Cell Metabolism

Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents

Graphical Abstract

Highlights

- High-fat diet induces low-grade bowel inflammatory changes in resident immune cells
- Altered gut immunity in obesity contributes to obesity-related insulin resistance
- Gut immunity alters gut barrier, fat inflammation, and oral tolerance in obesity
- Targeting gut inflammation is a novel treatment approach for metabolic disease

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In Brief

Luck and Tsai et al. report that altered gut immunity during obesity contributes to systemic insulin resistance through changes in intestinal barrier function, endotoxemia, fat inflammation, and oral tolerance to gut luminal antigen. Targeting gut inflammation with 5-ASA improves these parameters and represents a novel treatment approach for metabolic disease.
Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents

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SUMMARY

Obesity has reached epidemic proportions, but little is known about its influence on the intestinal immune system. Here we show that the gut immune system is altered during high-fat diet (HFD) feeding and is a functional regulator of obesity-related insulin resistance (IR) that can be exploited therapeutically. Obesity induces a chronic phenotypic pro-inflammatory shift in bowel lamina propria immune cell populations. Reduction of the gut immune system, using beta7 integrin-deficient mice (Beta7null), decreases HFD-induced IR. Treatment of wild-type HFD C57BL/6 mice with the local gut anti-inflammatory, 5-aminosalicylic acid (5-ASA), reverses bowel inflammation and improves metabolic parameters. These beneficial effects are dependent on adaptive and gut immunity and are associated with reduced gut permeability and endotoxemia, decreased visceral adipose tissue inflammation, and improved antigen-specific tolerance to luminal antigens. Thus, the mucosal immune system affects multiple pathways associated with systemic IR and represents a novel therapeutic target in this disease.

INTRODUCTION

Obesity and its associated metabolic abnormalities, including type 2 diabetes (T2D) and its precursor, insulin resistance (IR), have become global diseases that carry considerable morbidity and mortality (Johnson and Olefsky, 2013). Obesity-related IR can arise through multiple pathways, but chronic inflammation in visceral adipose tissue (VAT) has become a prominent pathological mechanism (Gregor and Hotamisligil, 2011; Odegaard and Chawla, 2013). Cells of both the innate and adaptive immune system residing in VAT have been shown to play a key role in IR. More specifically, M1 macrophages, interferon (IFN) γ-secreting Th1 T cells, CD8+ T cells, and B cells promote IR, in part, through secretion of pro-inflammatory cytokines (Lumeng et al., 2007; Nishimura et al., 2009; Winer et al., 2009a, 2011). In contrast, Foxp3+ regulatory T cells (Tregs), eosinophils, Th2 T cells, and type 2 innate lymphoid cells (ILC2) are associated with protection from IR through local control of VAT inflammation (Feuerer et al., 2009; Molofsky et al., 2013; Winer et al., 2009a; Wu et al., 2011).

In addition to VAT, recent evidence has pointed to the bowel as a key site that becomes altered in obesity-related IR (Johnson and Olefsky, 2013). These alterations include changes in the gastrointestinal flora, known as dysbiosis, which can impact body fat, systemic inflammation, and IR (Bäckhed et al., 2004, 2007; Membrez et al., 2008; Turnbaugh et al., 2006). Under normal physiological conditions, dysbiosis is kept in check through maintenance of an intact intestinal barrier, characterized by increased mucus, transforming growth factor (TGF)-β, interleukin (IL)-10, IL-22, and luminal secretion of IgA (Brown et al., 2013). Dysbiosis is believed to cause low-grade inflammation both systemically, through enhanced leakage of bacterial products such as lipopolysaccharides (LPS), and locally in the small bowel and colon (Cani et al., 2007; de La Serre et al., 2010). Systemically, some of these bacterial products, including gut-derived
antigens, are thought to accumulate and potentiate inflammation in the VAT (Caesar et al., 2012; Wang et al., 2010). Thus, manipulation of the gut barrier to reduce LPS leakage (e.g., with IL-22) improves insulin sensitivity (Wang et al., 2014). In the bowel, increased tumor necrosis factor alpha (TNF-α) and NF-κB activation occurs in the ileum, while IL-1β and IL-12p40 levels are elevated in colons of HFD-fed mice (Ding et al., 2010; Li et al., 2008). However, the local effects of HFD on most immune cell populations in the gut and their function in IR remain unclear.

