A single respiratory tract infection early in life reroutes healthy microbiome development and affects adult metabolism in a preclinical animal model

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In adult animals, acute viral infections only temporarily alter the composition of both respiratory and intestinal commensal microbiota, potentially due to the intrinsic stability of this microbial ecosystem. In stark contrast, commensal bacterial communities are rather vulnerable to perturbation in infancy. Animal models proved that disruption of a balanced microbiota development e.g., by antibiotics treatment early in life, increases the probability for metabolic disorders in adults. Importantly, infancy is also a phase in life with high incidence of acute infections. We postulated that acute viral infections in early life might pose a similarly severe perturbation and permanently shape microbiota composition with long-term physiological consequences for the adult host. As a proof of concept, we infected infant mice with a sub-lethal dose of influenza A virus. We determined microbiota composition up to early adulthood (63 days) from small intestine by 16S rRNA gene-specific next-generation sequencing. Infected mice underwent long-lasting changes in microbiota composition, associated with increase in fat mass. High-fat-high-glucose diet promoted this effect while co-housing with mock-treated animals overwrote the weight gain. Our data suggest that in the critical phase of infancy even a single silent viral infection could cast a long shadow and cause long-term microbiota perturbations, affecting adult host physiology.

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adult microbiota (Fig. S2a), most likely to the introduction of solid food into the diet and the lack of continuous milk intake. LefSe analysis\(^{12}\) revealed significant increase in Gammaproteobacteria in IAV-infected mice at this stage (Fig. S2c). By day 56-post infection (9-weeks-old mice) we found Enterobacteriaceae significantly increased in IAV-infected mice. Conversely, in mock-treated animals several groups of Firmicutes (mainly of the class Clostridia) were enriched. (Fig. 1e) Intriguingly, in mice, Enterbacteriaceae are favored under high-fat diet conditions\(^{13}\) or after low dose penicillin treatment, which both lead to obesity in mice\(^{5}\). In line with our findings from adult mice, in two American studies, populations of obese patients had enriched Enterobacteriaceae.
Fig. 1 Neonatal IAV infection changes microbiota composition in adult mice. Seven-days-old mice were infected intranasally with PBS or 40 pfu of VN/1203 in 5 μL of PBS. a At 3-, 7- and 14 dpi viral titers in lungs and snouts for IAV infected mice were determined by plaque assay. Each dot represents one animal (n = 7–8) and the median is represented by a black dash. Limit of detection is represented by a black dashed line. Statistical significance between groups was determined by Kruskal–Wallis test. P-values are indicated. b Body weight was followed for 14 days after infection and values for male mice are plotted as mean ± SEM (n = 29 for mock-treated and n = 38 for IAV-infected). Statistical significance between groups was determined by 2-way ANOVA test. P-value is indicated. c Individual Shannon H-index of small intestine microbiota at 7, 14, and 56dpi of mock-treated (black squares) and IAV-infected mice (red triangles) are depicted for indicated days of sampling. Mean ± SD per experimental group are shown. Statistical significance between groups was determined by unpaired students’ t-test. P-values are indicated. d Normalized individual 16 S/18 S qPCR results (n-fold relative to mean of mock samples) in small intestine at 7, 14, and 56 dpi are depicted for mock-treated (black squares) and IAV-infected (red triangles). Mean ± SD per experimental group are shown. Statistical significance between groups was determined by unpaired students’ t-test. P-values are indicated. e LefSe analysis of the composition of the microbiota in small intestine of mock-treated and IAV-infected mice based on 16S rRNA gene sequencing of samples collected at 56dpi. Linear discriminant analysis scores (LDA) are indicated for different taxonomic groups significantly overrepresented (p < 0.05) in mock-treated or IAV infected mice. (n = 15). Kruskall–Wallis statistical test was performed as described previously12. Cladograms of OTUs, as annotated by Qiime1, that are differentially represented in small intestine samples taken from mock and IAV infected animals on the right-hand side. Overrepresented taxonomy groups are given on legends next to the corresponding cladogram. f Scaled 3D principal coordinates analysis (PCoA) plots using a weighted-UniFrac distance matrix from small intestine microbiota of mock-treated or IAV-infected mice at indicated time points post-infection. Each symbol represents one individual mouse. Percentages explain variation in PC1 (x-axis), PC2 (y-axis) and PC3 (z-axis). PERMANOVA and PERMDISP statistical tests were performed and p-values are indicated in the figure and listed in Supplementary Table 1.

