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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Helminth-Induced and Th2-Dependent Alterations of the Gut Microbiota Attenuate Obesity Caused by High-Fat Diet

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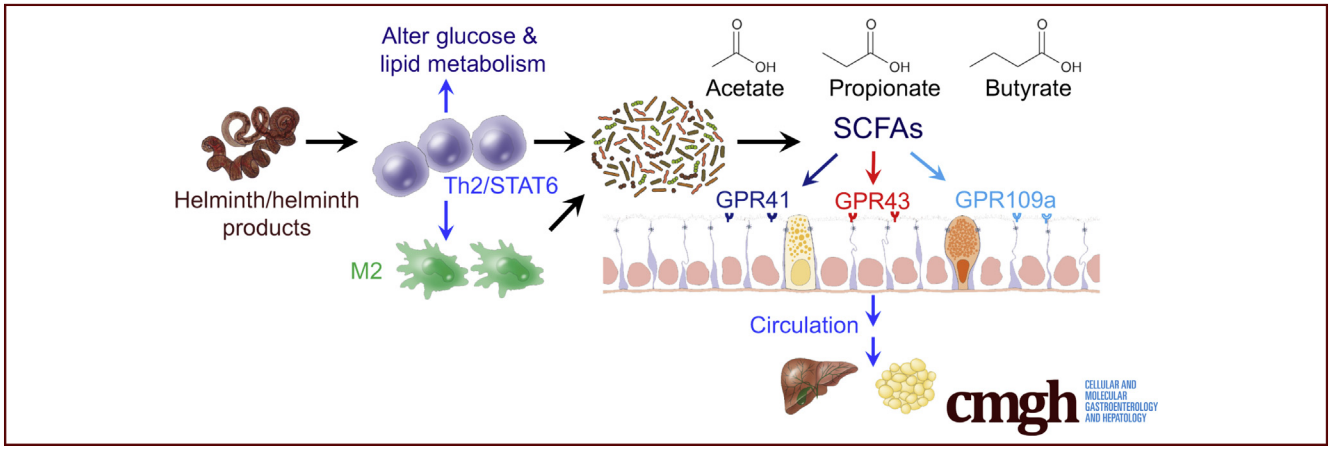

SUMMARY

Work from our laboratory and others show that helminth infection attenuates HFD-induced obesity. This current study demonstrates that helminth infection protects against HFD-induced obesity through helminth-induced, Th2-dependent alterations of the intestinal microbiota composition and functionality.

BACKGROUND & AIMS: Epidemiological and animal studies have indicated an inverse correlation between the rising prevalence of obesity and metabolic syndrome and exposure to helminths. Whether helminth-induced immune response contributes to microbiota remodeling in obesity remains unknown. The aim of this study is to explore the immune-regulatory role of helminth in the prevention of HFD-induced obesity through remodeling gut microbiome.

METHODS: C57BL/6J WT and STAT6$^{-/-}$ mice were infected with *Heligmosomoides polygyrus* and followed by high fat diet (HFD) feeding for 6 weeks. The host immune response, body weight, and fecal microbiota composition were analyzed. We used adoptive transfer of M2 macrophages and microbiota transplantation approaches to determine the impact of these factors on HFD-obesity. We also examined stool microbiota composition and short chain fatty acids (SCFAs) concentration and determined the expression of SCFA-relevant receptors in the recipient mice.

RESULTS: Helminth infection of STAT6$^{-/-}$ (Th2-deficient) mice and adoptive transfer of helminth-induced alternatively activated (M2) macrophages demonstrated that the helminth-associated Th2 immune response plays an important role in the protection against obesity and induces changes in microbiota composition. Microbiota transplantation showed that helminth-induced, Th2-dependent alterations of the gut microbiota are sufficient to confer protection against obesity. Collectively, these results indicate that helminth infection protects against HFD-induced obesity by Th2-dependent, M2 macrophage-mediated alterations of the intestinal microbiota.

CONCLUSION: Our findings provide new mechanistic insights into the complex interplay between helminth infection, the immune system and the gut microbiota in a HFD-induced obesity model and holds promise for gut microbiome-targeted immunotherapy in obesity prevention. *(Cell Mol Gastroenterol Hepatol 2020;10:763–778; https://doi.org/10.1016/j.jcmgh.2020.06.010)*

Keywords: Helminth; Microbiome; High-fat diet; Type 2 immunity; Obesity.

The worldwide increase in obesity remains one of the greatest threats to the health of adults and children. In 2015, more than one-third of U.S. adults suffered from obesity, and 17% of children were reported either overweight or obese.[1] Obesity is accompanied by chronic low-grade inflammation in adipose tissues, mainly owing to accumulated inflammatory cells (Th1/Th17 cells, macrophages, etc.), which exacerbate the transition from simple adiposity to metabolic diseases.[2,3] The increased rates of metabolic diseases (diabetes, steatosis, hypertension, heart disease, etc.) have imposed a large economic burden on individuals, families, and nations.[4] Therefore, reducing obesity is a public health priority that has substantial health and economic benefits.

Epidemiological studies have indicated an inverse correlation between the prevalence of the so-called Western diseases (obesity and metabolic syndrome) and exposure to parasitic helminths. Helminth infections form a major group of diseases that affect approximately 24% of the world's population, mainly in developing countries.[5] Helminths are known to modulate the immune system, promoting Th2-dominated as well as regulatory immune responses that are characterized by the production of interleukin (IL)-4, IL-5, and IL-13 by ILC2 and Th2 cells, and the induction of Tregs, eosinophils, mast cells, basophils, and alternatively activated (M2) macrophages.[6] The modulation of host immunity associated with helminth infection can also affect responses to concurrent infections and other antigens. For example, helminth-induced immune responses exacerbate concurrent bacterial infection,[7–11] whereas they ameliorate allergic conditions.[12–14] We have shown recently that infection of mice with the intestinal helminth parasite *H polygyrus* significantly attenuated high-fat diet (HFD)–induced obesity and that this attenuation was associated with enhanced Th2/Treg responses and M2 macrophage polarization.[15]

The intestinal microbiota is a complex and dynamic ecosystem, which is composed of trillions of microbes that reside in the gut and plays a significant role in health and disease.[16] It has been found that the composition of the intestinal microbiota differs between obese and healthy people and that altering the microbiota can alleviate obesity.[17] Published work from our laboratory and others has shown that *Heligmosomoides polygyrus* infection induces significant changes in the composition of the gut microbiota in mice.[10,18,19] The findings described previously raise the possibility that complex interactions between helminth infection, nutrition, immune function, and the gut microbiota influence the development of obesity. This idea is investigated in the current study.

Results

H polygyrus *Infection Protects Wild-Type But Not STAT6$^{-/-}$ Mice From HFD-Induced Obesity, Indicating the Involvement of a Th2-Dependent Mechanism*

Experiments from our laboratory and others have shown that helminth infections attenuate obesity induced by HFD in mouse models,[15,20–22] supporting the inverse correlation between helminth infections and obesity observed in clinical studies. In the current work, we investigated whether the protective effect of helminth infection against HFD obesity is dependent on the *H polygyrus*–induced Th2 immune response. To this end, control and *H polygyrus*–infected wild-type (WT) and Th2-deficient STAT6$^{-/-}$ mice were fed with HFD (Figure 1*A*) and the development of obesity was determined. WT mice gained weight by 40% whereas *H polygyrus*–infected WT mice were resistant to weight gain (Figure 1*B*). In contrast, the helminth infection failed to attenuate HFD-induced body weight gain in STAT6$^{-/-}$ mice (Figure 1*C*). Similar to our published results,[15] we found that food intake did not differ significantly between both helminth-infected and noninfected mice on HFD in the current study (data was not shown). In addition, we observed that *H polygyrus* infection had no visible effects on host growth performance in both WT and STAT6$^{-/-}$ mice when fed with a normal control diet (Figure 1*B* and *C*). Moreover, relative to the WT+HFD group, WT *Hp*+HFD mice had a lower circulating glucose level at 2 time points and a reduction of glucose area under the curve during a glucose tolerance test (Figure 1*D*). However, no significant differences in oral glucose tolerance test (OGTT) were observed between STAT6$^{-/-}$+HFD and helminth-infected STAT6$^{-/-}$ (STAT6$^{-/-}$ *Hp*+HFD) mice (Figure 1*E*). These observations suggest that the *H polygyrus*–induced Th2 immune response is essential for protection against obesity.

The helminth-induced Th2 response promotes the development of alternatively activated macrophages (AAM or M2). To further demonstrate the role of the helminth-induced Th2 response in the attenuation of HFD-induced obesity, we carried out macrophage transfer experiments using the methods that we recently developed.[15] Our results showed that adoptive transfer of M2 macrophages from helminth-infected WT mice conferred protection against HFD-induced obesity in the recipients (Figure 1*F* and *G*), confirming our recent observations.[15] Collectively, our results suggest that *H polygyrus* infection protects against HFD-induced obesity via the helminth-induced Th2 immune response.