In IR and T2D, treatment with systemic anti-inflammatory therapies such as salicylates and IL-1β antagonists has shown some efficacy in clinical trials (Goldfine et al., 2010; Larsen et al., 2007), and systemic targeting of T and B cells has shown effects in rodent models (Winer et al., 2009a, 2011). However, many systemic immune modulators carry potential serious side effects; thus, the development of locally active, well-tolerated, and efficient therapies is a principal goal of IR therapy research. One locally active, gut-specific anti-inflammatory agent is mesalamine (5-ASA), a first-line maintenance therapy for inflammatory bowel disease (IBD) (Rousseaux et al., 2005). 5-ASA is a salicylic acid derivative with anti-inflammatory properties that acts locally in the gut with minimal systemic absorption and side effects. As IBD is characterized by increased intestinal inflammation and altered permeability (Brown et al., 2013), we hypothesized that 5-ASA might have beneficial effects in obesity-related IR and may help elucidate roles of gut immune cells in this disease.

Here, we show that the gut immune system is an important modulator of IR. Diet-induced obesity (DIO) is accompanied by a low-grade pro-inflammatory shift in lamina propria (LP) immune cell polarity, consistent with changes previously described in response to an intestinal barrier defect (Brown et al., 2013). Targeting these changes may lead to new classes of potentially effective, minimal-side-effect therapies for IR.

RESULTS

HFD Induces a Pro-Inflammatory Shift in Intestinal Immune Cell Populations

To examine the effects of DIO on gut immunity, we first investigated if adaptive immune cell populations in the colon and small bowel LP are altered by HFD feeding in C57BL/6 mice at 3 or 12–16 weeks of HFD. After 3 weeks of HFD, changes in the proportions of bowel immune populations began in the colon, including a reduced percentage of Tregs and an increase in IL-17-producing γδ T cells (Figures S1A and S1B). However, after 12–16 weeks of diet, HFD induced a pro-inflammatory shift in immune cells in both the colon and small bowel. In colonic immune cells, there was an increase in the proportion and/or absolute number of IFNγ-producing Th1 T cells and CD8+ T cells and reduction in the proportion of CD4+ Foxp3+ Tregs (Figure 1A; Figure S1C). In the small bowel of HFD mice, there was an increase in the frequencies and/or numbers of Th1 CD4+ T cells and IFNγ-producing CD8+ T cells and a decrease in the proportion and absolute number of CD4+ Foxp3+ Tregs (Figure 1B; Figure S1D). We next assessed the effects of 12–16 weeks of HFD feeding on γδ T cells and innate lymphoid cell populations in the bowel. HFD increased the frequency and/or number of IL-17-producing but not IFNγ-producing γδ T cells in the colon and small bowel (Figures 1C and 1D; Figures S1C and S1D). Furthermore, there was an increase in total cell numbers of innate lymphoid cells in the colon, though the relative proportion of NKp46+ CD4− cells was reduced in HFD-fed mice (Figure S1E).

To determine if humans showed similar changes in gut immune populations during obesity, we correlated patient BMI with relative numbers of pro-inflammatory T-bet+ (Th1, ILC1 [Bernink et al., 2013]) T cells, anti-inflammatory Foxp3+ (Treg) T cells, and CD8+ T cells in the LP of colon and ileum resection specimens. Table S1 summarizes relevant clinical parameters of patients included in the study. Obese patients showed increases in colon and small bowel T-bet+ cells and CD8+ cells and a reduction in Tregs (Figures 1E and 1F).

Collectively, these results demonstrate a reduction in gut Tregs and a pro-inflammatory shift in some adaptive and innate T cell populations in the gut of HFD-fed mice, with a similar observation in our specific cohort of obese humans. Interestingly, this inflammatory shift was not associated with any apparent histological changes of chronic or active inflammation on H&E-stained sections of obese human (Figure S1F, top) or HFD-fed mouse colons (Figure S1F, bottom).