and decreased Clostridia14. In accordance with the compositional data, weighed PCoA analysis revealed a more dispersed beta diversity for mock-treated animals on D7 post-infection (Fig. 1f). By D14 post-infection, no obvious differences between the two mouse groups were apparent. Interestingly, a higher divergence along PC1 and PC2 was found for IAV-infected animals on D56 post-infection, which does not reach statistical significance, probably as a consequence of the large variance within this group (see Permdisp analysis in Supplementary Table 1). A direct comparison of D56 mock and IAV treated mice by Permanova analysis indicated a p-value of 0.178. Since we cannot monitor the severity of the infection in the young mice, due to lack of clinical signs, we propose that variability of infection could explain why few of the infected animals cluster with the majority of the mock-treated mice (bottom right corner of the graph in Fig. 1f). While IAV does not replicate in the intestine an increased interferon response was proposed to trigger microbiota changes in the intestinal tract after IAV infection9. We confirmed the absence of viral RNA in the small intestine by highly sensitive qPCR (Fig. S2d). Intriguingly we found 2-fold increased levels of IL28 mRNA but no changes in IP10 mRNA in the intestine of mice infected with IAV (Fig. S2e). This could explain the alterations of microbiota diversity and composition. At this stage, we speculate that the loss of rare OTUs might create a bottleneck early in life, which results in different trajectory of microbial community development. Likely, both direct and indirect mechanism play a role in this alteration. Obviously, this would open a therapeutic window for correction of infection-dependent microbiota depletion, e.g., by targeted application of probiotics. Strikingly, as previously shown in adult animals9, fecal microbiota were completely resistant to infection-related imprinting effects as shown by indistinguishable alpha and beta diversity and only minor differences in composition (Fig. S3a–c).

Intestinal microbiota are a driving force of host physiology (reviewed in15). In adult mice, acute IAV infection causes metabolic changes in adipose tissues, which eventually last until after IAV clearance16. We thus asked whether the long-term changes in small intestinal microbiota composition after early-life infection indeed affect the metabolism of adult mice. A striking observation arose from adult body weight curves. While there was overall no difference in the weight development of infected vs. mock-treated mice, gender-stratified data revealed a clear increase of absolute body weight in adult male mice infected with IAV in early in life starting around D42 post-infection (Fig. 2a), adding up to a median of about 3% body weight gain by D56 post-infection. We observed overall a similar tendency but with more variation in body weight among female mice, potentially due to the unsynchronized hormonal cycle of these animals (Fig. S4a). It further has to be taken into account, that female mice respond generally weaker to e.g., diet-induced weight gain7. Hence, we focused on male mice to further address the observed weight gain phenotype. Since both decreased activity or increased food uptake or processing could be a reason for the increased weight, we performed metabolic phenotyping of individually caged male mice 56d post-infection using TSE Labmaster system (TSE Systems, Germany). We found no difference in movement patterns, food, and water intake, or fatty acid oxidation18, which could have explained increased body weight (Fig. S4b–f). Similarly, caloric uptake, as determined by bomb calorimetry of consumed food and shed fecal matter, yielded no differences between the two mouse groups (Fig. S4g). We did, however, observe a decrease in specific energy expenditure (determined by indirect calorimetry19) after IAV infection, both during day- and night-time which could explain in part the increase body weight (Fig. 2b). We next determined lean and fat mass by Echo-MRI, and found a tendency for increased total fat mass in previously IAV-infected mice (p = 0.13) (Fig. 2c). Acute IAV infection was recently shown to affect white fat browning20. However, analysis of fat browning-related marker genes did not reveal important differences in mRNA expression as determined by specific qPCR (Fig. S4h). This makes involvement of reduced-fat browning in weight gain unlikely.

In order to address if intestinal microbiota were responsible for the weight gain, we co-housed mice in parallel to non-co-housed experiments described before, which were either treated with PBS or IAV in infancy, in equal proportion, after weaning and after viral clearance and followed weight gain until nine weeks of age. Importantly, we did not observe differences in median weight gain between PBS treated and IAV infected animals (Fig. 2d) or differences in selected organ weights (Fig. 2e), implicating that exchange of microbiota with PBS treated animals could compensate for the effects by IAV infection early in life. Co-housing resulted indeed in a significantly distinct microbiota as indicated by the shift in beta diversity away from both non-co-housed IAV-infected mice (Fig. 2f). Importantly, towards the non-co-housed mock-treated mice these differences were not significant. There was no difference in beta diversity between co-housed IAV infected and mock-treated mice, confirming the successful exchange of intestinal microbiota between infected and mock-treated mice. Accordingly, we did not find major differences in the compositional data (Fig. S5 and Supplementary Table 2). This implicates that microbiota exchange with mock animals can override the IAV-caused dysbiosis even after viral clearance.