Abbreviations used in this paper: **GPR, G protein–coupled receptor; HFD, high-fat diet; HFD+Cont-F, mice that received fecal material from control donors; HFD-Cont_mac, macrophages from control high-fat diet–fed mice; HFD+*Hp*-F, mice that received fecal material from *Heligmosomoides polygyrus*–infected donors; HFD-*Hp*_mac, macrophages from *Heligmosomoides polygyrus*–infected, high-fat diet–fed mice; IL, interleukin; LEfSe, linear discriminant analysis of effect size; mRNA, messenger RNA; OGTT, oral glucose tolerance test; PCA, principal component analysis; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SCFA, short-chain fatty acid; WT, wild-type.**

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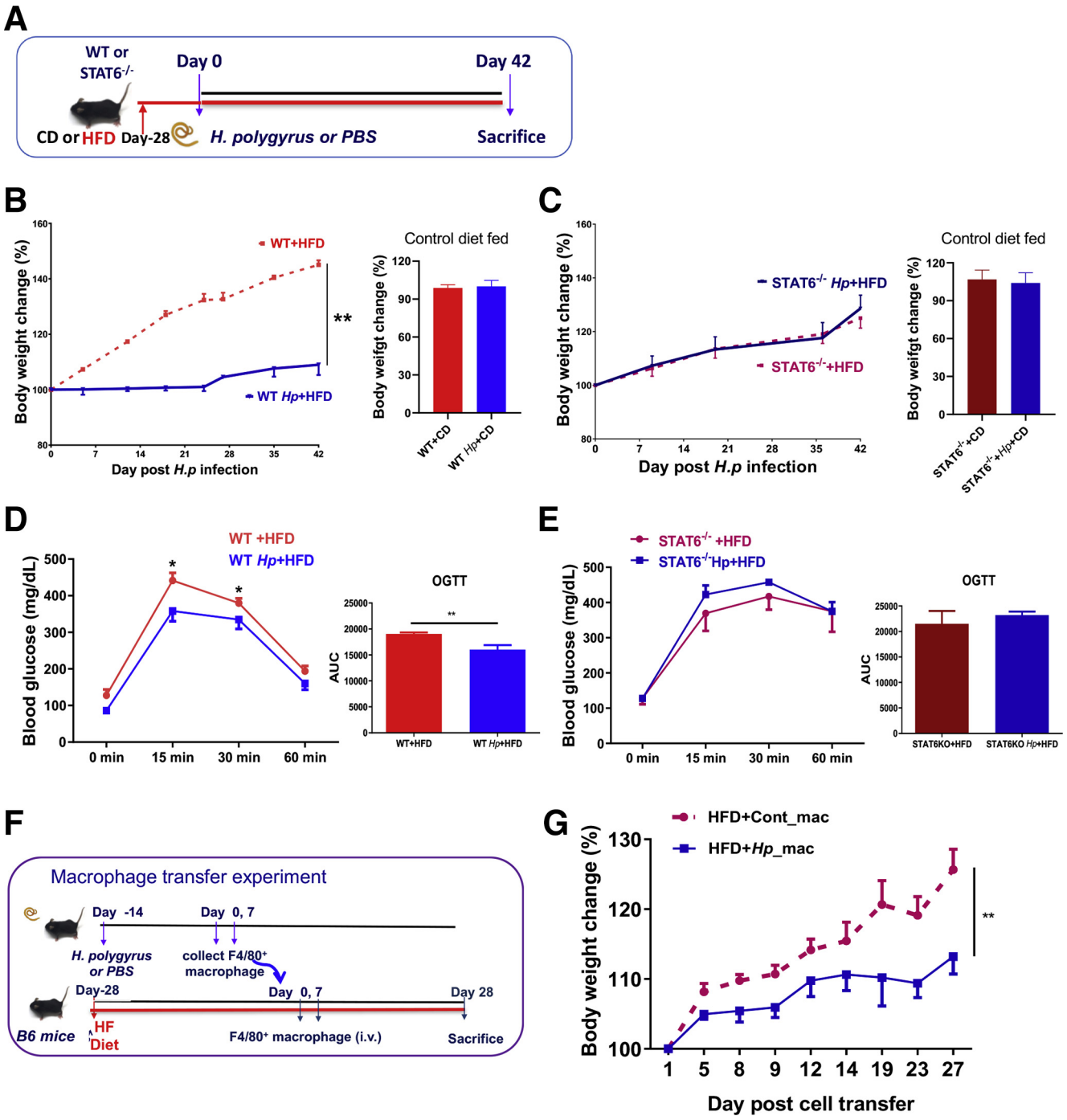
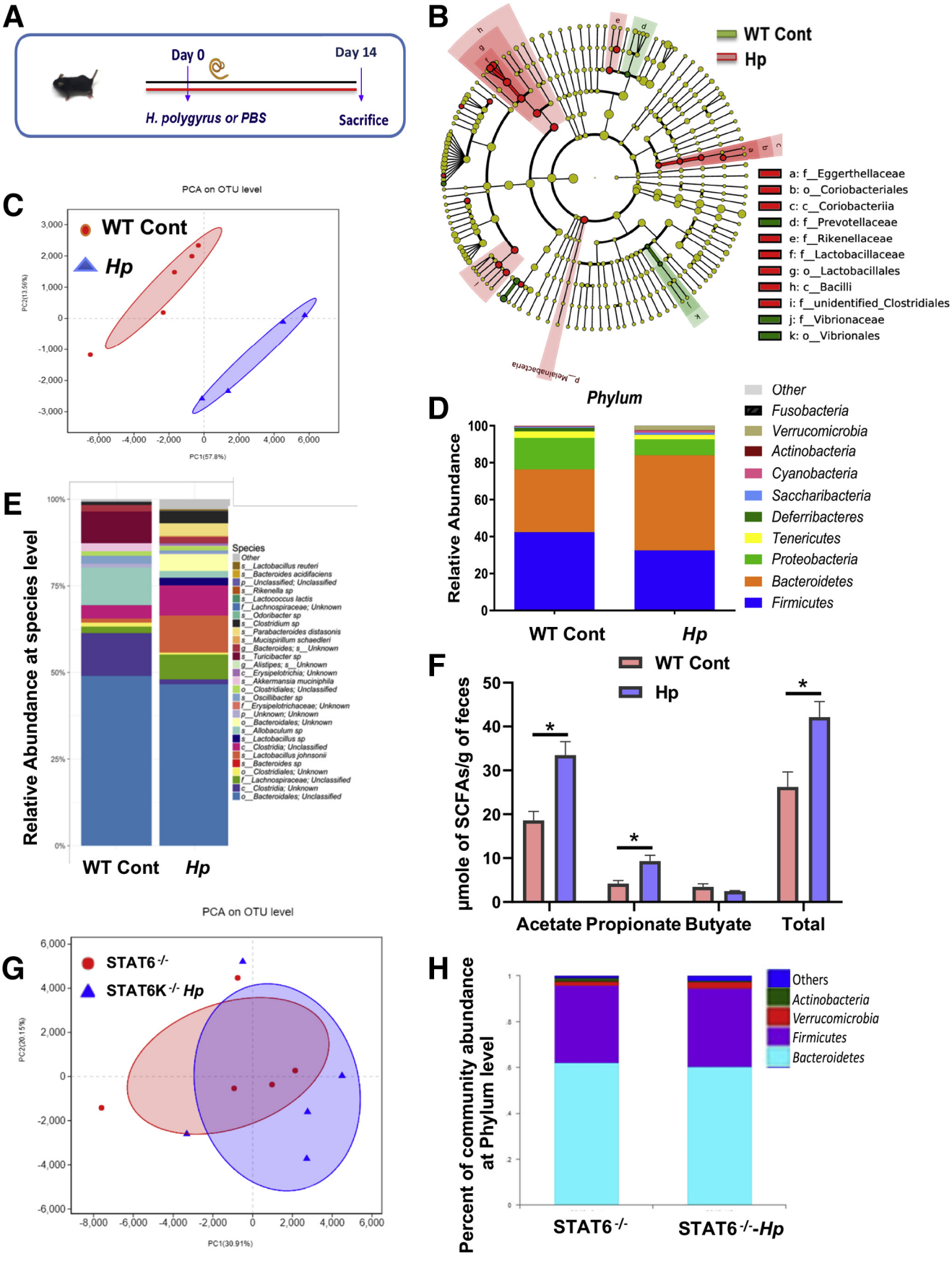