Beta7 Integrin-Deficient Mice Are Protected from HFD-Induced IR

We next determined if the gut immune system as a whole could contribute to the development of obesity-associated IR. We placed beta7 integrin-deficient C57BL/6 mice (Beta7null mice) on either normal chow diet (NCD) or HFD for 12 weeks and then assessed metabolic parameters. Beta7 pairs with alpha4 integrin on leukocytes to form the mucosal addressin molecule (LPAM-1), and mice deficient in beta7 show hypoplasia of gut lymphoid tissue due to reduced homing of leukocytes to colon and small bowel (Brown et al., 1996). Consistently, we observed a reduction mainly in the absolute numbers but not proportions of most immune cells, especially in IFNγ-producing T cell subsets in the LP of colons and small bowels of Beta7null mice after 12 weeks of HFD (Figure 2A; Figure S2A). There were no differences in the relative proportions of these subsets in the spleen (Figure S2B), suggesting that the lack of beta7 integrin does not attenuate systemic immunity.

In terms of metabolic parameters, there were no differences in weight gain during 12 weeks of HFD feeding between wild-type (WT) and Beta7null mice (Figure 2B). Interestingly, Beta7null mice demonstrated improved fasting glucose, glucose tolerance (using glucose tolerance test, GTT), and insulin sensitivity (using insulin tolerance test, ITT) compared to WT mice after 12 weeks of HFD (Figure 2C). These mice also showed similar food intake, oxygen consumption, and carbon dioxide production (Figure 2D). Histological analysis of bowels in HFD-fed Beta7null mice did not show signs of active colitis (Figure S2C). Interestingly, HFD-fed Beta7null mice presented no difference in adipocyte size, but showed a marked reduction in crown-like structures (CLS) in the VAT, along with reduced liver steatosis (Figure 2E; Figure S2D). Consistent with the reduced CLS, HFD Beta7null mice had overall less VAT immune cell infiltrates (Figures S2E–S2J). This change was likely not due to the beta7 integrin deficiency imparting an intrinsic defect on T cells to home to VAT, as Beta7null T cells were equally capable at trafficking and engrafing to VAT upon transfer as their WT counterparts.
Figure 1. HFD Is Associated with a Pro-inflammatory Shift in Intestinal Immune Cells

(A and B) Intracellular cytokine staining in T cells from colon (A) or small bowel (B) LP after 12–16 weeks of HFD (*p = 0.005 for Th1, *p = 0.02 for CD8, *p = 0.02 for Tregs, n = 3–4 experiments, 8–10 mice for colon; *p = 0.03 for Th1, *p = 0.046 for CD8, *p = 0.002 for Tregs, n = 5–6 experiments, 10–12 mice for small bowel).

(C and D) Intracellular cytokine staining in γδ T cells from colon (C) or small bowel (D) LP after 12–16 weeks of HFD (*p = 0.02 for colon, *p = 0.001 for small bowel; n = 4 experiments, 10 mice for colon and n = 4–5 experiments, 10 mice for small bowel).

(E) T-bet (far left), Foxp3 (middle), and CD8+ (far right) staining in colon and ileum of human subjects with lean or obese BMI (*p = 0.04, n = 7 for colon, *p = 0.02, n = 3–4 for ileum for T-bet; *p = 0.005, n = 7 for colon, *p = 0.047, n = 3–4 for ileum for Foxp3; *p = 0.03, n = 7 for colon, *p = 0.006, n = 3–4 for ileum for CD8+).

(F) Representative images of double staining for T-bet (red, examples indicated by blue arrows) and Foxp3 (brown, examples indicated by black arrows) (top and third row) and CD8+ (brown) (second and last row) in lean (left) versus obese (right) colons (top four quadrants) and ileums (bottom four quadrants).

Scale bar: 100 μm. HPF: high-power field. 40× objective. HPF = 0.237 mm². Data in bar graphs represent mean ± SEM.
Figure 2. Intestinal Immune Cells Influence Glucose Homeostasis

(A) Absolute cell counts, including CD45+ (top far left), CD3+ (top middle left), CD3+ CD4+ or CD3+ CD8+ (top far right), CD4+ subsets (bottom far left), IFNγ+ CD8+ (bottom middle), and γδ T cell subsets (bottom far right) from colon and small bowel (SB) LP after 12 weeks of HFD in WT and Beta7null (Beta7null) mice. Entire colons were processed, or the distal 10 cm of SB (jejunum + ileum). *p = 0.0008 for CD45 colon; *p < 0.0001 for CD45 SB; *p = 0.0045 for CD3 colon, *p = 0.0008 for CD45 colon; *p = 0.008 for CD4 IFNγ colon; *p = 0.001 for γδ IL-17 colon, n = 4 experiments, 8–11 mice.

