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https://doi.org/10.1038/s41467-021-21408-9

OPEN

Lysates of *Methylococcus capsulatus* Bath induce a lean-like microbiota, intestinal FoxP3⁺RORγt⁺IL-17⁺ Tregs and improve metabolism

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Interactions between host and gut microbial communities are modulated by diets and play pivotal roles in immunological homeostasis and health. We show that exchanging the protein source in a high fat, high sugar, westernized diet from casein to whole-cell lysates of the non-commensal bacterium *Methylococcus capsulatus* Bath is sufficient to reverse western diet-induced changes in the gut microbiota to a state resembling that of lean, low fat diet-fed mice, both under mild thermal stress (T22 °C) and at thermoneutrality (T30 °C). Concomitant with microbiota changes, mice fed the *Methylococcus*-based western diet exhibit improved glucose regulation, reduced body and liver fat, and diminished hepatic immune infiltration. Intake of the *Methylococcus*-based diet markedly boosts *Parabacteroides* abundances in a manner depending on adaptive immunity, and upregulates triple positive (Foxp3⁺RORγt⁺IL-17⁺) regulatory T cells in the small and large intestine. Collectively, these data point to the potential for leveraging the use of McB lysates to improve immunometabolic homeostasis.

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NATURE COMMUNICATIONS | (2021) 12:1093 | https://doi.org/10.1038/s41467-021-21408-9 | www.nature.com/naturecommunications
Gut microbes shape intestinal immunity\(^1\) and increase the bioavailability of otherwise indigestible nutrients\(^2\). A well-balanced community structure is therefore essential for immunometabolic homeostasis, whereas aberrant gut microbiota compositions associate with numerous diseases, both within and outside the gastrointestinal tract\(^3\).

While therapeutic implications of rebalancing a mistuned gut microbiota appear promising, inconsistent response rates in relation to both probiotics and fecal transfer studies, with occasional adverse events, emphasize the complexity of such approaches. One example relates to the otherwise promising probiotic candidate *Akkermansia muciniphila*\(^4\), where negative effects have been seen in immunocompromised recipients\(^5,6\). Similarly, *Prevotella copri* aids in metabolizing fibers in healthy individuals and protects against bacterial invasion in high fiber, chow-fed mice\(^7\), yet associates with insulin resistance in pre-diabetic obese individuals and precipitates glucose regulatory impairments in diet-induced obese (DIO) mice\(^8\).

An alternative to administering viable microbes is to utilize whole cell lysates, or selected cell components, of nonliving bacteria. Apart from alleviating global energy demands, if used as a nutrient source, such components may also potently affect host physiology as recently reported for *A. muciniphila*\(^9\) and *Bifidobacterium bifidum*\(^10\). In the latter example, cell surface poly-saccharides of *B. bifidum* were used to induce peripheral immune-tolerance via generation of regulatory T cells (T\(_{reg}\)). The authors reported a pronounced increase in Foxp3\(^+\) ROR\(\gamma^+\) T\(_{reg}\) (\(\gamma T_{reg}\)) specifically in lamina propria (LP) of the large intestine (LI)\(^11\). This cell type is believed to be induced by commensal microbes and has emerged as a potent T\(_{reg}\) subset, exhibiting increased lineage stability and enhanced immunosuppressive capacity during intestinal inflammation compared to conventional Foxp3\(^+\) ROR\(\gamma^+\) T\(_{reg}\) (\(\gamma T_{reg}\))\(^12\).

ROR\(\gamma^t\) is the canonical transcription factor controlling IL-17 expression; a pleiotropic cytokine with both proinflammatory and immune resolving actions depending on the eliciting cell type and physiological context\(^13\). Unfortunately, the studies describing \(\gamma T_{reg}\) function did not measure IL-17 secretion. It therefore remains unknown whether these cells exhibit normal, reduced or increased IL-17 levels, and how this translates to host physiology. The impact of this predominantly colonic cell subset on host metabolism also remains unknown. Still, mounting evidence points towards the importance of intestinal IL-17 for controlling metabolic homeostasis\(^14,15\). IL-17\(^+\) T\(_{reg}\) may therefore be leveraged as a ‘dual hit’ strategy to curb immunometabolic dysfunctions and gastrointestinal disturbances based on the immune-regulatory capacity of \(\gamma T_{reg}\) concomitant with the metabolic benefits of gut-delivered IL-17.

To this end, we hypothesized that environmental bacteria, who have not been under evolutionary scrutiny for host-microbe interactions, would provide an unexplored reservoir of immunomodulatory stimuli. In support of this hypothesis, the methanotrophic noncommensal bacterium *Methylococcus capsulatus* Bath (McB) has previously been shown to interact with human dendritic cells (DC) modulating T cell responses in vitro\(^16\), and to reduce inflammation and disease activity in dextran sulfate sodium (DSS)-induced mouse colitis\(^17\). However, the impact on host metabolism and intestinal immune cells as well as mucus dynamics were not addressed.

We accordingly explored the effect of using whole-cell lysates from McB as protein source to reshape immunometabolism and the aberrant gut microbiota of DIO mice. We show that McB lysates augment Foxp3\(^+\) ROR\(\gamma^+\) IL-17\(^+\) triple-positive \(\gamma T_{reg}\) in both SI- and LI-LP, and reset the obese microbiota concomitant with reversed key disease traits of diet-induced obesity.

### Results

**McB feeding reverses WD-induced gut microbiota changes and increases cecal SCFA levels.** To induce obesity and immunometabolic dysfunctions, C57BL/6J mice were initially fed an obesogenic WD. After 12 weeks of WD feeding, the mice were stratified into new groups based on weight, fat mass and glucose regulatory capacity (Supplementary Fig. 1a), and fed experimental WDs for an additional 6 weeks. While dietary fat is known to elicit reproducible and lipid-dependent alterations in the murine gut microbiome across a variety of different diet compositions\(^18\), less is known about the microbiota-modulating impact of protein. Thus, to investigate if dietary protein (i.e., casein versus whole-cell bacterial lysates) would affect gut microbiota community structures, we analyzed freshly collected fecal samples before and throughout the dietary intervention.

LFD and WD\(_{REF}\) fed mice showed distinct gut microbiota profiles after 12 weeks of feeding (intervention baseline, week 12 + 6; Fig. 1A, B), including ~10-fold lower abundance of the health-promoting genera, *Parasutterella*\(^19\) and *Parabacteroides*\(^20\), countered by an equally increased abundance of the obesity-associated genus *Desulfovibrio*\(^21,22\) (Fig. 1C) as well as a ~4-fold increase in the *Firmicutes to Bacteroidetes* (F/B) ratio (Fig. 1D).

Interestingly, WD\(_{CNTL}\) fed mice showed negligible changes in the microbiome signature during the 6 weeks of intervention (Fig. 1A–E, Supplementary Fig. 2a, b), suggesting that the added lipid source had limited influence on the intestinal ecology. In contrast to this observation, we noted a pronounced shift in bacterial composition in mice fed WD\(_{MB}\). Within the first 2 weeks of treatment, the general community structure in these mice shifted towards that of their LFD-fed counterparts (Fig. 1A–E, Supplementary Table 3). We next asked if the observed taxonomical differences between groups related to alterations in the functional potential. SCFAs are main end products of metabolized fibers, and to a lesser extent amino acids escaping digestion in the SI, with vast impact on host physiology.\(^23,24\) The highest levels of SCFAs are found in the cecum and proximal colon.\(^25\) We therefore investigated if cecal SCFA levels were different between groups. We found a consistent increase in the levels of the three major as well investigated if cecal SCFA levels were different between groups. We found a consistent increase in the levels of the three major acetate, propionate, and butyrate (Supplementary Table 4, 5). This was true regardless of dietary protein inclusion. Thus, we concluded that dietary protein modulates gut microbiota and SCFA levels, which may be an underlying mechanism contributing to the observed weight gain and metabolic alterations.\(^26\)

**WD\(_{MB}\) feeding stimulates induction of gut-specific regulatory T cells.** The intricate relationship between gut microbes and host immunity, combined with the immunoregulatory capacity of SCFAs\(^26\), prompted us to investigate if the observed changes mediated by WD\(_{MB}\) feeding were associated with immune alterations.

