–10 µg of nuclear extract proteins were used for Western blotting and enzymatic assays. Protein concentration was measured by colorimetric assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific). Western blotting was performed according a standard protocol previously described (28–31). Primary and secondary antibodies were DNA methyltransferase (DNMT)-1, DNMT3A, lamin A/C (Cell Signaling Technology, Danvers, MA, USA), and goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories). After 1 h of incubation in the secondary antibody, proteins were detected with the Luminata Forte Western horseradish peroxidase substrate (MilliporeSigma). Image acquisition was performed on a Molecular Imager ChemiDoc XRS+ (Bio-Rad Laboratories), and quantification was made with the Image Lab software (Bio-Rad Laboratories).

Enzymatic assays

Colorimetric ELISA-based enzymatic assays (Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit and EpiQuik DNMT Activity/Inhibition Assay; EpiGentek, Farmingdale, NY, USA) were used according to the manufacturer’s instructions, in replicates on 3–10 µg of nuclear extract. Absorbance (optical density, 450 nm) was measured on a Hitex Sense microplate reader and reported to the negative and positive controls provided by the manufacturer. For SAM measurements, duodenal and colonic cells were lysed in 50 µl of water in a Tissuedyser II (Qiagen) at 20 Hz for 1 min. Then the homogenate was split into 2 fractions. One fraction was used to measure protein concentration by bicinchoninic acid assay, and the other was used to measure the SAM concentration with a fluorescence assay relying on fluorescence resonance energy transfer technology (Bridge-It SAM; Mediomics, St. Louis, MO, USA). Fluorescence values were normalized to the amount of protein in each sample.

Methylated DNA capture (methyl-cytosine-phosphate-guanine-binding domain pulldown)

DNA samples were sheared with a Bioruptor Plus (Diagenode) sonication device to obtain an average DNA size of 250 bp, which was confirmed on a Bioanalyzer instrument (DNA 2100 kit; Agilent Technologies). Methylated DNA was isolated using the MethylMiner Methylated DNA Enrichment Kit (Thermo Fisher Scientific). This method uses the properties of the methyl-cytosine-phosphate-guanine (CpG)-binding domain to capture DNA fragments with CpG methylation. The total captured DNA fraction was used for preparation of the sequencing libraries (NEBNext Ultra DNA Library Prep Kit for Illumina; New England Biolabs, Ipswich, MA, USA) using the previously described protocol (32). Quality control of libraries was performed on a Bioanalyzer instrument (Agilent Technologies), and libraries were subjected to 100-bp single-end sequencing on a HiSeq 2500 system (Illumina, San Diego, CA, USA) at the Danish National High-Throughput DNA Sequencing Centre (Copenhagen, Denmark).

Bioinformatic analyses

Methyl-CpG-binding domain sequence reads were trimmed for adapters and low-quality flanking ends using Trim Galore! (v.0.3.7; Babraham Institute, Cambridge, United Kingdom) and Cutadapt (v.1.4.2; Massachusetts Institute of Technology, Cambridge, MA, USA). Processed reads were mapped to mm10 using Rsubread (33). Consensus peaks (value of P < 0.05) were called using Macs2 (34) on the full assembly of samples, including all 4 intestinal sections of both GF and Conv-R mice. We used macs2 callpeak--down-sample-seed -1 -g hs -f BAM -t InputFile for peak calling. The sample specific read coverage of each consensus peak was computed with featureCounts (33). The subset of the consensus peaks that survived an inclusion criterion of 0.5 reads per
kilobase million in ≥8 samples were used in the downstream analysis. Differential analysis between the GF and Conv-R mice was conducted using edgeR (35) with the glmQLFt/glmQLFTest modeling framework and the following model: “−0 + Treatment: Section + Treatment:Mouse.” Differential methylated regions were defined with a false-discovery rate (FDR) < 0.1, and biologic context was assigned based on most proximal gene using ChIPseeker (36). Data have been deposited with Gene Expression Omnibus (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/geo/) as accession GSE117434.