Figure 1. *H polygyrus* infection protects WT but not STAT6$^{-/-}$ mice from HFD-induced obesity. (*A*) The experimental model of HFD-induced obesity in WT and STAT6$^{-/-}$ mice with helminth infection. Phosphate-buffered saline (PBS) was used as control in the corresponding uninfected groups. (*B*) The *H polygyrus* infection group gained less body weight than did the WT control group (n = 5 mice/group). *$P < .05$; 2-tailed Student's *t* test. (*C*) *H polygyrus* infection failed to induce the attenuation of HFD-induced body weight gain in STAT6$^{-/-}$ mice (n = 5 mice/group). $P > .05$. (*B, C*) *H polygyrus* infection has no visible effects on host growth performance in both WT and STAT6$^{-/-}$ mice when fed with normal control diet (CD). (*D*) The oral glucose tolerance test revealed a significant difference between the WT-HFD fed and WT-*Hp*-HFD groups (left, blood glucose level at indicated time points; right, area under curve) (n = 5 mice/group). *$P < .05$; 2-tailed Student's *t* test. (*E*) The OGTT showed no difference between STAT6$^{-/-}$+HFD and helminth-infected STAT6$^{-/-}$ (STAT6$^{-/-}$ *Hp*+HFD) mice (left, blood glucose level at indicated time points; right, area under curve) (n = 5 mice/group). $P > .05$. (*F*) Experimental scheme for macrophage transfer. The macrophages were collected from either *H polygyrus*–infected or control donor mice at the indicated time points and intravenously injected into the corresponding recipients (HFD-Cont_mac and HFD-*Hp*_mac) pretreated with HFD for approximately 4 weeks. After macrophage transfer, the recipients continued receiving HFD for another 4 weeks. (*G*) The body weight change was less in HFD-*Hp*_mac than HFD-Cont_mac, indicating that adoptive transfer of helminth-induced M2 cells attenuates obesity in the recipients. The data are shown as the mean ± SEM (n = 3–5 mice/group) from 1 of 3 experiments performed showing similar results. *$P < .05$, ** $P < .01$; 2-tailed Student's *t* test.


A
Day 0
Day 14
H. polygyrus or PBS
Sacrifice
B
WT Cont
Hp
a: f__Eggerthellaceae
b: o__Coriobacteriales
c: c__Coriobacteriia
d: f__Prevotellaceae
e: f__Rikenellaceae
f: f__Lactobacillaceae
g: o__Lactobacillales
h: c__Bacilli
i: f__unidentified_Clostridiales
j: f__Vibrionaceae
k: o__Vibrionales
p__Melainabacteria
C
PCA on OTU level
WT Cont
Hp
PC2(13.56%)
PC1(57.8%)
D
Phylum
Relative Abundance
WT Cont
Hp
Other
Fusobacteria
Verrucomicrobia
Actinobacteria
Cyanobacteria
Saccharibacteria
Deferribacteres
Tenericutes
Proteobacteria
Bacteroidetes
Firmicutes
E
Relative Abundance at species level
Species
WT Cont
Hp
F
WT Cont
Hp
µmole of SCFAs/g of feces
Acetate
Propionate
Butyate
Total
G
PCA on OTU level
STAT6 -/-
STAT6K -/- Hp
PC2(20.15%)
PC1(30.91%)
H
Percent of community abundance at Phylum level
Others
Actinobacteria
Verrucomicrobia
Firmicutes
Bacteroidetes
STAT6 -/-
STAT6 -/--Hp

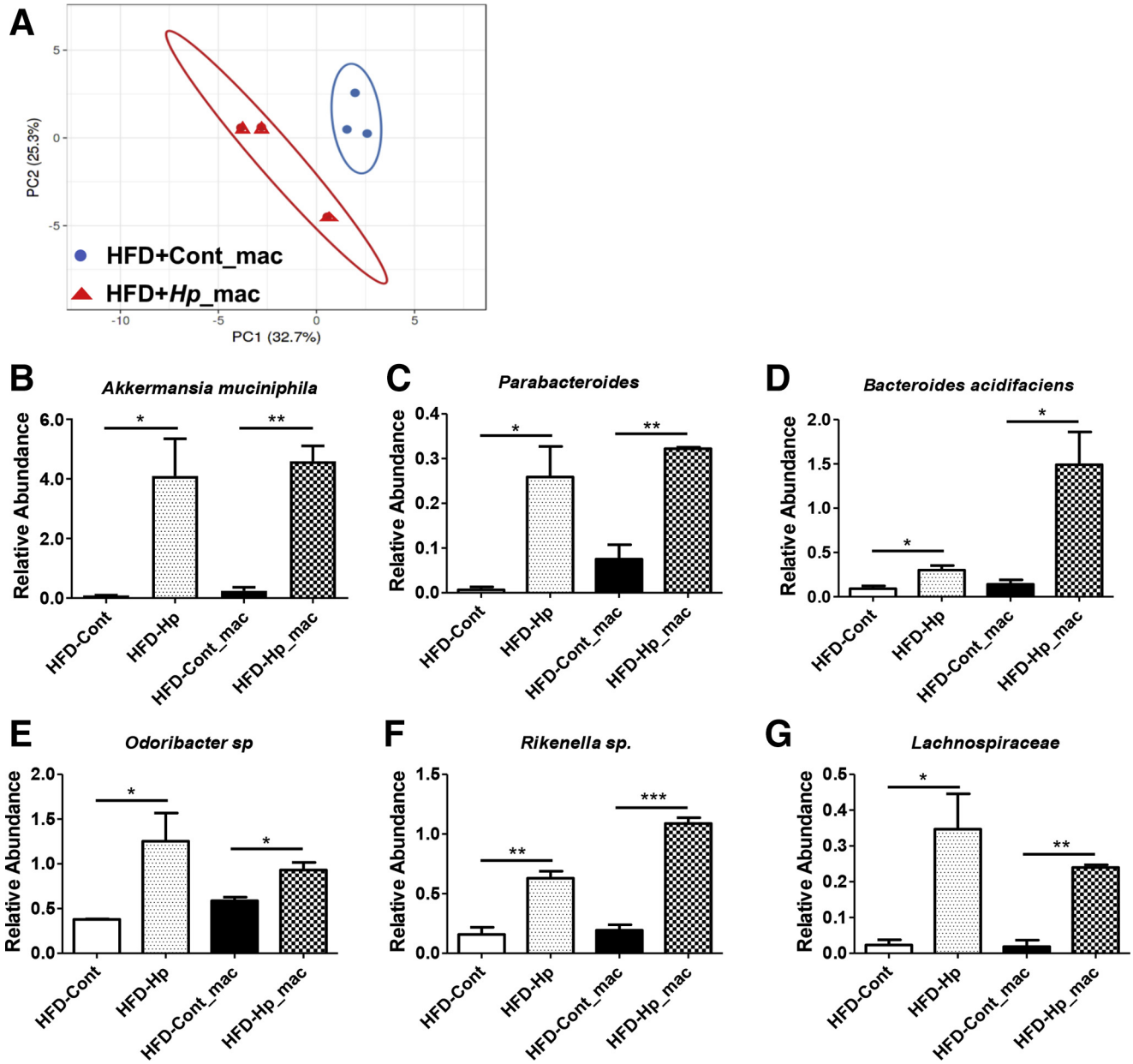
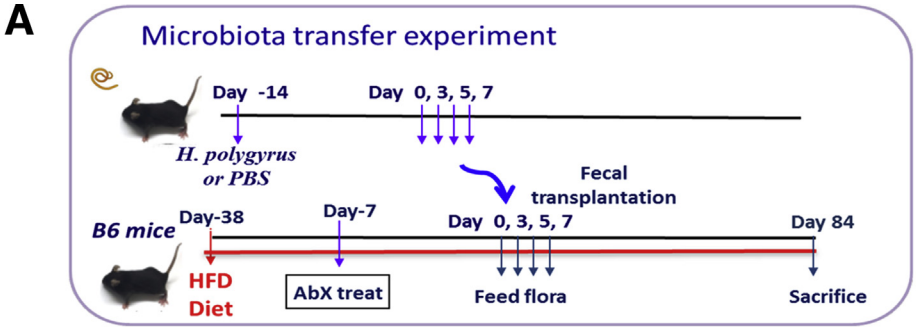
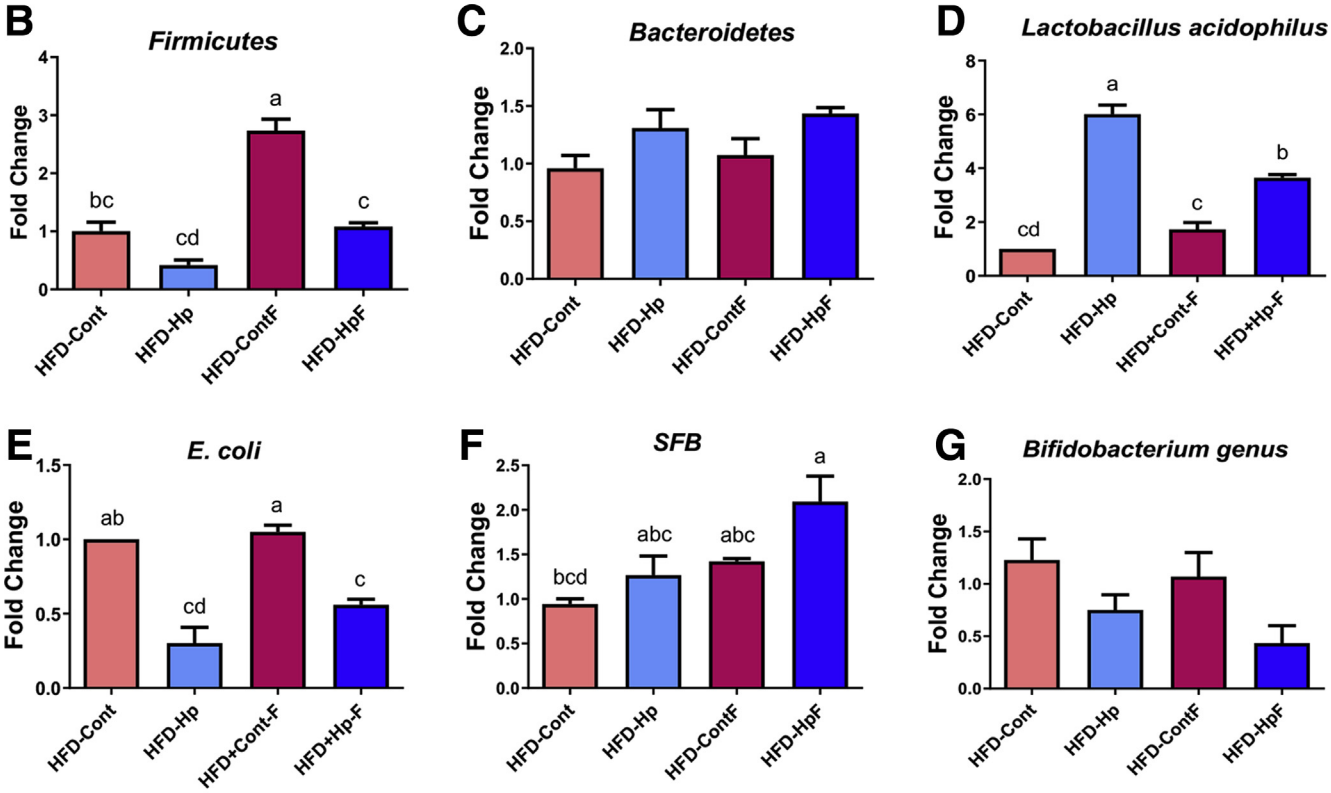