We accordingly analyzed the immune cell profile of SI-LP and LI-LP in a subset of experimental mice (\(n = 6–10\)/group) using multicolor flow cytometry focusing on phenotypic characterization of group 3 innate lymphoid cells (ILC3), natural killer (NK) cells and T cells (consult Supplementary Fig. 3a, b for gating strategies). Numbers of ILC3s, NK cells and T cell receptor (TCR)-\(\gamma\delta^+\) T cells, were similar between groups (Supplementary Fig. 3d–f). The same was true for the numbers of TCR\(\beta^+\) CD4\(^+\) T cells, as well as the proportion of T helper (T\(_{H1}\))–, T\(_{H7}\)–, and n\(\gamma T_{reg}\) cells (Fig. 2A, B; H, I). Interestingly, the proportion of \(\gamma T_{reg}\) was more than 2- and 3-fold increased in LI- and SI-LP, respectively of WD\(_{MB}\) fed mice compared to WD\(_{CNTL}\) fed counterparts (Fig. 2C, J; p < 0.001; S3G). Notably, this regulatory T cell subset has been shown to curb intestinal inflammation\(^12\) and mediate immunological tolerance to the gut pathobiont.
Helicobacter hepaticus, thereby protecting against T_{H}17-mediated barrier dysfunction and subsequent colitis\textsuperscript{27}. Because ROR\textsubscript{γ} is the hallmark transcription factor for TH17 cell differentiation and essential for their IL-17 production, we next assessed if the pTregs induced by the different diets were also capable of expressing IL-17. Indeed, ex vivo stimulated pTregs produced substantial and diet-dependent amounts of IL-17 protein, where WDMcB feeding increased the proportion of IL-17\textsuperscript{+} cells within L1-LP pTregs (Fig. 2D–G, p < 0.001; 2k-N; S3H). We confirmed gut-specificity of the WDMcB-induced pTregs as only negligible amounts were observed in single-cell suspensions of liver homogenates obtained from ‘weight-matched’ mice fed the respective diets.
Fig. 1 McB feeding reverses WD-induced gut microbiota changes and increases cecal SCFA levels. A Principle coordinate analysis (PCoA) using Weighted UniFrac distances of fecal microbiota sampled from first and second experiment biweekly during the dietary intervention period, as indicated by numbers post dietary intervention in centriods. The WDCNTL- and WDMcB-fed groups were similar in microbiota composition prior to dietary intervention week 12 + 0 (PERMANOVA p = 0.88 and 0.43 in Exp1 and Exp2, respectively). At the end of each experiment, the microbiota composition was significantly different between these groups (PERMANOVA p = 0.001 and 0.002 in Exp1 and Exp2, respectively). B Taxasummary of most abundant bacterial genera showing mean relative abundance in % of indicated family and genera in each group at indicated time points. C Deseq analysis of fecal bacterial genera abundances significantly regulated by McB intervention compared to WDCNTL (padj < 0.05). Relative abundance in % in each group and variation are shown for each regulated genus at the sampled time points. Fold-change and adjusted p values of individual genera are indicated in Supplementary Table 3. D Relative Firmicutes/Bacteroidetes ratio of fecal samples of individual mice before (12 + 0) change pmol per cecum. Isobutyrate and isovalerate data were tested by Kruskal statistical test. 

prophylactically for 7 weeks (Supplementary Figs. 1c, 4a). p1reg constituted <1% of all CD4+ T cells in the liver, hence contrasting the ~3 and 20% in SI- and LI-LP, respectively, of LFD- and WDREF-fed mice and starting ~12 and 30% in similar sites of WDMcB-fed mice (Supplementary Fig. 4b). Mechanistically, IL-1, but not SI-, LP-derived McB-induced pTreg exhibited enhanced secretory capacity of the hallmark suppressive cytokine, IL-10, upon ex vivo stimulation (Fig. 2O, consult Supplementary Fig. 3c for gating strategy). Augmented IL-10 secretion was identified in both pTregs and gTregs populations in LI-LP of WDMcB-fed mice (Fig. 2O, P, p < 0.05 and <0.001, respectively), pointing towards enhanced immune regulation following WD McB feeding. Although the absolute number of IL-10+ Tregs was similarly increased in SI-LP of WDMcB-fed mice (Supplementary Fig. 4c, d), the relative proportion of IL-10+ cells within these Tregs remained similar between groups (Fig. 2O, P). Collectively, these data corroborate that the phenotypic shift of enhanced secretory capacity was restricted to the colon of WDMcB-fed mice. The amount of Ki67+ cells followed the patterns of Treg abundances (Fig. 2Q, R; Supplementary Fig. 4e, f, consult Supplementary Fig. 3c for gating strategy).

WDMcB mitigates diet-induced obesity. The altered immune profile combined with a shift of the gut microbiota towards a state similar to that observed in lean LFD-fed mice, could potentially elicit crosstalk to glucoregulatory organs. To examine if McB lyses could reverse impaired glucose regulation, we performed OGTT and assessed GSIS concomitant with body mass composition in obese mice fed WDREF for 11 weeks and after 5 weeks of dietary intervention allowing for temporal analyses (Supplementary Fig. 1a). All mice were stratified into experimental groups based on their pre-intervention glucoregulatory capacity (Supplementary Fig. 4g, h). While the response to glucose challenge remained largely unaffected from week 11 to week 12 + 5, regardless of experimental diets (Fig. 3A–C), both 5 h fasted insulin levels and glucose-stimulated insulin responses were significantly increased in mice fed WDCNTL (Fig. 3E, p < 0.01 & p < 0.001, respectively) in accordance with our previous report on time-dependent alterations in glucose regulation28. LFD- and WDMcB-fed mice were fully protected against this detrimental trajectory (Fig. 3D–F; Supplementary Fig. 4i, p = 0.24 and 0.68, respectively), and WDMcB-fed mice further exhibited modestly improved insulin sensitivity based on 5 h fasted glycemia (Fig. 3C, p < 0.05) and intraperitoneal insulin tolerance test (Fig. 3G, p < 0.05).

Overall, weight development mimicked the glucoregulatory capacity. As such, WDMcB-fed mice exhibited stability of weight, fat mass and lean mass when changed to experimental diets, contrasting the continuous weight and fat mass development of WDCNTL-fed mice (Fig. 3H, I and Supplementary Fig. 4j). The absence of weight gain was not explained by decreased feed intake, but rather appeared to be associated with enhanced fecal energy secretion (Fig. 3J, K).

Since obesity and impaired glucose regulation are tightly associated with NAFLD29, we next subjected paraffin-embedded liver sections to histological evaluation. These analyses revealed both diminished steatosis and hepatocellular ballooning in WDMcB-fed mice compared to WDCNTL-fed counterparts, where especially hepatocellular ballooning was arrested in (or returned to) a state reminiscent that of lean LFD-fed mice (Fig. 3L, M, p < 0.05). Importantly, hepatocellular ballooning is instrumental in the development of the more severe liver disease, NASH29.