(B) Body weights of WT and Beta7null mice fed HFD over time, starting at 6 weeks of age (n = 13 WT, n = 11 Beta7null mice).

(C) Fasting glucose (left), GTT (middle), and ITT (right) of 12-week HFD-fed WT and Beta7null mice (*p < 0.05, n = 13–15 WT, n = 7–9 Beta7null mice).

(D) Food intake (left), metabolic cage analysis including oxygen consumption (left middle), carbon dioxide production (right, middle), and respiratory exchange ratio (RER) (right) of HFD WT and Beta7null mice (n = 7 for food intake, n = 7 WT and n = 6 Beta7null mice for metabolic cage analysis).

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showed improvements in fasting glucose (Figure 3C, left), fasting on metabolic disease (Figures S3G and S3H).

dose (150 mg/kg/day) of 5-ASA also exerted beneficial effects challenge (Figure 3E). Similar to the higher dose used, a lower
insulin tolerance, 5-ASA-treated mice showed increased phospho-
tion, carbon dioxide output, or respiratory exchange ratio
more, there was no difference in food intake, oxygen consump-
VAT or subcutaneous adipose tissue (SAT) (Figure S3D). Further-
5-ASA on expression of adipogenesis-related genes in either

tive protocol, 5-ASA did not change body weight (Figure 3F), but
to mice on only HFD from the beginning. Similar to the preventa-
onto a HFD with 5-ASA for 8 additional weeks and compared
8 weeks, with established metabolic disease, were switched
insulin tolerance (Figure 3G). To assess whether the beneficial
was little or no difference in body weight, fasting glucose,
glucose tolerance, or IR (Figure S3I). These results suggest
that the use of 5-ASA has specific therapeutic effects on glucose
homeostasis in the setting of DIO.

5-ASA Improves Bowel and VAT Inflammation in HFD Mice

to begin understanding the mechanisms by which 5-ASA can
exert effects on glucose homeostasis, we examined the effects
of 5-ASA on systemic and local immune function during HFD
feeding. 5-ASA treatment showed no effects on immune cell
populations in the spleen (Figure S4A), on stimulated spleen
immune cell cytokine secretion, or on circulating immune cell
polarity in the blood (Figures S4B and S4C). Similarly, serum
levels of cytokines in mice treated with 5-ASA were mostly un-
changed, though we did identify a significant but small increase
in RANTES and a reduction in TNF-α (Figure S4D).

Since obesity is associated with a pro-inflammatory shift in gut
immune populations, and the composition of a gut immune system
is important in the development of disease, we reasoned
that therapies aimed at targeting gut inflammation such as mes-
alamine (5-ASA) may have a role in the treatment of metabolic
disease. We first fed mice beginning at 6 weeks of age with either
HFD or HFD incorporated with 5-ASA (1,500 mg/kg/day). After
12–14 weeks of HFD, there was no significant difference in
body weight (Figure 3A, left), VAT weight (Figure 3A, right), adipocyte size (Figure 3B, left), number of crown-like structures in VAT (Figure 3B, right, and Figure S3A top), or organ weights between groups (Figure S3B). Although we did observe reduced liver steatosis in the 5-ASA-treated group (Figure S3A, bottom), we
could not detect significant changes in gluconeogenesis enzyme
gene expression, in spite of trends to lower expression with
5-ASA treatment (Figure S3C). There were also no effects of
5-ASA on expression of adipogenesis-related genes in either
VAT or subcutaneous adipose tissue (SAT) (Figure S3D). Further-
more, there was no difference in food intake, oxygen consump-
tion, carbon dioxide output, or respiratory exchange ratio
(RER) (Figures S3E and S3F). However, mice receiving 5-ASA
showed improvements in fasting glucose (Figure 3C, left), fasting
insulin (Figure 3C, right), glucose tolerance (Figure 3D, left),
and insulin tolerance (Figure 3D, right). Consistent with improved
insulin tolerance, 5-ASA-treated mice showed increased phos-
phorylated-Akt/Akt ratio in VAT, liver, and muscle with insulin
challenge (Figure 3E). Similar to the higher dose used, a lower
dose (150 mg/kg/day) of 5-ASA also exerted beneficial effects
on metabolic disease (Figures S3G and S3H).