Quantification of global DNA methylation levels by liquid chromatography–tandem mass spectrometry

Global DNA methylation was analyzed using an adjusted protocol developed previously by Quinlivan and Gregory (37). Briefly, DNA samples were digested by adding 100 ng of DNA in 10 μl of ultrapure water into 10 μl of buffer [250 U of benzonase (SRP6593), 300 mU of phosphodiesterase I from Crótilus adamanteus venom (P92322) and 200 U of alkaline phosphatase from bovine intestinal mucosa (A2256)] into 5 μl of 20 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl and 20 mM MgCl₂. All salts were purchased from MilliporeSigma at analytic grade and incubated at 37°C for 6 h. A positive control (containing a synthetic sequence with 50% CpG) and a negative control without CpG (EpiGentek) were digested in parallel with the genomic DNA. Samples were stored at −20°C until the analysis. All chemicals used in the standard solution and calibration curve (A, C, T, G, U, dC, dG, dA, 5mdC) were obtained from MilliporeSigma, except for 5mdC (Sc-278256), which was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Before liquid chromatography–tandem mass spectrometry (LC-MS/MS), 20 μl of hydrolysis product was mixed with 20 μl of ultrapure water kept on wet ice; 20 μl of the diluted sample was set in a prechilled LC-MS glass vial. Samples were maintained at 6°C throughout the analysis. Chromatographic separation was performed with Ultra HPLC Dionex Ultrasphere 3000 (Thermo Fisher Scientific) with an EVO C18 analytic column (1.7 μm, 2.1 × 100 mm; Phenomenex, Torrance, CA, USA) and an EVO C18 guard column (sub-2 μm, 2.1 mm; Phenomenex) kept at 40°C. Gradient elution and the flow rate were applied as specified by Zhang et al. (34). LC was coupled with a QToF Impact II mass spectrometer (Bruker Daltonics, Billerica, MA, USA) operating in positive ion mode. The spectrometer was set to acquire at 3.00 Hz spectra rate using the source settings for tandem mass spectrometry. The m/z range was 100–1000, with an ion acceptance threshold forgate of 3000. The acceleration voltage of the MS was 4.0 kV, and the capillary voltage was 500 V. The typical cone and collision energies were 40 eV and 15 eV, respectively. The detector was operated in the selected ion monitoring mode. Global DNA methylation was analyzed using the adjusted protocol developed previously by Quinlivan and Gregory (37). Briefly, DNA samples were digested by adding 100 ng of DNA in 10 μl of ultrapure water into 10 μl of buffer [250 U of benzonase (SRP6593), 300 mU of phosphodiesterase I from Crótilus adamanteus venom (P92322) and 200 U of alkaline phosphatase from bovine intestinal mucosa (A2256)] into 5 μl of 20 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl and 20 mM MgCl₂. All salts were purchased from MilliporeSigma at analytic grade and incubated at 37°C for 6 h. A positive control (containing a synthetic sequence with 50% CpG) and a negative control without CpG (EpiGentek) were digested in parallel with the genomic DNA. Samples were stored at −20°C until the analysis. All chemicals used in the standard solution and calibration curve (A, C, T, G, U, dC, dG, dA, 5mdC) were obtained from MilliporeSigma, except for 5mdC (Sc-278256), which was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

RESULTS

High DNA methylation in intestinal cells from GF mice

To determine whether the presence of gut microbiota influences the epigenome of intestinal cells, we first investigated global DNA methylation levels in 4 distinct portions of the intestine (Supplemental Fig. S1A) from GF vs. Conv-R mice. Global methylation was markedly higher in the proximal colon from GF mice compared with Conv-R mice, whereas it was unchanged in the duodenum, jejunum, and ileum (Fig. 1A). Given the important crosstalk between the liver and the intestine, we also investigated global DNA methylation in the liver but found similar global DNA methylation levels in GF and Conv-R mice (Supplemental Fig. S2).

The gut microbiota may have a role in the DNA methylation footprint of the intestinal cell through modulation of folate, a precursor for the synthesis of the methyl donor SAM (18) (Supplemental Fig. S3). To determine whether a relationship exists between global hypermethylation and folate levels, we tested whether GF mice had altered levels of folate compared with Conv-R. Although we found only a trend for lower plasma folate levels in the portal vein of GF mice (Fig. 1B), folate content was decreased in venae cava and in colonocytes (Fig. 1C, D). Intriguingly, despite the altered folate levels in colonocytes, we found similar levels of SAM in colonocytes from GF mice compared with Conv-R mice (Fig. 1E).