WDMcB feeding resets the hepatic lipidome and decreases hepatic immune infiltration alleviating NAFLD. Based on the decreased NAFLD in WDMcB-fed mice housed at T22°C we designed a new experiment (study outline, Supplementary Fig. 1b) using a recently described30 method where thermonutral housing (T30°C) potentiates NAFLD in WT C57BL/6 J mice fed an obesogenic diet for 20–24 weeks. To more thoroughly investigate the effect of WDMcB-feeding, we also redesigned the diets and omitted macadamia oil in the WDCNTL group, as this might lead to progression of obesity (Fig. 3) and related disorders. This new diet design entailed an increased fat/protein ratio in WDMcB compared to WDREF, due to phospholipids inherently present in bacterial lyses31 (Supplementary Table 1). Despite the lower protein content in WDMcB compared to both other diets, the relative amounts of indispensable amino acids were similar between groups (Supplementary Fig. 1f) and protein availability was well beyond critical levels, corroborated by similar lean mass to WDREF-fed mice post diet intervention (Supplementary Fig. 5a). Still, WDMcB-fed mice exhibited significantly improved 5 h fasting insulin levels and decreased fat mass (Fig. 4A, B), despite weight maintenance and significantly increased energy intake compared to both LFD and WDREF-fed mice (Supplementary Fig. 5b–d). The decreased body fat mass was accompanied by a diminished NAS, supported by both pathological evaluation of H&E stained liver sections (Fig. 4C, D) and hepatocytic lipid content assessed by Oil-Red-O staining (Fig. 4E–G). We additionally observed augmented adiponectin secretion (Fig. 4H),
**Fig. 2** WD<sub>MB</sub> feeding stimulates induction of gut-specific regulatory T cells. **A–D** Number of indicated cells in colon. **E–G** Representative plots of colonic TCR<sup>β</sup>CD4<sup>+</sup> FoxP3<sup>+</sup> ROR<sup>γ</sup>T<sup>+</sup> p<sub>Tregs</sub> (left) and IL-17<sup>+</sup> p<sub>Tregs</sub> (right) in LFD (E), WD<sub>CNTL</sub> (F), and WD<sub>MB</sub> (G) group. **H–K** Number of indicated cells in small intestine. **L–N** TCR<sup>β</sup>CD4<sup>+</sup> FoxP3<sup>+</sup> ROR<sup>γ</sup>T<sup>+</sup> p<sub>Tregs</sub> (left) and IL-17<sup>+</sup> p<sub>Tregs</sub> (right) in LFD (L), WD<sub>CNTL</sub> (M), and WD<sub>MB</sub> (N) group. **O–R** Percentage of indicated cells in SI- and LI-LP from ‘weight-matched’ mice housed at thermoneutrality. **A–D, H–K, O–R** Bars indicate group mean ± SEM and dots indicate individual data points. All p-values < 1 × 10<sup>-1</sup> between WD<sub>CNTL</sub> and indicated group by one-way ANOVA with multiple comparisons and Dunnett post-hoc are depicted.
pointing towards improved insulin sensitivity in the WD_{McB} group, further supported by the assessment of insulin tolerance and hepatic gene transcription activity of key metabolic enzymes (Fig. 4I, J). Of interest, we observed a >10-fold down-regulation of Scd1 in the liver of WD_{McB}-fed mice, the hepatic expression of which is (a) regulated by the microbiota\textsuperscript{32} and (b) instrumental in de novo lipogenesis at the onset of metabolic syndrome\textsuperscript{33}.

We next assessed the hepatic lipidome by tandem mass spectrometry to elucidate if diminished NAFLD was associated with an altered lipid profile. Through comparison of WD_{McB} and WD_{REF} we identified 57 and 279 differentially regulated peaks in
negative and positive ionization mode, respectively (Fig. 5A, B; Supplementary Fig. 5e, f, all FDR < 0.05). Of these, most classified lipids were changed with WDMcB in the direction of LFD-fed mice (Fig. 5C, D). Notably, 57% of upregulated species were odd-chain fatty acids, whereas 80% of downregulated species represented lipids with even carbon numbers (Fig. 5C, D and Supplementary Table 4), hence supporting previous reports where odd- rather than even-chain fatty acids are inversely associated with human insulin resistance and type 2 diabetes.

To estimate the functional consequences of an altered lipid profile, we used the Lipidmaps database to identify affected pathways and plotted the observed changes on a log2 scale comparing both LFD and WDMcB to WDREF. The majority of affected pathways was similarly regulated in both direction and magnitude in LFD and WDMcB mice compared to WDREF mice (Fig. 5E; Supplementary Fig. 5g). Notably, bile acids and ceramides, both of which were significantly downregulated in WDMcB-fed mice compared to WDREF-fed counterparts, have been shown to mediate steatohepatitis by upregulation of IL-6 and TNF-α, respectively. We therefore measured these hepatic cytokines and observed similarly reduced levels in both LFD and WDMcB compared to WDREF (Fig. 5F).

A key feature of diet-induced liver pathologies, including NAFLD, is recruitment of newly activated immune cells capable of eliciting a proinflammatory immune response. This process is generally hampered in mice housed at mild thermal stress, which therefore fail to phenocopy human pathophysiology. However, thermoneutral housing recapitulates some human disease traits, which combined with HFD-feeding accentuates intrahepatic infiltration of proinflammatory Ly6high monocytes. These monocytes interact with tissue resident T cells and play a central role in the pathogenesis of liver injury, hence representing an attractive therapeutic target to mitigate NAFLD development and to curtail associated pathologies.

We therefore subjected liver tissues from representative mice to immunological evaluation by immunohistochemistry and observed a marked decrease in both CD3+ T cells and Ly6G+ neutrophils in WDMcB-fed mice compared to their WDREF-fed counterparts (Fig. 5G). Diminished hepatic immune infiltration was mirrored by increased levels of circulating IL-22, IL-18, and IL-17 in WDMcB-fed mice compared to their WDREF-fed counterparts (Fig. 5H, p < 0.01, <0.05, and <0.05, respectively).

We next initiated a short term experiment in ‘weight-matched’ mice to evaluate the hepatic immune profile before obesity onset (Supplementary Figs. 1C, 5h, i). Surprisingly, this experiment revealed increased numbers of tissue resident Tim4+ macrophages (i.e., Kupffer cells) in WDMcB-fed mice, suggesting that McB feeding either recruits or stimulates in situ proliferation of this key cell subset driving hepatic homeostasis (Fig. 5I). Kupffer cells are central to innate immunity and responsible for containment and clearance of foreign particles. Inflammatory activation of hepatic Kupffer cells potentiates obesity-associated insulin resistance, in part by recruiting neutrophils and T cells. Yet, Kupffer cells exhibit tremendous plasticity in their activation program, with anti-inflammatory properties in their alternative activation state ameliorating hepatic steatosis. While our staining panel did not allow us to identify the activation state of the enhanced Kupffer cell proportions, it is pertinent to note that none of the classically recruited cell types were altered in numbers (Supplementary Fig. 5j, k). Instead, we observed increased proportion of Ly6C+ monocytes. The mean fluorescence intensity (MFI) within these monocytes was marginally lower in WDMcB-fed mice than in their WDREF-fed counterparts (Fig. 5I). Newly recruited monocytes express high levels of Ly6C in their inflammatory state; an expression that is gradually downregulated in immune resolving alternatively activated cells. Despite weight maintenance, WDREF-fed mice exhibited ~50% increase in hepatic IL-17γ γ5 T cells (Fig. 5K), an immunological precursor for subsequent NAFLD controlled by the gut microbiota. WDMcB-fed mice were fully protected from this trait, pointing towards extraintestinal regulation of innate immunity key to metabolic homeostasis.

WDMcB feeding reverses prolonged gut microbial dysbiosis and markedly improves colonic mucus production. The improved hepatic phenotype prompted us to further investigate potential target traits in the gut-liver axis. We initially assessed if the observed cytokine responses associated with improved gut health in mice housed at T37°C where inflammation is expected to be increased. Indeed, WDMcB-fed mice were resistant to WD-induced colonic shortening closely associated with colonic inflammation (Fig. 6A).