Figure 3. Adoptive transfer of helminth-induced M2 cells alters gut microbiota in the recipients. The macrophages were collected from either *H polygyrus*–infected or control donor mice and intravenously injected into the corresponding recipients (HFD-Cont_mac and HFD-*Hp*_mac) pretreated with HFD for approximately 4 weeks. Fecal samples were collected for microbiome profile analysis at 3 weeks after the last cell transfer. (*A*) PCA identified distinct clustering of microbiota in HFD-*Hp*_mac and HFD-Cont_mac. (*B–G*) Analysis of the relative abundance of *Akkermansia muciniphila*, *Parabacteroides*, *Bacteroides acidifaciens*, *Odpribacter sp.*, *Rikenella sp.*, and *Lachnospiraceae* between groups (n = 3–5/group). **P* < .05, ***P* < .01, ****P* < .001; 2-tailed Student's *t* test. The results show that adoptive transfer of helminth-induced M2 cells produces alterations in the gut microbiota of the recipients that are similar to those seen in the corresponding donors.

Figure 2. (***See previous page***). ***H polygyrus*** **infection of WT but not STAT6$^{-/-}$ mice leads to alterations in the gut microbiota.** (*A*) C57BL/6J WT mice received either helminth infection through oral gavage or PBS as control (n = 4–5 in each group). Fecal samples were collected for microbiome profile analysis and SCFA quantitation at 4 weeks postinfection. (*B*) LEfSe on clustered group revealed that Vibrionaceae and Prevotellaceae were more abundant in the control group while Rikenellaceae, Eggerthellaceae, and Lactobacillaceae were more abundant in the *H polygyrus* (*Hp*)–infected group. (*C*) PCA identified significant clustering of noninfected (Cont.) and *Hp* group based on Bray-Curtis distances (with the 97% confidence interval around group centroids). (*D, E*) Relative abundance at the phylum and species level of WT control and WT *Hp*-infected mice using relative abundance (%). (*F*) Total SCFAs differed significantly between groups of WT control and WT *Hp*-infected mice. **P* < .05; 2-tailed Student's *t* test. (*G, H*) Microbiota analysis of STAT6$^{-/-}$ mice with/without *Hp* infection. (*G*) No difference in PCA was identified between the microbiota of STAT6$^{-/-}$ control and *Hp*-infected STAT6$^{-/-}$ mice (n = 4–5). (*H*) Relative abundance (%) of microbiota communities of STAT6$^{-/-}$ control and *Hp*-infected STAT6$^{-/-}$ mice at phylum level, showing the similar microbiota composition.


A
Microbiota transfer experiment
Day -14
Day 0, 3, 5, 7
H. polygyrus
or PBS
Fecal
transplantation
B6 mice
Day-38
Day-7
Day 0, 3, 5, 7
Day 84
HFD
Diet
AbX treat
Feed flora
Sacrifice


B
Firmicutes
Fold Change
bc
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C
Bacteroidetes
Fold Change
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D
Lactobacillus acidophilus
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HFD-Cont
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HFD+Cont-F
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E
E. coli
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ab
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HFD-Cont
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F
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bcd
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abc
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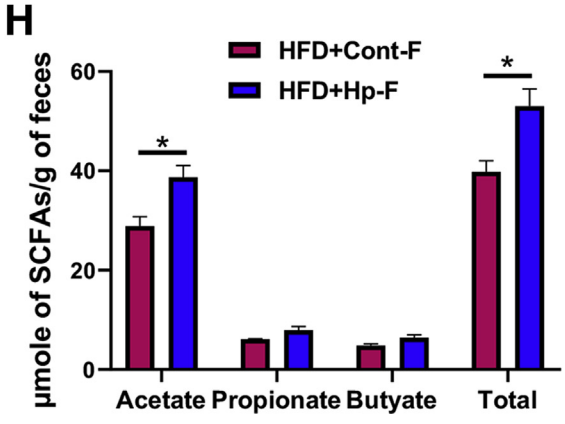

H
HFD+Cont-F
HFD+Hp-F
µmole of SCFAs/g of feces
*
*
Acetate
Propionate
Butyate
Total

H polygyrus *Infection of WT But Not STAT6$^{-/-}$ Mice Leads to Alterations in the Gut Microbiota in Association With Increased Levels of Fecal Short-Chain Fatty Acids*

To determine whether helminth-induced protective effect on HFD-obesity might be caused by helminth-induced alterations in the gut microbiota, we used bacterial 16S rRNA gene sequencing to compare the stool microbiome composition of control and helminth-infected WT mice. Our data revealed that *H polygyrus* infection induced significant changes in the gut microbiota composition (Figure 2*B*–*E*). Principal component analysis (PCA) revealed distinct clustering of the *H polygyrus*–infected group and uninfected control group (Figure 2*C*). Due to the significant difference in PCA clustering between *H polygyrus*–infected and uninfected groups, linear discriminant analysis of effect size (LEfSe) was performed on these 2 groups as a biomarker discovery approach to identify taxa that were possibly contributing to differences between the groups. LEfSe analysis showed that the control group had greater abundance of the families Vibrionaceae and Prevotellaceae, and the *H polygyrus*–infected group had greater abundance of the families Rikenellaceae, Eggerthellaceae, and Lactobacillaceae, contributing to the differences between the groups (Figure 2*B*). At the relative abundance of phylum level, our results confirmed that helminth infection induced significant changes in intestinal microbiome composition as indicated by a marked increase in Bacteroidetes and a decrease in Firmicutes (Figure 2*D*). At the relative abundance of species level, *H polygyrus* infection increased *Lactobacillus sp.,* and *Parabacteroides distasonis*, and decreased *Turicibacter sp.*, as well as *Oscillibacter sp.* when compared with the control group (Figure 2*E*).

We next determined whether the helminth-induced alterations of the gut microbiota result in changes in microbial metabolites. Specifically, we determined whether helminth infection affects bacterial short-chain fatty acids (SCFAs) production by measuring concentrations of the major SCFAs acetate, butyrate, and propionate in stool of control and *H polygyrus*–infected mice. Our results showed that relative to the control group, the levels of total SCFAs, as well as acetate and propionate, were significantly higher in stool of *H polygyrus*–infected hosts (Figure 2*F*) ($P < .05$), while no statistically significant difference in butyrate was detected between infected and control groups. These results demonstrated the role of *H polygyrus* infection in the modulation of microbiota-derived SCFAs. Together with our finding that helminth infection attenuates HFD-induced obesity in mice, both in this work and our previously published study,[15] our results suggest that the helminth-induced alteration in the gut microbiota might be one of the contributors to the attenuation.