To gain insight into the link between folate and methylation, we performed a correlation analysis between

Immunofluorescence

Female Swiss Webster mice were perfused with 4% (w/v) paraformaldehyde in PBS. Intestines were dissected, postfixed in 4% paraformaldehyde, and transferred to 30% (w/v) sucrose in PBS overnight at 4°C. Parts of the duodenum, ileum, and colon were embedded in optimal cutting temperature (OCT) compound (HistoLab, Gothenburg, Sweden), frozen, and kept at −80°C until sectioned using a cryostat. For CD56 and CD45 staining, 10-μm-thick sections were incubated sequentially for 10 min each in 0.3% (w/v) glycine solution and 0.3% (w/v) SDS solution. Sections were blocked in 10% donkey serum for 1 h at room temperature, incubated with primary antibodies against CD56 (AF2048, dilution 1:1000; R&D Systems, Minneapolis, MN, USA) and CD45 (ab25386, dilution 1:250; Abcam, Cambridge, United Kingdom) overnight at 4°C, and washed in PBS–Tween. Sections were then incubated with secondary donkey anti-goat antibody conjugated to Alexa Fluor 488 dye and secondary donkey anti-rabbit antibody conjugated to Alexa Fluor 594 dye (Alexa Fluor, Thermo Fisher Scientific) for 1 h at room temperature. Hoechst solution (dilution 1:10,000, H1399; Thermo Fisher Scientific) was used to visualize cell nuclei. Fluorescence images were captured with an AxioPlan 2 (Carl Zeiss, Oberkochen, Germany) imaging system equipped with an AxioCam digital camera HRc (Carl Zeiss) using the program AxioVision 4.8.2.0 (Carl Zeiss). Three ×10 pictures/condition were used to count double-positive fluorescent cells using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistics

Statistical analysis was performed using Prism software (GraphPad Software, La Jolla, CA, USA). Statistical difference between 2 groups was tested using nonparametric Mann–Whitney tests. Data from GF and Conv-R mice, treated with or without folic acid, were tested by 2-way ANOVA, with a Sidak multiple-corrections posttest. Normal distribution and homogeneity of variance was achieved, when appropriate, by log2 transforming the data before 2-way ANOVA. A value of P < 0.05 was considered statistically significant.
creased DNA methylation levels of colonocytes from GF mice. We next tested the potential role of DNMTs and enzymes in colonocytes from GF mice. TET and DNMT enzymatic activities are lower in colonocytes from GF mice only (low plasma folate was associated with low colonocyte methylation in GF, but not in Conv-R, mice; Supplemental Fig. S4).

Microbial-derived folate is unlikely to contribute to global DNA methylation

Previous reports have established that microbiota-derived folate is predominantly produced in the colon (16, 39). To further investigate the role of folate-producing bacteria on DNA methylation of intestinal cells, we supplemented Conv-R mice for 7 d with succinylsulfathiazole (SST). SST is a poorly absorbed antibiotic that is described to cause decreased circulating folate levels through the inhibition of the conversion of phosphoaminobenzoate (p-aminobenzoate) to folate by the gut microbiota (40–42) (experimental design and mode of action described in Fig. 3A). Compared with control animals, mice treated with SST showed 15% lower systemic folate levels, confirming the role of the gut microbiota in folate levels (Fig. 3B). This difference in folate levels is comparable to the difference we detected between Conv-R and GF mice (Fig. 1B; 217 ± 37 vs. 184 ± 15.1 nM, respectively). Importantly, in contrast to GF mice, in SST-treated animals, nuclear amounts of DNMT1 and DNMT3A (Fig. 3C, D) and overall DNMT (Fig. 3E) and TET activity (Fig. 3F) were unchanged in colonocytes. In addition, global DNA methylation was not changed in IECs from the small intestine or the proximal colon (Fig. 3G).

To further investigate the role of folate homeostasis on global DNA methylation, we supplemented GF and Conv-R mice with 2 μg/d/kg of folic acid or vehicle (experimental design depicted in Supplemental Fig. S5A). Folic acid supplementation led to a marked increase in circulating folate levels in both GF and Conv-R animals (Supplemental Fig. S5B). Global DNA methylation differences in the colon of Conv-R and GF mice were abolished by folic acid supplementation (Supplemental Fig. S5C), which indicates that dietary folate can modulate global methylation in the colon.