To gain additional insights into the immunomodulating properties of McB lysates, we focused on mucus production and function. Because mucin production is a constitutive process...
where both secretion and adherence are constantly ongoing and rapidly adjust to environmental changes, immunohistochemical labelling for various MUC epitopes may not fully recapitulate the physical properties of the mucus. We therefore applied specialized mucin histochemistry staining allowing us to differentiate between neutral and acidic mucins according to the net charge of each molecule. Acidic mucins were further separated into sulfomucins and sialomucins. Sections revealed that neutral mucins in crypt-residing goblet cells were consistently downregulated in WDREF-fed mice compared to LFD-fed counterparts in three well defined segments of the colon; i.e. proximal, middle and distal area (Fig. 6B, D). WDMcB-feeding not only reversed this pattern in all three segments but even also enhanced the production of neutral mucins exceeding the levels found in LFD-fed counterparts. This is a remarkable finding considering the continuous intake of a westernized diet high in fat and sucrose, known to hamper goblet cell function. We next evaluated crypt depth (CD) in stained sections. While the CD of the proximal and distal part of the colon was largely unaffected by diet, we observed increased CD in the middle segment of WDMcB-fed mice (Fig. 6C). WDMcB-feeding further enhanced the glycosylation pattern, particularly in the middle segment of colon, where this group exhibited a 3-fold increase in sulfomucins balanced by a similar (~2-fold) decrease in sialomucins compared to WDREF-fed mice (Fig. 6E–G). No differences were observed between WDREF and LFD-fed mice, indicating that the reciprocal regulation of mucin glycosylation status was specific to WDMcB-feeding.

With focus on the dynamic interactions between gut immunity, mucin glycosylation and commensal microbes, we next assessed the gut microbiota composition in temporally separated samples. This was done to determine if the changes towards a gut
microbiota resembling that of lean LFD-fed mice was recapitulated in this intensified setup. In contrast to the first set of experiments, where we used cohoused mice shown to exhibit resilient microbiota profiles, we now employed single-housed mice to explore if the WD McB-mediated community structures were persistent enough to induce consistent changes in the more dynamic communities of single-housed mice. Similar to our first experiments at T2-3°C, we observed a normalization of the gut microbiota of WD McB-fed mice, despite prolonged WD feeding prior to intervention (Fig. 6H-K). WD McB-induced changes were surprisingly consistent with the first set of experiments, including a significantly lower F/B ratio (Fig. 6J), and a substantial reduction of Desulfovibrio abundance, countered by a similar bloom of the Parasutterella and Parabacteroides genera (Fig. 6K, Supplementary Table 5). Of note, the age-related increases in Desulfovibrio abundances recently reported was confirmed in this study where the general magnitude in both WD REF-feeding and LFD-fed mice increased 2-3-fold over the 5 week intervention (Fig. 6K). WD McB-feeding fully prevented this trajectory and paired analyses even revealed a diminished relative abundance of Desulfovibrio in these mice. McB lysates rely on adaptive immunity to favor Parabacteroides blooms. To assess if WD McB-induced microbiota alterations were a result of diminished obesity or altered immunity we next evaluated the impact of WD McB-feeding in ‘weight-matched’ C57BL/6/N mice fed the respective diets for 7 weeks (Supplementary Fig. 1c). In agreement with previous reports, WD REF feeding induced a ~3-fold decrease in Parabacteroides abundance independent of obesity, a feature that has been reported in several immune-competent mouse strains. WD McB feeding not only prevented this trajectory but selectively enhanced the relative abundance of this genus > 10-fold (Fig. 7A, C; Supplementary Fig. 6a). The WD McB-induced change in Parabacteroides abundance shifted the microbiota consortium away from their WD REF-LFD-fed counterparts (Fig. 7D, G). Considering the marked $\gamma_T_{reg}$ phenotype, we next evaluated microbial structures in RAG2 $−/-$ mice deficient in adaptive immunity. Here, mice on LFD (Week 0) exhibited diminished Parabacteroides abundance as compared to their WT counterparts (Fig. 7A-C, E). Strikingly, WD McB feeding was incapable of boosting the relative abundance in RAG2 deficient mice, indicating that the selective increase reported above was mediated by WD McB through adaptive immunity, hence potentially by $\gamma_T_{reg}$ induction (Fig. 7B, E). The reciprocal actions on Desulfovibrio abundance was on the contrary independent of adaptive immunity (Fig. 7A, B; Supplementary Fig. 6a, b).

Despite some synergies between McB and T/B cells in affecting specific gut microbes (i.e., Parabacteroides), the global changes to microbial community structures induced by WD McB-feeding in both WT and RAG2 $−/-$ mice was generally mitigated by WD McB-feeding. These traits include a diminished F/B ratio in WDMcB-fed RAG2 $−/-$ mice (Supplementary Fig. 6c). Collectively, these findings suggest that the vast majority of WD REF-induced changes and the McB-mediated protection against these, were independent of adaptive immunity (Fig. 7A-H; Supplementary Fig. 6a, b).

Altered gut microbiota by WD feeding affects glucose regulation. Despite a general protection against WD-induced global changes in the gut microbiota, the specific and substantial changes in key driver species (e.g., Parabacteroides) by WD McB-feeding solely in WT mice, could suggest that some of the metabolic effects reported above was mediated by the selective increase in $\gamma_T_{reg}$. We thus evaluated glucoregulatory capacity in RAG2 $−/-$ mice fed either diet for 6 weeks and observed a partial protection against WD-induced 5 h fasted hyperinsulinemia, insulin secretion during OGTT, body weight gain, and total fat mass (Fig. 8A-G). Both cecum weight and SCFAs were further enhanced to levels resembling those in the above-reported WT mice (Fig. 8H, I). Collectively, these data suggests that at least some of the metabolic effects observed in WD McB-fed mice may occur independent of adaptive immunity.

We therefore designed CMT experiments in ABX treated mice and assessed the glucoregulatory capacity in cohorts fed either LFD or WD REF (Supplementary Fig. 1e). While we failed to observe a donor-dependent effect of CMT in WD-fed mice (Fig. 8I, K; Supplementary Fig. 6d–g), we did observe transient tendencies of metabolic improvements, notably on fat mass and GSIS, in WD-fed mice receiving cecal microbes from LFD- or WD McB-fed donors (Fig. 8K–O).

Discussion
In this report, we explored the relationship between dietary nutrients and host-microbe interactions with a focus on immunometabolic response rates in the context of high fat, high sucrose, WD feeding. We found whole-cell lysates from the noncommensal methanotrophic bacterium, McB, capable of reversing hallmark signatures of WD feeding despite continued
intake of an obesogenic diet. The signatures included diminished fat mass, improved intestinal immunity and glucoregulatory capacity accompanied by gut microbial community structures resembling that of lean LFD-fed counterparts.

The aberrant microbiota composition associated with obesity was recently shown to reflect HFD intake rather than obesity per se. It is therefore worth noting that we were able to normalize the dysregulated gut microbiota in mice remaining on a westernized, high fat, high sucrose diet; especially considering that it remains a clinical challenge to change dietary habits in individuals with lifestyle-related obesity. This remarkable change was reproduced in four substrains of mice originating from two different vendors, corroborating a robust phenotype not notably affected by baseline microbiota composition. To this end, a
previous report on the resilience of the microbiota, argued for prolonged normalization. This study found that mice transferred to a low fat, fiber-rich chow diet after 12 weeks of HFD feeding, shifted their microbiota towards age-matched chow-fed control mice within 4 weeks, but only fully converged 10 weeks post diet change. This is particularly interesting as dietary fibers are known to be the most potent dietary regulator of the gut microbiota, by far exceeding that of dietary fat. Still, in our hands, WDMcB feeding was able to reverse the obese microbiota at a higher pace than chow diet was in the previous report, despite similar fiber content in the two WDs. We further showed that the reversal of the microbiota traits was reproducible at different temperatures, altered reference diets, changed experimental duration, and in both cohoused and single-housed mice; all of which are prominent modulators of gut microbiota community structures. While many genera were similarly affected between experiments, others were either exclusively regulated at the species level resembling that of WDREF-fed WT mice, hence pointing toward a role for the McB-induced microbiota changes in situ differentiation to Ly6Clow cells facilitate tissue repair and inflammation in obese WDMcB-fed mice. Importantly, while Ly6Chigh cells were importantly regulated in both WT and RAG2−/− mice. Interestingly, Desulfovibrio was most recently shown to flourish in aged, immunocompromised, obese mice with impaired glucose regulation. Both T cell loss and Desulfovibrio administration per se, precipitated obesity-induced insulin resistance. Still, in our hands, Desulfovibrio abundances neither were notably affected by the absence of T cells in RAG2−/− mice, nor were the suppressive capacity of our McB lystate. This bacterium has moreover been shown to thrive in desulfonated colonic mucosa, associated with human and mouse colitis. Combined, these observations lend credence to the hypothesis of a mechanistic link between WDMcB-mediated reductions of Desulfovibrio and the observed improvements in mucin chemotype and metabolic response rates.