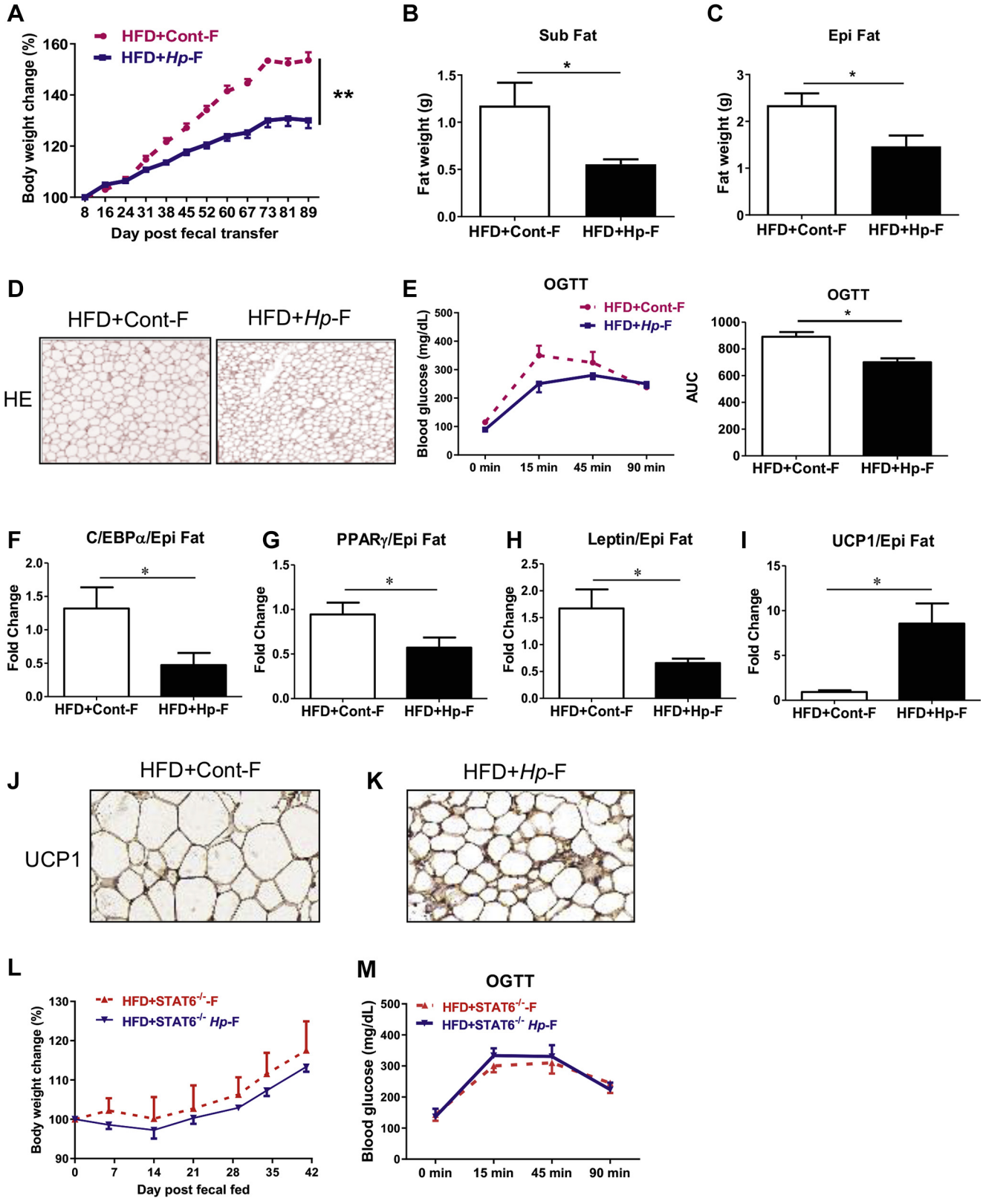
It has been well recognized that the mammalian gut microbiota has a role in the development and activation of the host immune system. Much less is known about how host immunity regulates the gut microbiota. To directly test the causative role of helminth-induced Th2 immune status on gut microbiota composition, we compared the fecal microbiota composition in control and *H polygyrus*–infected STAT6$^{-/-}$ mice. In contrast to PCA showing a clear separation of the microbiota of control and infected WT mice (Figure 2*C*), there was no statistically significant difference between the microbiota of the control and infected STAT6$^{-/-}$ mice (Figure 2*G*). In contrast to the results from WT mice (Figure 2*D*) showing the clear differences in microbiota composition between control and helminth-infected mice, at the relative abundance of phylum level, *H polygyrus* infection of STAT6$^{-/-}$ mice did not induce marked changes in gut microbiota composition compared with uninfected STAT6$^{-/-}$ mice (Figure 2*H*). These data suggest that *H polygyrus* infection remodels the gut microbiome, at least in part, in a Th2-dependent manner.

Recipients of Adoptively Transferred M2 Macrophages From H polygyrus*–Infected WT Mice Have Alterations in the Gut Microbiota That Are Similar to Those Seen in the Donors*

Work from our laboratory and others showed that *H polygyrus* infection promotes M2 polarization of macrophages. We have also recently demonstrated a role for helminth-induced M2 cells in modulating HFD-induced obesity.[15] In the current study, we further addressed the question of whether helminth-induced immune cells may contribute to helminth-induced alterations in the gut microbiota and protection against obesity. Accordingly, the macrophage transfer techniques that we recently developed[15] were used to determine the functional roles of helminth-induced macrophages in the modulation of intestinal microbiota composition. As revealed by PCA, recipients of macrophages from *H polygyrus*–infected, HFD-fed mice (HFD-*Hp*_mac) had clear differences in gut microbiota composition from recipients of macrophages from control

Figure 4. (***See previous page***). High similarity of the microbiota community in microbiota donors and corresponding recipients. (*A*) The microbiota transfer experimental scheme was shown. Fecal material was collected from either *Hp*-infected or control donor mice at the indicated time points and orally gavaged to the corresponding recipients pretreated with HFD for approximately 5 weeks followed by a 7-day antibiotic cocktail. (*B*–*G*) qPCR results showing the abundance of Firmicutes, Bacteroidetes, Lactobacillus, *Escherichia coli*, segmented filamentous bacteria, and *Bifidobacterium* between 4 groups (donors: HFD-Cont and HFD-*Hp*; corresponding recipients: HFD-Cont-F and HFD-*Hp*-F). Data were shown as mean ± SEM (n = 4–5). Different letters indicate statistically significant differences. $P < .05$; 1-way analysis of variance (Tukey HSD multiple comparison test). (*H*) Analysis of SCFAs in the HFD-fed recipient mice that were colonized with control microbiota (Cont-F) and the microbiota from helminth-infected donor (*Hp*-F) revealed significant differences between groups (n = 4–5). *$P < .05$; 2-tailed Student's *t* test.

HFD-fed mice (HFD-Cont_mac) (Figure 3*A*). Analysis of the most changed bacterial communities revealed that the HFD-*Hp*_mac group had increased relative abundance of *Akkermansia muciniphila*, *Parabacteroides*, *Bacteroides acidifaciens*, *Odpribacter sp.*, *Rikenella sp.*, and *Lachnospiraceae* relative to the HFD-Cont_mac group, reflecting the abundances of these species in the corresponding macrophage donors, HFD-*Hp* and HFD-Cont, respectively



(Figure 3*B*–*G*). These observations further confirm the role of the helminth-induced immune response in the modulation of gut microbiota composition.

Recipients of the Altered Microbiota of H polygyrus*–infected WT Mice Are Resistant to HFD-Induced Obesity*

To determine whether the helminth-altered gut microbiota was involved in attenuation of obesity, we collected fecal material from either helminth-infected or control donor mice and transferred fecal microbiota by oral gavage into the corresponding recipients, which were prepared by HFD feeding for 5 weeks followed by treatment with an antibiotic cocktail for 7 days to deplete the endogenous microbiota (Figure 4*A*). To confirm that the microbiota of the recipient mice reproduced the gut flora of their donors, we collected fecal pellets from the recipient mice 1 week after the last transfer and analyzed fecal bacteria. Our quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of representative bacteria demonstrated a clear concordance between the microbiota of the recipients and the corresponding donors even though long-term HFD feeding caused increased Firmicutes in recipients compare with donors (Figure 4*B*–*G*). Moreover, the total fecal SCFA concentrations of the recipients of the helminth-altered microbiota were higher than the recipients of the control microbiota (Figure 4*H*), mirroring the corresponding donors (Figure 2*F*). As we reported recently,[10] these results confirm that the fecal transplantation approach produced high similarity in the pattern of the microbiota community between the donors and the corresponding recipients.