TET and DNMT enzymatic activities are lower in colonocytes from GF mice

We next tested the potential role of DNMTs and enzymes participating in DNA demethylation pathway on the increased DNA methylation levels of colonocytes from GF mice. Although intranuclear expression of DNMT3A was not changed, DNMT1 was markedly lower in GF, compared with Conv-R mice (Fig. 2A, B). DNA methyltransferase activity was lower in GF, compared with Conv-R mice (Fig. 2C). These results indicate that the gut microbiota influences DNA methyltransferase activity in colonocytes; however, our results, which show that lower DNMT activity in colonocytes is associated with higher methylation levels in GF mice, indicate that higher methylation is not caused by a change in DNMT1 activity. Thus, we next investigated whether increased global DNA methylation levels could be linked to lower activity of the TET enzymes, which participate in active DNA demethylation (38). Strikingly, TET activity was virtually absent in colonocytes extracted from GF mice, whereas it was readily detected in Conv-R (Fig. 2D). Thus, our results suggest that increased methylation in the proximal colon from GF animals may be caused by the loss of TET activity.

Intestinal cells from GF mice show gene-specific DNA methylation remodeling

Global DNA methylation does not indicate whether specific genes are differentially methylated. Thus, we established the DNA methylation signature of IECs from the...
duodenum, jejunum, ileum, and colon of GF mice using genomewide analysis of DNA methylation and compared it with the epigenetic signature in IECs from Conv-R mice. Using methylated DNA-capture sequencing, we identified a total of 947 differentially methylated regions (DMRs) in all intestinal segments investigated (FDR < 10%). Of the different intestinal segments tested, the duodenum and the colon carried the most of the unique DMRs, with, respectively, 148 and 92 unique DMRs (Fig. 4A). Most (n = 149; 65.6%) of the 227 DMRs common to all segments were hypermethylated in the colon of GF mice, which is consistent with our global DNA methylation results (Fig. 4B).

Analysis of the genomic localization of the DMRs in colonic cells revealed specific enrichment outside gene bodies and promoters (odds ratio > 1 in intergenic regions; Fig. 4C), with greater frequency of DMRs between 100 and 250 kb (Fig. 4D). Pathway analysis of the genes proximal to the DMRs using g:profiler (43) returned an enrichment for the Kyoto Encyclopedia of Genes and Genomes (Kyoto University, Kyoto, Japan) pathway NK cell–mediated cytotoxicity. Of interest, we found that the genes enriched in that pathway were common to all 4 segments of the intestine (Fig. 4E).

Of the 10 genes enriched under the NK cell–mediated cytotoxicity pathway, we found that 6 were members of the subfamily of killer cell lectin-like receptor (Klra). We also identified protein tyrosine phosphatase, nonreceptor type 11 (Ptpn11), son of sevenless homolog 2 (Sos2), UL16 binding protein 1 (Ulbp1), and retinoic acid early transcript 8 (Raet1d) (Fig. 4E). These results are consistent with our previous transcriptomic analysis in GF and Conv-R mice showing that numerous genes related to adaptive and innate immunity are expressed at low levels in IECs from GF mice (26). Specifically, we reported earlier that, in GF mice, Ptpn11 is significantly down-regulated in the duodenum (0.88-fold), whereas Sos2 is up-regulated in the ileum and colon (1.34- and 1.14-fold, respectively) (26). Here, we found a marked hypermethylation signal in all intestinal segments on a region located within Chr10 and including the cluster of Raet1 genes, a family of ligands of the NK cell receptor NKG2D (Fig. 5A).

Previously, we reported that DNA methylation is reprogrammed in immune and somatic cells, notably in the context of intestinal surgery (44–46). To rule out that a contamination with inflammatory cells in our preparation of IECs caused, at least in part, detection of DMRs proximal to genes related to inflammation, we measured the mRNA expression of the leukocyte receptor CD45. We found similar Cd45 expression in the duodenum and colon and lower expression in the jejunum and ileum in cell preparations from GF, compared with Conv-R, mice (Supplemental Fig. S6). In contrast, analysis of 2 DMRs near the Raet1a/Raet1e/H60b cluster transcription start site returned similar magnitudes in fold change between Conv-R and GF mice across intestinal segments (Supplemental Table S1), supporting the idea that the presence of CD45+ cells did not drive differential methylation of the Raet1a/Raet1e/H60b cluster in GF mice.