In support of this notion, we observed a partial protection against WD-induced metabolic impairments in WDMcB-fed RAG2−/− mice concomitant with Desulfovibrio suppression. Further pointing toward a role for the McB-induced microbiota changes to modulate host metabolism, WDREF-fed, ABX treated recipient mice in CMT studies temporally exhibited a phenotype visually resembling that of their donors. Despite following a protective trajectory, the phenotype remained insidious, likely due to a combination of the inherent variation in such experiments, a rather low n-number based on sample availability, and only a partial involvement of the gut microbial community structure in the precipitated phenotype.

Future studies are warranted to elucidate host-microbe mutualism upon WDMcB feeding. On this note, mucin-producing goblet cells are capable of delivering luminal antigens to LP-residing DCs instrumental for T cell polarization. As McB potently induce DC maturation markers in vitro—even exceeding the effects of the well-described probiotic strain, Escherichia coli Nissle 1917—acting subsequent T cell responses, we predict a direct link between McB intake and the corresponding immune profile, which might subsequently precision edit certain microbes, e.g., Parabacteroides.

In addition to the direct link proposed here, McB intake might also indirectly promote Treg polarization through augmented SCFAs, a trait persisting in RAG2−/− mice. These important metabolites also sustain mucus production and facilitate tissue crosstalk in the gut-liver axis. In keeping with this notion, we observed decreased hepatic bile acids and TNF-α levels combined with a pronounced reduction of intrahepatic CD3+ and Ly6G+ cells in obese WDREF-fed mice. Importantly, while Ly6C+ cells represent proinflammatory, fibrogenic macrophages, their in situ differentiation to Ly6C+ cells facilitate tissue repair and improved NASH prognosis. It is therefore encouraging to note, that the MFI of Ly6C was nominally decreased in ‘weight-matched’ mice fed WDMcB short term. These data indicate that, despite being increased in absolute numbers, Ly6C+ cells in WDMcB-fed mice were in the process of differentiating from...
Ly6\textsuperscript{high} cells to Ly6\textsuperscript{low} cells with immune resolving capabilities, although further studies are warranted to fully describe the kinetics in such responses. In agreement with an immune resolving phenotype, WD\textsubscript{MCB}-fed mice were fully protected from the ~50% increase in hepatic IL-17\textsuperscript{+} γδ T cells observed in WD\textsubscript{REF}-fed counterparts before the onset of obesity. This cell subset has been shown to (a) be regulated by the gut microbiota and (b) both precede and causally induce NAFLD\textsuperscript{43}. The above affected targets are all proposed as relevant strategies to curb NAFLD and the more terminal liver disease, NASH\textsuperscript{36}.

The substantial, consistent, and global changes in the immunometabolic profile found in this study point towards clinical
potential if further developed. Notably, only a single study has previously succeeded in inducing pTregs, and this was limited to LI-LP11. Apart from inducing this cell subset throughout the gastrointestinal tract, we further show that these cells exhibited enhanced secretory capacity of both the hallmark immunosuppressive cytokine, IL-10, as well as IL-17 in IL-LP of WD McB-fed mice compared to their WD CNTL fed counterparts. To this end, emerging evidence indicates that the non-regulatory counterparts to pTregs, i.e. T H17 cells, are purged from SI-LP in DIO mice14 and that re-introducing ex vivo differentiated gut tropic T H17 cells curtail obesity development, hence improving insulin sensitivity15. Notably, despite intergroup similarities in the relative proportion of SI-LP IL-10+ /IL-17+ pTregs the absolute numbers of these cells were markedly increased in WD McB-fed mice, thus advocating enhanced immune regulation.

That IL-17 in this intervention is produced by Tregs, and not T H17 cells might further improve the safety profile of a putative medication, as the physiological impact and properties of T H17 cells are context-dependent. As such, T H17 cells may potentiate inflammatory bowel disease (IBD) in individuals with compromised barrier function56, whereas pTregs have shown enhanced suppressive capacity against the same disease12. This is a pertinent feature given the relatively high proportion of subjects with metabolic complications co-suffering from IBD57. While our study was not designed to assess IBD susceptibility, it is well described that purified diets compromise barrier function47,58,59 and thus represent clinical IBD-features (disease-cause) preceding inflammation (symptom)60. In addition to the immunomodulating phenotype, WD McB-feeding augmented neutral mucus production in all segments of the colon while enhancing IBD-protective sulfomucins specifically in the middle segment. In this segment, we also detected increased CD in WD McB-fed mice, collectively pointing towards increased barrier function.

pTregs were most recently described to be transferred from mother to offspring via nongenetic inheritance through breastmilk IgA; a trait transmitted during a tight age window after birth but stable for life, and resistant to many microbial or cellular perturbations61. It is thus striking to note the pronounced and reproducible induction we here report in both SI- and LI-LP in three substrains of mice from different vendors. Unfortunately, we have not investigated the B cell component of the immune system in this study, and thus, we cannot conclude on the extent to which pTregs induction relies on B cells, McB lysate per se or the McB-induced SCFAs and/or gut microbial changes. It is, however, worth noting that apart from IgA-induced pTreg-control in early life, other reports have also convincingly demonstrated that this cell subset can be induced by bacterial metabolism of bile acids62,63 as well as microbe-derived SCFAs in general54 and butyrate in particular64. The latter of which we found abundantly increased upon WD McB feeding.

In summary, this study demonstrates a consistent activation of IL-10+ /IL-17+ pTregs throughout the gastrointestinal tract and a reversion of gut dysbiosis in response to WD McB feeding, even in the context of high dietary fat and sucrose intake. The prospects of using bacterial lysates as an alternative to traditional live probiotics merit further investigation, just as future studies are warranted to assess to what extent our results can be translated to humans. Future studies are also urgently needed to identify the biological effector molecule(s) in this bacterial lysate, with a potential for being developed into a medical product(s) rebalancing intestinal immunity while targeting gut-related dysbiosis and metabolic abnormalities.

Methods
Mice and ethical statements. All experiments were conducted in accordance with the EU directive 2010/63/EU as approved by the Danish Animal Experiments Inspectorate (#2014-15-2934-01,027). Six- to seven-week-old male C57BL/6 BomTac, C57BL/6/N, C57BL/6-Arg2/2 (B6.Rag2 mice were acquired from Taconic Laboratories, Denmark, or Janvier Labs, France, respectively, as described below. All mice were allowed to acclimatize on regular chow diet for the first week upon arrival and then fed a low fat diet (LFD) for
2 weeks prior to study initiation. Mice were kept under specific pathogen free conditions at 22 °C (T22°C) or 30 °C (T30°C), as indicated, in 12 h light/dark cycle (6AM–6PM).

Housing, diets, and experimental setup. Standard pelleted diets as well as protein-free western diet (WD) powder were obtained from Ssniff Spezialdiäten GmbH, Germany, and stored at −20 °C throughout the duration of the experiment. Mice were fed a compositionally defined low fat diet (LFD, S8672-E050) until study initiation. A subgroup remained on LFD, whereas the remaining mice were transferred to a soy oil based reference WD (S8672-E025) (high fat, high sucrose, containing 0.15% cholesterol) for the run-in period (12 or 21 weeks dependent on the experiment). Experimental WDs were lot-matched and produced without protein (S9552-E021), which was subsequently added, and then pelleted, by the investigators as indicated; WDREF = containing 19.5% (w/w) casein, WD2NTL = containing 16.5% (w/w) casein and 3% Macadamia oil, WD2NTLC = 19.5% (w/w) whole-cell bacterial lysates (predominantly protein but up to ~15% phospholipids31). Diet composition is more extensively described in Supplementary Table 1 with amino acid composition presented in Supplementary Fig. 1f. Detailed description of bacterial lysates is provided in the designated section below.