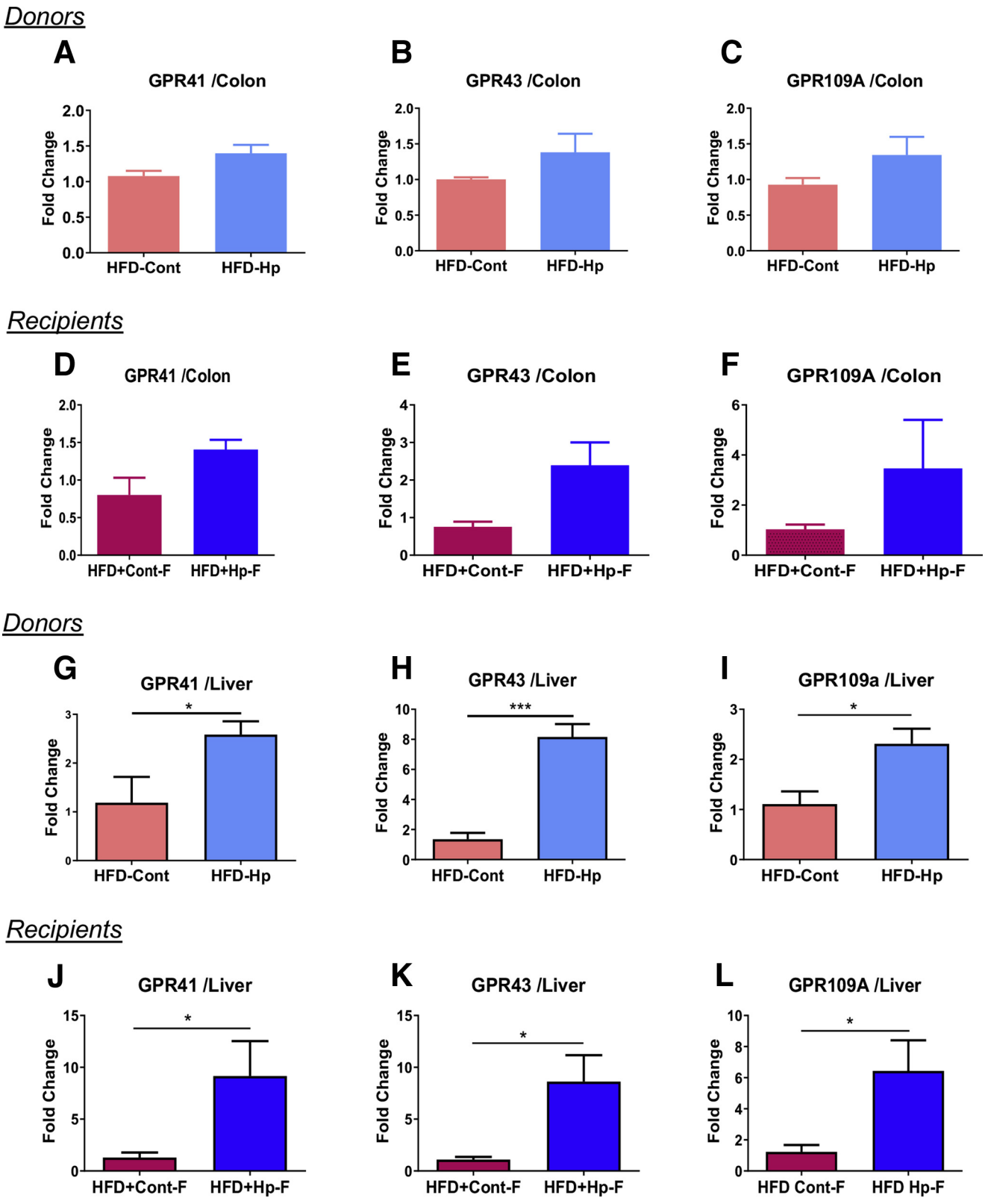
We next determined the functional significance of helminth-induced microbiota in the modulation of HFD-induced obesity in the recipient mice (Figure 4*A*). The mice that received fecal material from *H polygyrus*–infected donors (HFD+*Hp*-F) showed resistance to obesity as evidenced by less body weight gain ($P < .01$) (Figure 5*A*), a marked reduction in subcutaneous and epididymal fat pads (Figure 5*B* and *C*), and smaller adipocyte sizes in both fat pads (Figure 5*D*) when compared with mice that received fecal material from control donors (HFD+Cont-F). Moreover, relative to the HFD+Cont-F group, HFD+*Hp*-F mice had a lower circulating glucose level at 2 time points and a reduction of glucose area under the curve during a glucose tolerance test ($P < .05$) (Figure 5*E*). Moreover, we analyzed several key lipid metabolism regulators in epididymal fat using qRT-PCR, including leptin, a hormone produced in adipose tissue, which regulates fat storage in the body by balancing both appetite and energy usage, PPARγ, as well as C/EBPα, the critical transcription factors in adipogenesis.[23] Relative to the HFD+Cont-F group, HFD+*Hp*-F mice had significantly lower messenger RNA (mRNA) levels of *C/EBPα*, *PPARγ*, and *Leptin* (Figure 5*F*–*H*). Intriguingly, the expression of the fat-burning marker UCP1, which is involved in uncoupling-mediated energy expenditure,[24] was found to be significantly elevated in the HFD+*Hp*-F group (Figure 5*I*–*K*). Interestingly, however, no significant differences in body weight gain and OGTT were observed between recipients of microbiota from STAT6$^{-/-}$ control (HFD+STAT6$^{-/-}$-F) and helminth-infected STAT6$^{-/-}$ (HFD+STAT6$^{-/-}$ *Hp*-F) mice (Figure 5*L* and *M*). No significant differences in food intake were detected between the HFD+Cont-F and HFD+*Hp*-F mice (data was not shown). Collectively, the results of our fecal transplantation experiments suggest that *H polygyrus*–induced, Th2-dependent alterations in the microbiota are major contributors to the protective effects of helminth infection on HFD-induced obesity.

Helminth Infection and Helminth-Altered Microbiota Upregulate Expression of G Protein–Coupled Receptors for SCFAs

The intestinal bacteria-produced metabolites like SCFAs (eg, acetate, propionate, butyrate) are frequently reduced in patients with obesity, diabetes, autoimmune disorders, and cancers. SCFAs, as key mediators of the host-microbe mutualism that influence host energy homeostasis and immune function, can activate G protein–coupled receptors (GPRs), including free fatty acid receptors GPR41, GPR43, and GPR109A, and can also inhibit histone deacetylases.[25,26] The results described previously demonstrated that helminth infection resulted in an increased fecal SCFA level (Figure 2*F*) and mice that were colonized with helminth-induced microbiota also showed enhanced levels of total fecal SCFAs compared with the mice that received microbiota from control donors (HFD+Cont-F) (Figure 4*H*). The detection of increased SCFAs in these mice corresponds with helminth-induced protective effects on obesity. These

Figure 5. (***See previous page***). **Transfer of *H polygyrus*–altered microbiota protects against HFD-induced obesity.** (*A*) HFD-fed recipients of helminth-altered fecal microbiota (HFD-*Hp*-F) exhibited less body weight gain than recipients of control microbiota (HFD-Cont-F) (n = 5 mice/group). *$P < .01$; 2-tailed Student's *t* test. (*B, C*) The weights of subcutaneous and epididymal fat in HFD-*Hp*-F group were less than those in HFD-Cont-F group. (*D*) Hematoxylin and eosin staining showed the difference in adipocyte size between HFD-*Hp*-F and HFD-Cont-F mice. (*E*) The results of oral glucose tolerance test were significantly different between the 2 groups (left, blood glucose level at indicated time points; right, area under curve) (n = 5 mice/group). *$P < .05$; 2-tailed Student's *t* test. (*F–I*) qRT-PCR results showing the mRNA levels of C/EBPα, PPARγ, leptin, and UCP1 between the 2 groups. (*J, K*) A representative image of UCP1 staining of adipose tissue from the 2 groups. (*L, M*) The similar body weight gain and oral glucose tolerance test were detected in the WT recipient mice that were colonized with microbiota from control STAT6$^{-/-}$ (HFD+STAT6$^{-/-}$-F) and helminth-infected STAT6$^{-/-}$ (HFD+ STAT6$^{-/-}$ *Hp*-F) mice. Data were shown as mean ± SEM (n = 5–6) from 1 of 2 experiments performed showing similar results. *$P < .05$, ** $P < .01$; 2-tailed Student's *t* test.

results suggest that the increased SCFA levels may contribute, at least in part, to the effects of helminth infection on obesity. We next determined whether helminth infection and helminth-induced microbiota may affect the expression of GPRs in the hosts. Our qRT-PCR results showed a high similarity of intestinal and liver GPR



expression in the microbiota donors and corresponding recipients. A clear trend of increased mRNA expression of *GPR41*, *GPR43*, and *GPR109* was detected in the colon (Figure 6*A–C*) and liver (Figure 6*G–I*) of HFD-fed mice with helminth infection (HFD-*Hp*), and also in the colon (Figure 6*D–F*) and liver (Figure 6*J–L*) of the recipients of *H polygyrus*–altered microbiota (HFD+*Hp*-F) compared with their corresponding control animals. These results further support the idea that the SCFA-GPR signaling pathway may be involved in *H polygyrus*–induced protection against HFD-induced obesity, and that the altered microbiota are a key mediator of this effect through increased production of SCFAs and increased expression of SCFA receptors.