Figure 2. Lower DNMT and TET activity in colonocytes from GF mice. A) Western blot detection of nuclear DNMT1 and DNMT3A content. B) Quantification of DNMT3A (left) and DNMT1 (right) relative to lamin A/C. C) Total DNMT activity, representing cumulative activity of all the DNMTs in nuclear extracts. D) Total TET activity, representing cumulative activity of all the TET enzymes in nuclear extracts. Conv-R (n = 7). GF (n = 6). Data represent means ± sd. *P < 0.05, **P < 0.01 (nonparametric Mann-Whitney test).
Figure 3. Lower folate levels after SST treatment does not alter global DNA methylation. Control Swiss Webster, male mice were given a control diet, with or without 1% SST for 7 d. A) Graphic representation of experimental setting. This illustration has been partly generated using fully free-license–rights images from Servier Medical Bank (http://www.servier.com/Powerpoint-image-bank). B) Plasma folate concentration in portal vein. C) Western blot detection of nuclear DNMT1, DNMT3A, and lamin A/C. The vertical, dotted line corresponds to the removal of an empty well from the original image, where no nuclear extract was loaded. D) Quantification of DNMT1 (left) and DNMT3A (right) expression relative to lamin A/C. E) Total DNMT activity, representing cumulative activity of all the DNMTs in nuclear extracts. F) Total TET activity, representing cumulative activity of all the TET enzymes in nuclear extracts. G) Global DNA methylation as assessed by the ratio of methylated deoxycytidine (5mC) to total deoxycytidine (dC), in various intestinal segments. Control, n = 10; SST treated, n = 10. Data represent means ± sd. **P < 0.01 (nonparametric Mann-Whitney test).
Lower infiltration of NK cells in the intestine epithelium of GF mice

To determine whether differential methylation was associated with changes in gene expression, we measured mRNA expression of genes near to the DMRs in the Raet1a/Raet1e/H60b cluster. Although expression of H60B, Raet1a, Raet1b, and Raet1d was not detected by qRT-PCR in our IECs preparation, we found that Raet1e expression was markedly lower in the ileum from GF mice compared with Conv-R mice (Fig. 5B), suggesting that transcriptional activity of the Raet1e gene is regulated by DNA methylation. Because the Raet family members regulate the crosstalk between NK and target cells (Fig. 5C) (47), we investigated the link between epigenetic and expression difference for the Raet1e gene and the infiltration of NK cells in intestinal epithelium from GF mice. Using immunofluorescence and the quantification of CD56+/CD45+ cells, we found a markedly lower presence of NK cells within the epithelium of GF, compared with Conv-R mice (Fig. 6).

Thus, our results collectively suggest that epigenetic remodeling in the Raet1a/Raet1e/H60b cluster has a functional role on the infiltration of NK cells in the intestine of GF mice.

DISCUSSION

Here, we demonstrate that a lack of microbiome in GF mice is associated with marked epigenetic remodeling in intestinal cells. We provide insight into the possible implication of gut microbiome-dependent epigenetic signatures on immune cell infiltration.
Our results suggest that dietary folate, but not folate produced by the microbiota, is associated with global DNA methylation levels in colonic intestinal epithelial cells. We detected global DNA methylation changes in the proximal colon and not upper portions of the intestine. The fact that global DNA methylation is greater in colonic IECs from GF mice, but not from the other parts of the intestine, can potentially be explained by the important concentrations of folate in the colon (48, 49). Indeed, not only are bacteria-producing folate predominantly present in the colon (16, 39) but also the colon wall expresses the folate transporters reduced carrier folate and folate receptor 1.

Intriguingly, our results showing that global DNA hypermethylation in the colon is associated with decreased intracellular levels of folate in colonocytes are in contradiction with the consensus that low folate levels are associated with low DNA methylation levels. However, several studies have reported a paradoxical relationship
between low folate and high DNA methylation. For example, a folate-deficient diet induces a 56% increase in global DNA methylation in the mouse liver (38). Folic acid deprivation in cell cultures increases global DNA methylation in a cell-type–specific manner (50), and folate-deprived neurons exhibit higher global DNA methylation compared with control cells, but with SAM, the levels are elevated after folate deprivation (51). Our results on SAM levels, showing no significant increase of SAM in GF mice, could be caused by a lack of statistical power or sensitivity of the assay. Alternatively, the steady-state colonocyte content of SAM could be truly unchanged in GF mice, which would not argue in favor of a mechanism by which folate alters SAM levels. Consistent with that, several studies have shown that moderate folate depletion does not change SAM (or the ratio of SAM to S-adenosylhomocysteine) in colonocytes (52, 53). Because SAM is also contributing to a methyl donor for protein methylation, folate depletion could affect the allocation of SAM to DNA methylation without altering the steady-state pools. Our correlation analysis, showing that DNA methylation is correlated with plasma folate in GF mice only, suggests that GF mice have specific mechanisms controlling intracellular folate homeostasis and 1-carbon metabolism. Yet, more experiments are needed to elucidate the specific relationship between plasma folate and DNA methylation in intestinal cells in GF mice.