Fig. 7 McB lysates rely on adaptive immunity to boost Parabacteroides. A, B Taxasummary of most abundant bacterial genera showing mean relative abundance of indicated genera in each group at indicated timepoint in WT (A) and RAG2−/− (B) mice. C, E Relative abundance of Parabacteroides in fecal samples of indicated genotype at indicated timepoint. D, F Weighted UniFrac distance (instability test) between paired samples from indicated 2-weeks interval post dietary intervention in WT (D) and RAG2−/− (F) mice. C–F Bars represent group mean ± SEM and dots indicate individual data points. All p-values <1 × 10−1 between WDREF and indicated group are depicted. Statistical significance compared to WDREF by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post-hoc. G, H PCoA of fecal microbiota composition of indicated group at baseline (Week 0, all mice fed LFD) and after 2, 4, and 6 weeks of dietary intervention with group mean indicated as centroids.
All diets were freshly made for each experiment with independent batches of bacterial lysates to substantiate robustness and reproducibility of the observed findings. Mice were fed ad libitum, and feed intake was measured thrice a week. Water was changed weekly.

12 + 6 protocol T22°C. Experimental setup is outlined in Supplementary Fig. 1a. C57BL/6JRj mice were purchased from Janvier Labs, France, and housed three mice per cage, reproduced in two independent experiments. For transparency, all data are depicted in respective graphs with mean ± SEM plus individual dots for the combined results of the two independent experiments. Experiment-dependent shapes enable visual discrimination of respective experiments as well as data distribution within and between experiments.

21 + 5 protocol T30°C. Experimental setup is outlined in Supplementary Fig. 1b. C57BL/6JBomTac mice were purchased from Taconic Laboratories, Denmark, and initially housed 10 per cage to accelerate weight gain. Twelve weeks into the run-in period, mice were single caged to allow accurate assessment of food intake, and also monitor if the more sensitive microbiota of single-housed mice would uniformly change towards a composition typical for LFD-fed mice, or if such phenomenon relied on a few highly responding mice transferring their microbiota to cage mates under group housed conditions. The timing of single housing allowed affected mice to adapt to social isolation and stabilize their weight development before intervention (Supplementary Fig. 5b).

Prophylactic protocols. Experimental setup is outlined in Supplementary Fig. 1c, d. C57BL/6N-Rag2Tm1/CipheRj (RAG2−/− mice) and matched C57BL6/N WT mice were purchased from Janvier Labs, France, and housed three mice per cage at thermoneutrality, acclimated on LFD and from 9 weeks of age fed the experimental diets for 6–7 weeks depending on genotype (see Supplementary Fig. 1c, d for details). This was reproduced in two independent experiments analogous to the 12 + 6 protocol described above.

Cecal microbiota transfer (CMT) protocol. Experimental setup is outlined in Supplementary Fig. 1e. C57BL/6JBomTac mice were purchased from Taconic Laboratories, Denmark, and housed three mice per cage at thermoneutrality, reproduced in two independent experiments. Experiment-dependent shapes enable visual discrimination of respective experiments as well as data distribution within and between experiments. We used mice from the 21 + 5 protocol as donors of their cecal microbiota, ensuring transfer between genetically identical mice hence limiting obvious confounders. Cecal microbiota transfer (CMT) was performed as
Fig. 8 Altered gut microbiota by WD feeding affects glucose regulation. A, C OGTT in 5 h fasted RAG2−/− mice after 1 and 6 weeks of dietary intervention as indicated. Statistical significance compared to WDREF by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post-hoc. n = 12 mice per group. B, D Glucose stimulated insulin Secretion during OGTT after 1 and 6 weeks of dietary intervention. Statistical significance compared to WDREF by mixed effects analysis RM, adjusted for multiple comparisons by Dunnett post-hoc. n = 12 mice per group except for WDmB donor group at 120 min in B, WDmB donor group at 60 min in D, and WDmB group at 15 min in D where n = 11 due to insufficient sample material. E–G Body weight, fat mass and lean mass, as indicated, at indicated time points post dietary intervention. Statistical significance of body weight compared to WDREF by mixed effects analysis RM, adjusted for multiple comparisons by Dunnett post-hoc, and fat and lean mass by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post-hoc. n = 12 mice per group except for WDmB donor group Week 1 in E where n = 9. H Cecum weight at termination. Statistical significance compared to WDREF by one-way ANOVA, adjusted for multiple comparisons by Dunnett post-hoc test. n = 7 (LFD and WDREF) or 11 (WDmB). I Concentration of short chain fatty acids in cecum from H. Statistical significance compared to WDREF by Kruskal–Wallis test, adjusted for multiple comparisons by Dunn’s post-hoc. n = 12 (LFD and WDmB) or 10 (WDREF). J, K Body weight and fat mass development in ABX treated WT mice fed either LFD (grey bars) or WDREF (blue bars), receiving cecal microbiota from indicated donor mice. Statistical significance compared to WDREF by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post-hoc. WD-fed mice n = 11 (LFD donor), 9 (WDREF donor), and 12 (WDmB donor). LFD-fed mice n = 12 (LFD donor), 9 (WDREF donor), and 12 (WDmB donor). N OGTT in 5 h fasted WDREF-fed WT recipient mice 1 and 5 weeks after first cecal microbiota transfer, as indicated. Statistical significance compared to WDREF by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post-hoc. LFD donor n = 11, WDREF donor n = 9, and WDmB donor n = 12. M, O Glucose stimulated insulin Secretion during OGTT (L + N) 1 and 5 weeks after first cecal microbiota transfer. Statistical significance compared to WDREF by two-way ANOVA-RM, adjusted for multiple comparisons by Dunnett post-hoc. In M, LFD donor n = 11 except at 0 min where n = 4, WDREF donor n = 9 except at 0 min where n = 6, and WDmB donor n = 12 except at 0 min where n = 9. In O, LFD donor n = 10, WDREF donor n = 8 except at 90 min where n = 9, and WDmB donor n = 11 except at 90 min where n = 12. Reduced n-size reflects insufficient sample material at indicated timepoint. A–G, I–O Lines represent group mean and dots indicate individual data points. F, H, I, K Bars represent means ± SEM. Dots indicate individual data points. A–O All p-values < 1 × 10−1 between WDREF and indicated group are depicted.

one after week of antibiotic (ABX) treatment. Specifically, mice were housed in disposable IVC cages with free access to sterilized water and LFD during the 2 weeks of acclimatization. They were then treated with a two-leg ABX mixture (1 and 0.5 g ampicillin and neomycin, respectively, per liter water, changed thrice weekly) for seven days while remaining on LFD. After ABX treatment, experimental mice were fasted for 2 h and subsequently gavaged with Polyethylene Glycol (Gangiden, Sandoz) to clean the intestines from potential remnants. Six hours later, mice received the first of three boluses of cecal microbiota from either LFD-, WDREF-, or WDmB-fed donors. Cecal microbiota were pooled from all mice of the respective donor group before transfer. Immediately after first CMT, mice were divided into either LFD- or WDREF-fed recipients and fed their respective diets for additionally 5 weeks.

Cultivation of M. capsulatus Bath and preparation of bacterial lysate. Methylococcus capsulatus Bath (NCIMB 11132) was cultivated in nitrate mineral salts (NMS) medium to produce the single-strain bacterial lysate. NMS medium was composed from 5 stock solutions; 10× NMS Salts (98.9 mM KNO3, 43.8 mM MgSO4·6H2O and 9.0 mM CaCl2), 1000× NaMoO4·5H2O, 1.8 mM FeSO4·7H2O, 1.4 mM ZnSO4·7H2O, 0.24 mM H3BO3, 0.21 mM CoCl2·6H2O, 0.74 mM EDTA-Na2, 0.1 mM MnCl2·4H2O, 42.07 μM NiCl2·6H2O and 10× phosphate buffer pH 6.8 before the NMS medium was sterile filtered using a 0.45-μm filter. Medium was stored in the dark.