Discussion

Work from our laboratory and others has demonstrated that helminth infection results in the attenuation of HFD-induced obesity and that infection of diet-induced obese mice with *H polygyrus* ameliorates whole-body insulin resistance and glucose intolerance.[15,22,27] The pathophysiology of obesity is multifactorial, and not completely understood. The protective effects of helminths on metabolic disorders have been attributed to the immunomodulatory effects of worms driving Th2 and regulatory immune responses,[15,22,27] which have been linked to the amelioration of Th1- or Th17-mediated diseases.[27–29]

It has become increasingly clear that multiple facets of the Th2-associated immune response promote metabolic homeostasis.[30,31] In the current study, we determined the effect of the helminth-induced Th2 immune response on host microbiota composition and functionality. Our results revealed that *H polygyrus* infection of STAT6$^{-/-}$ mice failed to induce a significant alteration in the gut microbiome and infection of HFD-fed STAT6$^{-/-}$ mice did not attenuate obesity. These results demonstrated that the effects of helminths on intestinal microbiota composition are dependent on the helminth-induced Th2 immune response. This notion is further supported by the results showing that even in the absence of live *H polygyrus* infection, adoptive transfer of *H polygyrus*–induced immune cells (M2 macrophages) resulted in attenuated obesity, which corresponded with altered intestinal microbiota composition in the recipient mice. Our results are supported by a study showing that treating mice with IL-25, an initiating factor for type 2 immunity, altered the gut microbiota.[32] By utilizing the microbiota transplantation approach, we showed for the first time that transplantation of gut microbiota of helminth-infected WT, but not STAT6 knockout, mice was sufficient for the protective effects of helminths on HFD-induced obesity. Overall, our results suggest that helminth infection protects against HFD-induced obesity through Th2-dependent alterations of the intestinal microbiota.

The intestinal microbiota has received increasing attention as a metabolic interface between the outer environment and the host, modulating intestinal barrier function, inflammation, energy metabolism, and body weight homeostasis. A link between intestinal microflora and host metabolism was first provided by a study demonstrating that transplantation of the intestinal microflora from obese mice replicated the obese phenotype in germ-free mice.[33] It has also been shown that germ-free mice were resistant to HFD-induced obesity[34] and gained less weight than conventional mice when consuming a sugar- and fat-rich Western diet.[35] Western diet feeding alters the gut microbiota by increasing Firmicutes and reducing Bacteroidetes in conventional mice,[36] in addition to inducing excessive weight gain. Functional analysis of the Western diet–altered intestinal metagenome showed the enrichment of bacterial genes involved in the transport and fermentation of sugars and host glycans.[36] In line with these observations, it was also reported that obese mice lacking leptin, a lipid metabolism regulator (*ob/ob* mice), have a distinct gut microbiota compared with (normal) lean mice, with a shift toward fewer Bacteroidetes and more Firmicutes in the obese animals. In line with these observations, our analysis showed increased abundance of Bacteroidetes and reduced Firmicutes in helminth-infected lean mice. Our results further indicate that the helminth-induced microbiota can modulate the metabolic processes of the host independently of live parasite infection, establishing a new mechanism by which helminths modulate host responses to diets.

The results of our microbiota transfer experiments show that the recipients of helminth-induced microbiota have enhanced UCP1 expression in adipose tissues. UCP1, which is exclusively expressed in adipose tissue, is involved in uncoupling-mediated energy expenditure and is a marker of fat browning. UCP1 allows brown adipose tissue to enhance thermogenesis via uncoupling oxidative phosphorylation at the expense of adenosine triphosphate production. The effects of the microbiota transfer on UCP1 expression are consistent with our recently published observations showing that helminth infection upregulated UCP1, activated brown adipose tissue and increased browning of white adipose tissues in HFD-fed mice.[15] The current study thus suggests that the helminth-induced increase in UCP1 is mediated by the microbiota, and that the attenuation of HFD-induced obesity is the result of increased energy

Figure 6. (*See previous page*). Similarity of intestinal and liver GPR expression in the microbiota donors and corresponding recipients. (*A–F*) Colonic GPR expression. (*A–C*) qRT-PCR results showing the colon mRNA levels of GPR41, GPR43, and GPR109a in the 2 donor groups, HFD-Cont and HFD-*Hp*. (*D–F*) qRT-PCR results showing the colon mRNA levels of GPR41, GPR43, and GPR109a in the 2 recipient groups: HFD-Cont-F and HFD-*Hp*-F. (*G*) Liver GPR expression: qRT-PCR results showing the liver mRNA levels of GPR41, GPR43, and GPR109a in the 2 donor groups: HFD-Cont and HFD-*Hp*. (*J–L*) qRT-PCR results showing the liver mRNA levels of GPR41, GPR43, and GPR109a in the corresponding recipient groups: HFD-Cont-F and HFD-*Hp*-F. Data were shown as mean ± SEM (n = 4–5) from 1 of 2 experiments performed showing similar results. *$P < .05$, *** $P < .001$; 2-tailed Student's *t* test.

expenditure. The question of how precisely helminth-induced microbiota regulates UCP1 expression warrants future investigation.

Our results also suggest that one of the mechanisms by which helminth-altered intestinal microflora modulates HFD-induced obesity may be through the production of microbial metabolites. SCFAs (acetate, butyrate, and propionate) have emerged as major mediators in linking nutrition, gut microbiota, physiology and pathology. Evidence indicates that SCFAs can participate in energy, glucose, and lipid homeostasis, playing an overall beneficial role in preventing obesity through interaction with GPR receptors.[37] Accordingly, we observed that the helminth-altered microbiota has an increased level of SCFAs, which is supported by a report indicating that mice receiving a *H polygyrus*–modulated microbiota[14] had elevated amounts of cecal SCFA (mainly acetate). Acetate has been found to reduce the appetite through the interaction with the central nervous system.[38] It has been reported that butyrate and propionate can induce the production of gut hormones, reducing food intake.[39,40] A recent study showed that dietary SCFA-induced protection against HFD-induced obesity is mediated by downregulation of PPARγ, a regulator of adipocyte differentiation, promoting a change from lipid synthesis to lipid oxidation.[26] In line with this, we found that mice that were colonized with helminth-induced microbiota displayed reduced PPARγ expression. SCFAs can also promote a regulatory immune response by stimulating the differentiation and suppressive capacity of Foxp3^{+}Tregs in the intestine, which may modulate the development of obesity by decreasing the chronic low-grade inflammation characteristic of this condition.[3] The increased stool SCFA levels in helminth-infected mice probably results from changes to microbiota structures that favor SCFA-producing bacterial communities, which may improve the metabolic profile in mice.[41] However, it may be also possible that the increased stool SCFA levels result from reduced absorption of SCFAs in the gut, contributing to reduced weight gain and fat deposition in infected mice, which is consistent with the notion that excessive SCFAs produced by gut microbiota may also represent an additional energy source, causing an imbalance in energy regulation that contributes to obesity.[42] Our analysis showed that helminth-infected mice and the recipients of helminth-infected donors displayed increased expression of GPR41, GPR43, and GPR109a in the liver and colon compared with the corresponding control animals. The published studies on knockout mice implicated the role of GPR41 and GPR43 in chronic inflammatory disorders such as obesity, colitis, asthma, and arthritis,[43] as well as in glucose tolerance.[44] However, the potential functional significance of the changed microbiota, SCFA concentrations, and host GPR expression induced by helminth parasites in modulating host metabolism and dietary induced disorders remains to be established. The increased level of SCFAs and the number and availability of GPRs on the cell surface that are detected in our study may affect the magnitude and duration of the effect of SCFAs in helminth-infected mice or mice colonized with helminth-altered microbiota.

Conclusion

The results from the current study suggest that *H polygyrus* infection protects against obesity by helminth-induced and Th2-dependent alterations of the gut microbiota. This finding provides new mechanistic insights into the complex interplay between helminth-induced immune deviation, gut microbiota alterations, and HFD-induced obesity in mice. These insights will facilitate the development of novel strategies for gut microbiome–targeted immunotherapy in obesity prevention.

Materials and Methods

Mice and Diet

C57BL/6 and STAT6$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), fed a normal control diet (10% of kcal from fat) or given a HFD (60% of kcal from fat; Research Diets, New Brunswick, NJ) to induce obesity. The animals were maintained in a specific pathogen-free facility at Massachusetts General Hospital. Animal care was provided in accordance with protocols approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. At the fourth week of dietary intervention, some of the control diet–fed mice and HFD-fed mice were infected with *H polygyrus*. The dietary intervention was continued, and the body weight was measured every week throughout the experimental period. The design of different experiments is illustrated in Figures 1*A*, 1*F*, 2*A*, and 4*A*.