Finally, we asked, if mice, which underwent an IAV infection in infancy were more prone to diet-induced alterations of body mass. We hence fed a high fat/high glucose (HFHG) diet ad libitum.
from 35d post-infection onwards to IAV infected or PBS treated mice. While the overall body mass did not differ between PBS treated and infected mice on D56 or D80 post-infection (Fig. 2g), we found an increase in visceral and subcutaneous fat in adult mice (D80 post-infection), which were infected with IAV in infancy (Fig. 2h) with about 30% median effect size in VAT. This suggests that early life infections could sensitize to adulthood food-induced gain of fat mass in specific depots. The mechanistic link between the altered microbiota after early life IAV infection and the metabolic changes will require additional investigations.
**METHODS**

**Animal experiments, metabolic phenotyping, and organ collection**

C57BL/6J mice (male/female, 7-8 weeks of age) were purchased as breeders from Charles River Laboratories (France). Animals were housed at specific pathogen free (SPF)/biosafety level 2 (BSL2) animal facility of Central Medical University (CMU) at University of Geneva under a strict 12-hour light/dark cycle and fed ad libitum with standard chow diet (RM3 (E) FG, 3 Special Diets Service, UK) unless otherwise stated. Breeding couples were time-mated and 7-day old pups, born in house to IAV naïve parents, were used for the experiments indicated in this study. At age 21d, pups were weaned and housed separately based on sex and experimental group. All animals were introduced to a new clean cage with fresh bedding and enrichment every two weeks.

For infection, 7-day-old pups were inoculated with 5 µl of PBS (1X) or IAV/A/Viet Nam/1203/2004 (VN/1203, 40 pfu) HAOs10 (low pathogenic version) into intranasal route without anesthesia. Body weights of the animals were followed daily for two weeks following infection, then weekly up to 56 days post-infection, if not every two days for animals under HFHG diet. At 42 days post-infection, animals were introduced to clean cages in pairs. Food intake and feces production over 48 hours were measured for each cage. Fecal pellets were subsequently collected, vacuum-dried, and grounded to a fine powder. Carbohydrate content of food and feces was measured by an oxygen bomb calorimeter (Parr, 6100, USA)25. Average caloric uptake of two animals was calculated based on caloric content of consumed food and excreted feces. At 56 days post-infection, body composition of the animals, i.e., fat mass, lean mass, and free fluids, were analyzed through the use of EchoMRI-700 (EchoMIR, Houston, USA), without anesthesia. For experiments including high caloric diet, animals were fed high fat/glucose (HFDG) diet (HCD, 22.1 M/kg Gross Energy; 45 kJ/kg Fat, 20 kJ/kg Protein, 35 kJ/kg Carbohydrates, D12451, Ssniff, Germany) for 3 weeks starting from 35 days post-infection, at age of 6 weeks.

Upon reaching experimental endpoints, animals were euthanized using controlled CO₂ exposure. Organs were sampled following euthanasia using sterile tools, sterilized between groups, under aseptic conditions. Tissue samples were collected from visceral adipose tissue (epididymal WAT, unilateral), subcutaneous adipose tissue (inguinal WAT, unilateral), liver (medial lobe), or muscle (quadriceps, unilateral). Samples were weighed where necessary and immediately stored at −80 °C to be used in viral titer determination, metabolic processing or extraction of DNA. Fecal pellets were freshly sampled on the day indicated before euthanasia. Infectious virus particles in indicated organs were quantified by standard plaque assay on MDCK cells24. Briefly, organ homogenates were preincubated using 200 U/mL of serologically diluted and transferred to confluent monolayers of MDCK cells. These were subsequently overlaid with 2% agar-containing medium to limit free viral diffusion and allow plaque formation. Plaques were stained with crystal violet solution and counted to determine infectious virus titers.

All animal procedures were in accordance with federal regulations of the Bundesamt für Lebensmittelsicherheit und Veterinärwesen (BLV) Switzerland (Tierschutzgesetz) and approved by cantonal authorities (License number: GE/159/17).
Bacteria DNA Extraction, Library construction, and Bioinformatic analysis

Total DNA extraction from small intestine and fecal pellets was performed using QIAyen Pathogen Cador Mini kit (USA) and PowerLyzer PowerSoil DNA isolation kit (MoBio, QIAyen, USA), respectively, according to manufacturer’s instructions with slight modifications. Together, these methods facilitate the processing of DNA for downstream analyses.

Statistical analysis

In order to determine statistical significance, we applied unpaired t-tests for parametric comparison of two experimental groups, or Kruskal–Wallis or 2-way ANOVA for comparison of longitudinal data sets, and multiple corrected t-tests for comparison of more than two parameters from the same two experimental samples, using Graph Pad Prism 7.0. T-Statistic tests are indicated in each figure legend. Analysis of microbiome composition was performed using LefSe. Statistical analysis of beta diversity was done with PERMANOVA and PERMADISP.

DATA AVAILABILITY

NGS sequencing data are deposited under NCBi Bioproject PRJNA768309. Material is available through the lead contact.

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AUTHOR CONTRIBUTIONS

S.Y., B.M.S., J.P.P.B., M.S.: Conceptualized study, performed experiment, analyzed and interpreted data, wrote the manuscript. S.Y., B.M.S., and J.P.P.B. contributed equally to this manuscript. M.S. acquired funding for this project.
COMPETING INTERESTS
The authors declare no competing interests.

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