The 1000× Trace Element Solution was made from the following stock solutions and stored in the dark: 400.5 mM CuSO4·5H2O, 3.6 mM FeSO4·7H2O (pH 3.0), 347.8 mM ZnSO4·7H2O, 161.7 mM H2BO3, 42.0 mM CoCl2·6H2O, 50.5 mM MnCl2·4H2O and 42.0 mM NiCl2·6H2O. McB culture aliquots were frozen in liquid nitrogen and total RNA was extracted by TRIzol (Sigma–Aldrich, USA) according to the manufacturer’s protocol using PReCELLYS® 24 for homogenization. One microgram of RNA was transcribed into cDNA using the Stratagene Mx3000P qPCR System. Standard curve and no template control (NTC) were included on every 96-well plates. Validation of each target was done. Primer sequences are summarized in Supplementary Table 2.

Glucose and insulin tolerance tests. Mice were subjected to magnetic resonance (MR)-scan using EchoMRI 410t (Texas, USA) to determine fat- and lean mass at indicated time points (Supplementary Fig. 1a–d). Mice were fasted 5 or 2 h prior to any oral glucose tolerance (OGTT) or intraperitoneal insulin tolerance test (ITT), respectively. Glucose was measured using the Bayer Contour glucometer (Bayer Health Care). Mice were subsequently gavaged with 3 μg glucose/g lean mass (OGTT) or intraperitoneally injected with 0.75 μM insulin/g lean mass (ITT). Blood were sampled at specified time points for blood glucose and insulin measurements to assess glucoregulatory capacity including glucose-stimulated insulin secretion (GSIS) as described in detail elsewhere28.

Short chain fatty acid measurements. Short chain fatty acids (SCFAs) were determined by gas chromatographic analysis. Feces were suspended in MilliQ water (1:1 (w/v)), and subsequently homogenized in a FastPrep-96 (MP Biomedicals) sample preparation unit. The homogenates were diluted with 0.4% formic acid (1:1 (v/v)), transferred to Eppendorf tubes and centrifuged at 16,200 ×g for 10 minutes. 300 μl of the supernatants were applied to spin columns (VWR, 0.2 μm pore size) and centrifuged at 9560 ×g for 5 minutes. The eluates were transferred to 300-μl GC vials. Split injection mode was used with an injection volume of 0.2 μl. The gas chromatograph was a Trace 1310 (Thermo Scientific) equipped with an autosampler and a flame ionization detector. Helium was used as carrier gas, and the column was a 30 m long Stabilwax (Restek) with polyethylene glycol as stationary phase. Injector temperature: 250 °C, temperature intervals: 2 min at 90 °C before a 6 min increase to 150 °C, then a 2 min increase to 215 °C and a hold at this temperature for 4.9 min. Detector temperature: 275 °C.

RNA extraction and quantitative RT-PCR. Frozen liver tissue was cryo-grinded by mortar and pestle on liquid nitrogen and total RNA was extracted by TRIRagent (Sigma–Aldrich, USA) according to the manufacturer’s protocol using PReCELLYS® 24 for homogenization. One microgram of RNA was transcribed into cDNA using the Stratagene Mx3000P System standard curve and no template control (NTC) were included on every 96-well plates. Validation of each target was done. Based on R2 of standard curve >0.995, amplification efficiency of 100 ±10% and singular peak, Ct values for each target were related to 18 S reference Ct values of the same sample by 2−ΔCt and visualized as fold-change to the LFD reference group mean. Primer sequences are summarized in Supplementary Table 2.

Histology. Liver tissue was sampled promptly after euthanasia, fixed in 10% formalin and subsequently embedded in paraffin prior to sectioning according to standard procedures for light microscopy. Colon was emptied for content and subsequently rolled around a 27 G needle after which the generated Swizz Rolls were carefully preserved in liquid nitrogen (wrapped in aluminum foil) until paraffin embedding. Embedded tissues were cut 2-μm thick, and sections mounted onto glass slides. These sections were processed further and stained either with hematoxylin and eosin (H&E), Oil Red O, PAS, or HID-AB, or labelled with antibody of interest for immunohistochemical investigations, as detailed below. Samples were randomized and blinded to the pathologists performing the histological analyses.

Liver. Nonalcoholic fatty liver disease activity score (NAS). Liver sections were prepared by standard protocols and subsequently assessed by two independent observers in a blinded fashion, using the established NAFLD activity score (NAS) for nonalcoholic steatohepatitis (NASH). In short, score of 0–2 excludes nonalcoholic steatohepatitis (NASH), a score of 3–4 defines “borderline NASH”, and score ≥5 is considered as NASH.
Oil Red O staining. Liver biopsies were fixed in paraformaldehyde, cryoprotected in polyvinylpyrrolidone/saccharose, and frozen in liquid nitrogen. Cryosections were prepared and stained using the Lipid Tracer Kit (Abcam) and analyzed under an Axios Imager M2e microscope. Images were captured with the Axioscim 506 color camera (Zeiss, Jena, Germany). Areas of stained lipids were determined using ImageJ-1.30 software (https://imagej.nih.gov/ij/index.html).

Colon. The morphological and morphometrical evaluations of the colonic tissues were performed in a blinded fashion, and all measurements were made by the same trained pathologist. One H&E stained section of colon from each individual was evaluated histologically for any sign of pathology. To assess the quality of the colonic crypts, neutral mucins were stained by Periodic Acid Schiff (PAS). Acidic mucins were stained through a combination of High Iron Diamine (HID), for sulfated mucins (sulfomucins), and Alcian Blue (AB) for carboxylated mucins (sialomucins). Crypt depth (CD) measurements, as well as analyses of mucin type and amount, were performed on HID/AB- and PAS-stained sections in three histologically distinct areas of the colon, i.e., the proximal, middle and distal area. At each of these three locations, three longitudinally sectioned crypts were measured. Each data point is thus presented as an average of three (mucin type and amount) or six (CD) measures. Crypts were selected only when the entire crypt epithelium was visible from the lamina muscularis mucosae to the lumen. The histological sections of the colon were examined in an Axios Imager Z2 microscope (Zeiss, Jena, Germany), and digital images were obtained using an Axioscim 506 color camera (Zeiss, Jena, Germany). Micrographs were captured with the same ×20 objective magnification. Crypt depth measurements were performed using the software program Image J-1.48v (https://imagej.nih.gov/ij/) using the same criteria as described by van der Spek et al.18,19. For quantitative evaluation and automated scoring of the different mucin types, a plugin for color deconvolution for the Image J-Fiji program was used20.

Hepatic lipodomics. Samples were randomized and lipid extracted following standard protocols. In short, 50 mg of frozen tissue were homogenized in a pre-chilled 2 ml tube added 0.5 ml of ice-cold 50% MeOH with −40 µM D5-tryptophan following standard protocols and stored at −80 °C until LCMS analysis. The pentan-2-one obtained tLC-MS/MS processing (features defined by their mass/charge value, retention time and peak area) was analyzed in MetaboAnalyst 4. Data were then auto-scaled (mean-centering and division by the square root of standard deviation of each variable) to enforce Gaussian distribution enabling relative comparison. Univariate and multivariate analysis were performed with t-tests, volcano plot, analyses of variance (ANOVA) followed by Tukey’s HSD test, and principal component analysis (PCA) to detect significant hits and to visually separate trends between groups. Features showing a similar pattern between LFD and WD,B,N were represented by pattern matching function. The top 25 features obtained were then processed for identification using the online database Lipidmaps (http://lipidmaps.org) with a mass tolerance between the measured m/z value and the exact mass of 3 ppm.