Figure 6. Lower representation of CD56+/CD45+ cells in duodenum, ileum, and colon from GF mice, compared with Conv-R mice. A, B) Representative immunofluorescence images showing CD56+/CD45+ cells in the colon from Conv-R (A) and GF (B) mice. Scale bars, 50 μm. C) Total counts of cells expressing both CD56 and CD45 in Conv-R and GF mice. Conv-R, n = 5; GF, n = 5. Data represent means ± SD.*P < 0.05 (nonparametric Mann-Whitney test).
intestinal fractions in Conv-R mice, indicate that, if differential absorption has a role, other factors are likely to be involved. Our observation that SAM content in colonocytes is not affected by the low folate levels and that folate content in colonocytes is not associated to global methylation changes, suggests complex and dynamic links between the various pools of methyl donors. Several studies have reported that folate transporters are up-regulated in the colon in situations of folate deficiency (55, 56), suggesting that mild folate depletion causes an enhanced efficiency in folate use in colon epithelial cells from GF mice. Folate supplementation and deprivation experiments, performed in a time-course fashion, as well as the analysis of folate transporter expression and folate absorption rate across the colonic epithelium, are requested to fully elucidate the influence of folate on DNA methylation in intestinal cells of GF mice.

We found a marked difference in global DNA methylation in colon from GF, compared to Conv-R, mice. Given the importance of epigenetic information in normal cell function, notably IECs (57, 58), the dramatic difference in global DNA methylation that we observed in GF mice could have a role in a modified intestinal tract function. Using a genomewide approach, we identified DNA methylation changes proximal to the genes controlling the crosstalk between intestinal cells and NK cells, notably the Raet1 genes. The Raet1 genes are members of the NK group 2 member D (NKG2D) ligand superfamily that allows crosstalk between intestinal cells and NK cells through cell surface receptors, such as during inflammation (59). Intriguingly, our genomewide DNA methylation analysis showed an enrichment of regions near genes encoding both the receptors expressed at the surface of NK cells and the ligand expressed at the surface of the intestinal cells. This may indicate that epigenetic differences at proximity to genes specifically expressed in NK cells are due to the differential presence of NK cells in the colon from GF, compared with Conv-R, mice. Indeed, we found NK-specific genes, such as KlrA genes, were hypermethylated in GF mice, which would be consistent with a lower infiltration of NK cells in GF mice (because regulatory genomic regions of NK-specific genes are hypomethylated in NK cells). However, given the small representation of NK cells within the intestinal epithelium (<1%), differential presence of NK cells in GF vs. Conv-R mice is unlikely to account for the large difference in methylation signal at KlrA gene loci. Differential methylation on Raet1 genes is even more likely to be specific to intestinal cells because Raet1 genes are almost exclusively expressed in IECs (60).

Our targeted DNA methylation results, showing a specific epigenetic signature proximal to genes regulating NK cell function, suggest an altered regulation in GF mice. The markedly lower abundance of NK cells that we report in the intestinal epithelium of GF mice may, therefore, be partly due to epigenetic alterations in the Raet1a/Raet1e/H60b gene cluster in IECs, which, in turn, affect the homeostasis of NK cell population in the intestinal epithelium. Previously, accumulation of invariant NK T cells, a cell type sharing properties with both NK and T cells, was found to be altered in the intestine of GF mice (61). This study revealed an increased presence of invariant NK T cells in the lamina propria of GF mice, which was associated with a greater allergic response. In the light of this study, our results, therefore, support the notion that a lack of a gut microbiota dysregulates the interaction between enterocytes and specific immune cells, thereby leading to an altered immune response, for example a chronic susceptibility to intestinal inflammation.

In summary, our study shows specific DNA methylation signatures in the intestine from GF animals that potentially affect the crosstalk between enterocytes and NK cells. More studies are needed to understand the mechanisms by which the gut microbiome controls the epigenome of the intestine and its role on intestinal function.

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AUTHOR CONTRIBUTIONS

A. Poupeau, M. Arumugam, D. Simar, L. E. Olofsson, F. Bäckhed, and R. Barrès designed the research; A. Poupeau, K. Sulek, K. Citirkikaya, and L. E. Olofsson performed the research; A. Poupeau, C. Garde, K. Sulek, J. T. Treebak, M. Arumugam, D. Simar, L. E. Olofsson, F. Bäckhed, and R. Barrès analyzed the data; R. Barrès is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; and all authors approve the final version of the manuscript.

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