H polygyrus *Infection*

H polygyrus were propagated as previously described and stored at 4°C until use.[7,8] Mice were inoculated orally with 200 third-stage larvae.

Gut Microbiome Analysis

Fecal pellets were collected from colons of uninfected and *H polygyrus*–infected mice (2 weeks after infection) at necropsy. DNA was extracted using the QIAamp DNA stool kit (QIAGEN, Chatsworth, CA). We used 16S ribosomal RNA gene sequencing to characterize bacterial populations and perform grouping by shared sequence characteristics (taxonomical assignment). Sequencing and analysis were performed at the Research and Testing Laboratory (Lubbock, TX) using 454 methods as described previously.[45]

Operational taxonomy units were clustered with 97% identity by Usearch (version 7.0, http://drive5.com/usearch/manual7/), and chimeric sequences were identified and removed. PCA was conducted using the representative sequences of operational taxonomy units for each sample. Differentially significant features between combinations of paired group for both taxonomic and functional assignments were identified using LEfSe.[46] Linear discriminant analysis scores derived from the LEfSe analysis were used to show the relationship between taxa using a cladogram of significantly increased or decreased bacterial taxa in the gut microbiota between groups.[46] Levels of the cladogram represented phylum, class, order, family, and

Table 1. Primer Sequence

Primer	Sequence
Akkermansia muciniphila For	CAGCACGTGAAGGTGGGGAC
Akkermansia muciniphila Rev	CCTTGCGGTTGGCTTCAGAT
Universal For	TCCTACGGGAGGCAGCAGT
Universal Rev	GGACTACCAGGGTATCTAATCCTGTT
SFB For	AGGAGGAGTCTGCGGCACATTAGC
SFB Rev	CGCATCCTTTACGCCCAGTTATTC
Bifidobacterium genus For	TCGCGTCCGGTGTGAAAG
Bifidobacterium genus Rev	CCACATCCAGCATCCAC
Firmicutes Fc	GGAGCATGTGGTTTAATTCGAAGCA
Firmicutes Ft	GGAGTATGTGGTTTAATTCGAAGCA
Firmicutes Rev	AGCTGACGACAACCATGCAC
Bacteroidetes Fa	GGAACATGTGGTTTAATTCGATGAT
Bacteroidetes Fg	GGAGCATGTGGTTTAATTCGATGAT
Bacteroidetes Rev	AGCTGACGACAACCATGCAG
Prevotella Fa	CACAGTAAACGATGGATGCC
Prevotella Fb	CACGGTAAACGATGGATGCC
Prevotella Rev	GGTCGGGTTGCAGACC
Lactobacillus acidophilus For	GAGGTAGTAACTGGCCTTTA
Lactobacillus acidophilus Rev	GCGGAAACCTCCCAACA
PPARγ For	CCCAATGGTTGCTGATTACAAAT
PPARγ Rev	CTACTTTGATCGCACTTTGGTATTCT
Leptin For	GAGACCCCTGTGTCGGTTC
Leptin Rev	CTGCGTGTGTGAAATGTCATTG
C/EBPα For	GAGCCGAGATAAAGCCAAACA
C/EBPα Rev	GCGCAGGCGGTCATTG
UCP1 For	GTGAAGGTCAGAATGCAAGC
UCP1 Rev	AGGGCCCCCTTCATGAGGTC
GAPDH-For	CCTGCACCACCAACTGCTT
GAPDH-Rev	ATGACCTTGCCCACAGCCT
GPR109a-For	ATGGCGAGGCATATCTGTGTAGCA
GPR109a-Rev	TCCTGCCTGAGCAGAACAAGATGA
GPR43-For	ACAGTGGAGGGGACCAAGAT
GPR43-Rev	GGGGACTCTCTACTCGGTGA
GPR41-For	TTGCTAAACCTGACCATTTCGG
GPR41-Rev	GATAGGCCACGCTCAGAAAAC

genus from the inner to outer rings. Colors indicated the groups, and letters indicated the taxa that contribute to the uniqueness of the corresponding groups at an linear discriminant analysis of >2.0. All analyses were performed in R, version 3.6.3 (R Foundation for Statistical Computing. Vienna, Austria), the vegan,[47] and phyloseq packages.[48]

Antibiotic Treatment and Microbiota Transfer

C57BL/6 recipient mice were pretreated with a cocktail of antibiotics (kanamycin 0.4 mg/mL, gentamicin 0.035 mg/mL, colistin 850 U/mL, metronidazole 0.215 mg/mL, vancomycin 0.045 mg/ml) (Sigma Aldrich, St. Louis, MO) in drinking water for 1 week, a treatment regime that has been shown to effectively eliminate intestinal bacteria.[10,49] One week after antibiotic treatment, recipient mice were administered intestinal contents (including cecal, colonic, and fecal materials) collected from the control or *H polygyrus*–infected donor mice 3 times a week. HFD treatment was sustained for 10–11 weeks after microbiota transfer. The HFD was introduced to the recipient mice and maintained throughout the experimental period (for a total of 122 days) (Figure 4*A*).

Macrophage Isolation and Adoptive Transfer

Spleen and peritoneal cells from *H polygyrus*–infected mice (2–3 weeks after helminth infection) and noninfected mice were collected aseptically into complete Dulbecco's modified Eagle medium as previously described.[50] $F4/80^+$ macrophages were purified using our published methods[15] and transferred into HFD-fed recipient mice ($2–3 \times 10^6$ cells per mouse) via tail vein injection 4 weeks after dietary intervention. HFD treatment was sustained for 4 weeks after macrophage transfer. Immunostaining with anti-F4/80 and anti-CD206 was used to confirm the M2 phenotype of macrophages from helminth-infected mice.

Glucose and Insulin Tolerance Tests

The OGTT and insulin tolerance test were carried out according to our published methods.[15] Briefly, the mice were fasted overnight for 15 hours and challenged with glucose solution (2 g/kg of body weight) by oral gavage. Prior to glucose load, blood glucose level was measured and then monitored at times 15, 30, 45, 60, and 120 minutes, using a Bayer contour (Bayer Healthcare, Mishawaka, IN). For insulin tolerance test, the mice were fasted for 6 hours and challenged with insulin (0.75-U/kg body weight; 2% [v/v] in phosphate-buffered saline) by intraperitoneal injection. The baseline blood glucose level was measured and monitored at time 10, 20, 30, 40, and 60 minutes following insulin injection.

Real-Time PCR Analysis of Gut Microbiota

Fecal material of donor mice (*H polygyrus*–infected and uninfected) and the corresponding recipient mice were collected. DNA was extracted using the QIAamp DNA stool kit (QIAGEN). The abundance of bacteria that have been reported to be involved in obesity was determined by quantitative PCR using the primers listed in Table 1.

Measurement of Gene Expression

Total RNA was isolated from gonadal adipose tissue. qRT-PCR was used to determine the expression levels of adipocyte specific genes (PPARγ, leptin, and C/EBPα), thermogenesis-related gene (UCP-1), and several GPRs (GPR109a, GPR43, and GPR41). GAPDH was the housekeeping control. The primer sequences are listed in Table 1.

SCFA Measurement

Fecal SCFAs were measured using gas chromatography according to a previously described method.[51] Briefly, fecal samples were vortex-mixed in Milli-Q water with 1:4 dilution, and incubated at room temperature for 10 minutes prior to centrifugation at 14,000 rpm for 20 minutes. Then, the supernatants were centrifugated twice to remove impurities (14,000 rpm, 20 minutes, 4°C) and 2-ethylbutyric acid (Sigma-Aldrich) was added as an internal standard at final concentration of 1 mM, followed by passage through 0.22-μm filters before being transferred to clean vials. GC 6890N (Agilent Technologies, Santa Clara, CA) equipped with DB-FFAP capillary column (30-m length by 0.25-mm internal diameter, with a 0.25-μm film thickness; Agilent Technologies) and a flame ionization detector was used for identification and quantification of SCFAs. Data were collected using the ChemStation software (Agilent Technologies). SCFAs (acetate, propionate, i-butyrate, and n-butyrate) were identified by comparison of their retention times with those of the corresponding standards. The peaks were quantified as relative abundances with respect to the internal standard. The concentration (in μmole range) of each SCFA was calculated using the linear regression equations ($R^2 \geq 0.99$) from the corresponding standard curves.

Statistical Analysis

All results were expressed as the mean ± SEM. N refers to the number of mice used. Statistical differences were determined using a 2-tailed Student's t test or 1-way analysis of variance (Tukey honestly significant difference multiple comparison test) using GraphPad Prism (GraphPad Prism 8 Software, San Diego, CA). A P value of $<.05$ was considered significant.

Ethics approval and consent to participate

All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (protocol number 2004N000044, animal welfare assurance number A3596-01).

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Conflicts of Interest

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