Microbiome analysis and bioinformatic processing. Fresh feces samples were collected 3–4 h into the light cycle, snap frozen and stored at −80 °C until downstream processing. Bacterial DNA from fecal samples was extracted using a NucleoSpin soil kit (Macherey-Nagel) according to manufacturer’s instructions. 16S rRNA gene amplification was performed using 515F and 806 R primers and sequenced on the 250PE on Illumina MiSeq (Germantown, Germany) and analyzed under an Axios Imager M2e microscope. Images were captured with the Axioscim 506 color camera (Zeiss, Jena, Germany). Areas of stained lipids were determined using ImageJ-1.30 software (https://imagej.nih.gov/ij/index.html).

Isolation of small intestine (SI) and large intestine (LI) lamina propria (LP) cells. Feces and mucus from LI were scraped off while SI was flushed with 1× HBSS (Gibco) containing 15 mM HEPES (Thermo Fisher Scientific). Perey’s patches were carefully excised from SI. SI and LI were opened longitudinally, cut into approx. 1 cm pieces, and homogenized three times with HBSS (Gibco), ending up with 15 mM HEPES (Thermo Fisher Scientific), 5% FCS (ViraBx™, PAA Laboratories), 50 µg/ml gentamycin (Gibco), and 2 mM EDTA (Invitrogen, Life Technologies) for 15 min at 37 °C. During the first incubation step, 0.15 mg/mL DL-dithiothreitol (Sigma–Aldrich) was added to LI samples, LI, but not SI samples, were shaken on an orbital shaker at 50 rpm during incubation. After each incubation step, SI, but not LI samples, were shaken by hand for 10 s. Media containing epithelial cells and debris were discarded by filtration through a 250 µm mesh (Teknika Precisionsifter, JR AB). The remaining tissue was digested for 20–28 min at 37 °C under magnetic stirring (350–500 rpm) in R-10 media (RPMI 1640 (Gibco) with 10% FCS (ViraLux, PAA Laboratories), 10 mM HEPES (Thermo Fisher Scientific), 100 µM penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 50 µg/ml gentamycin (Gibco), 50 µg 2-mercaptoethanol (Gibco), and 1 mM sodium pyruvate (Gibco)) containing 1 mg/ml collagenase IV (Roche) and 0.05 mg/ml DNase I (Roche). After digestion, SI-LP and LI-LP cells were purified by density gradient centrifugation (600 ref for 20 min at 22 °C, acceleration 5 and brake 0) with 40/70 % Percoll (GE Healthcare). Cell suspensions were subsequently filtered through 100 µm cell strainers (BD Biosciences) and restimulated in vitro prior to the flow cytomery analysis.

Isolation of liver cells. To isolate liver cells, livers were collected from 1× PBS (Gibco)-perfused mice, cut into small pieces and digested for 40 min at 37 °C in R-10 media (RPMI 1640 (Gibco) with 10% FCS (ViraLux, PAA Laboratories), 10 mM HEPES (Thermo Fisher Scientific), 100 µM penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 50 µg/ml gentamycin (Gibco), 50 µg 2-mercaptoethanol (Gibco), and 1 mM sodium pyruvate (Gibco)) containing 0.3 mg/ml collagenase IV (Roche), 0.21 mg/ml collagenase D (Roche) and 0.05 mg/ml DNase I (Roche) on an orbital shaker (370 rpm). After digestion, liver cells were filtered through 100 µm cell strainers (BD Biosciences) and subsequently purified by density gradient centrifugation (600 ref for 20 min at 22 °C, acceleration 5 and brake 0) with 40/70 % Percoll (GE Healthcare). Cell suspensions were either restimulated in vitro prior to or directly used for flow cytometric analysis.

Ex vivo stimulation of SI-LP, LI-LP, and liver cells. SI-LP, LI-LP, and liver cells were restimulated in vitro in R-10 media in the presence of either 20 ng/ml IL-23 (R&D Systems) or 250 ng/ml PMA (Sigma–Aldrich) in combination with 0.5 µg/ml ionomycin (Sigma–Aldrich) for 4 h at 37 °C. After 1 h incubation, 10 µg brefeldin A (BioLegend) was added.

Flow cytometry. Flow cytometry was performed according to standard procedures. Cell aggregates (identified in FSC-H or FSC-W vs. FSC-A scatter plots) and dead cells identified using Zombie Aqua or Zombie UV Fixable Viability Kit (BioLegend) were excluded from analyses. Intracellular staining was performed using the eBioscience FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. Data was acquired on a LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Antibodies (Abs) and reagents. The following mAbs and reagents were used in the study: anti-CD3e (17A2), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (6D5 and ID3), anti-CD45 (30F11), anti-CD45RB/B20 (RA3-6B2), anti-CD90.2 (30-H12), anti-CD127 (A7R34), anti-Ly-6a (A50.2), anti-Ly-6c (AL-21), anti-Ly-6G (1A8), anti-TER-119 (TER-119), anti-F4/80 (BM8), anti-CD11c (E5) (M5/114.15.2), anti-Siglec-F (E50-2440), anti-TIM-4 (M4R/T5M-4), anti-TCRβ (H57-597), anti-TCRγδ (GL3), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-FoxP3 (FJK-16s), anti-ROTY1 (B2D), anti-T-bet (AB10), anti-Ki67 (B62), anti-IL-10 (JES5-16E3), anti-IL-17A (TC11-18H10.1), anti-IL-12 (IL22JOP), and anti-IFNγ (XMG1.2). All Abs were purchased from eBioscience, BioLegend or BD Biosciences. PE/CF594-conjugated and BV421-conjugated streptavidin were purchased from BD Biosciences and BioLegend, respectively.

Multiplex cytokine quantification. Liver tissues were crushed with a mortar and pestle on liquid nitrogen. 30–40 mg of tissue were transferred to a clean tube and 400-µL T-PER tissue protein extraction reagent (Thermo Scientific) buffer including proteinase inhibitors (Sigma–Aldrich) were added. Protein concentration was assessed by BCA (Thermo Scientific) according to manufacturer’s instructions. Cytokines were quantified using LUMi-plex technology with combinations of Bio-Plex Pro (BioRad) premixed mouse cytokine panels, and the analyses were carried out on a Bio-Plex 200 system and the Bio-Plex Manager Software package.

Statistical analysis. Omics data were acquired as described in their appropriate sections. Normality residuals of remaining data were assessed by D’Augousto-Pearson omnibus (k2), Graphpad Prism 8, and subsequently evaluated by parametric (k2), non-parametric (k2) or mixed models (k2) tests to the relevant WDREF or WDCNTL group. Data are expressed as indicated in individual figure legends, and all groups are compared by two-sided tests to the relevant WDDEG or WDNTL group as indicated.
ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-21408-9

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RECEIVED: 7 November 2019; ACCEPTED: 27 January 2021;
Published online: 17 February 2021

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Acknowledgements

This work was supported by the Norwegian Research Council (project 267655). B.A.H.J. was supported by Lundbeck Foundation (grant number: R232-2016-2425) and Novo Nordisk Foundation (grant number: NNFI7OC0026698). A.-L.G. and A.M. were supported by Canadian Institutes for Heart Research and Sentinel North from the Canada First Research Excellence Fund.

Author contributions

B.A.H.J., J.B.H., C.R.K., K.K., and T.E.L. conceived and designed the study. B.A.H.J., J.B.H., I.S.L., S.B.S., and M.T.F.D. conducted the in vivo experiments. B.A.H.J., K.K., and T.E.L. supervised all parts of the study. T.T.T., A.M., L.M., W.A., and C.S. supervised parts of the study. N.v.B., S.I.P., and A.R. performed flow cytometry analyses. B.A.H.J., J.B.H., I.S.L., S.D., C.P.A., N.v.B., A.L.A., A.R., S.A.I., Y.J.A., K.S., E.F., M.T.F.D., and S.I.P. performed key experiments and analyzed the data. B.A.H.J. integrated the data and wrote the manuscript with inputs from J.B.H., I.S.L., K.K., and T.E.L. All authors edited, revised, and approved the final version of the manuscript.

Competing interests

B.A.H.J., J.B.H., I.S.L., K.K., C.R.K., and T.E.L. are co-inventors of International (PCT) Patent Application No. PCT/EP2018/071076 based on the enclosed data. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-21408-9.

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Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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