We next assessed whether 5-ASA could be used to treat
established obesity-associated IR. C57BL/6 mice on HFD for
8 weeks, with established metabolic disease, were switched
onto a HFD with 5-ASA for 8 additional weeks and compared
to mice on only HFD from the beginning. Similar to the preventa-
tive protocol, 5-ASA did not change body weight (Figure 3F), but
did produce significant improvements in glucose tolerance and
insulin tolerance (Figure 3G). To assess whether the beneficial
metabolic effects of 5-ASA require a HFD-induced milieu, we
placed 6-week-old C57BL/6 mice on either NCD or NCD with
5-ASA (1,500 mg/kg/day). After 12 weeks of treatment, there
was little or no difference in body weight, fasting glucose,
glucose tolerance, or IR (Figure S3I). These results suggest
that the use of 5-ASA has specific therapeutic effects on glucose
homeostasis in the setting of DIO.
Figure 3. 5-ASA Improves Systemic Metabolic Parameters during HFD Feeding

(A) Left: body weights of HFD and HFD 5-ASA (1,500 mg/kg/day)-fed C57BL/6 mice over time, starting at 6 weeks of age (n = 10). Right: VAT weights of mice, after 14 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) (n = 10).

(B) Relative fat cell diameter of mice (left) or number of VAT “crown-like structures” (CLS) (right) per 100× low power field, after 14 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) (n = 3).

(C and D) Fasting glucose (C, left), fasting insulin (C, right), glucose tolerance test (GTT, D, left), and insulin tolerance test (ITT, D, right) of mice after 14 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) (*p = 0.001 for glucose, n = 10 mice, *p = 0.02 for insulin, n = 8–9 mice, *p < 0.05 for tolerance testing, n = 10 mice for GTT, n = 13–15 mice for ITT).

(E) Left: insulin signaling in VAT, liver, and muscle of NCD, HFD, and HFD 5-ASA-fed mice injected with (+) or without (−) insulin and immunoblotted for pAkt and total Akt proteins (image shown is representative of 1 of 3 experiments with similar results, n = 3–4 mice). Right: fold change of pAkt/Akt protein ratios in mice fed HFD 5-ASA relative to HFD-fed controls (*p ≤ 0.01, n = 4 mice for VAT, 3–4 mice for liver and muscle).

(F and G) Body weights (F), GTT (G, left), and ITT (G, right) after 8 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) in mice switched over from 8 weeks of HFD (*p < 0.05, n = 5 mice, GTT was performed with an i.p. glucose challenge at a dose of 1.0 g/kg). Data in bar graphs represent mean ± SEM.
Figure 4. 5-ASA Improves Gut and VAT Inflammation in Mice during HFD Feeding

(A and B) Intracellular staining of cytokines and Foxp3 in LP T cell populations in the colon (A) or small bowel (B) of mice after 16 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) (*p = 0.001 for Th1, *p = 0.01 for CD8, *p = 0.045 for γδ T cell IL-17, n = 2–3 experiments, 9 mice for colon; *p = 0.01 for Th1, *p = 0.02 for CD8 IFNγ, *p = 0.005 for Treg, *p = 0.03 for γδ T cell IFNγ, *p < 0.0001 for γδ T cell IL-17, n = 2–4 experiments, 6–8 mice for small bowel).

(C and D) Flow cytometry analysis of T cell (C) and M1 macrophage subset (D) in VAT of mice after 16 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) (*p = 0.03 for Th1, *p = 0.02 for CD8, *p = 0.03 for Treg, *p = 0.01 for macrophages, n = 2 experiments, 8 mice). Data in bar graphs represent mean ± SEM.
Figure 5. 5-ASA Targets Adaptive Gut Immunity in a PPARγ-Dependent Manner during HFD Feeding

(A and B) Body weights (A), GTT (B, left), and ITT (B, right) of Rag1 null mice after 8 weeks of HFD or HFD 5-ASA (1,500 mg/kg/day) (n = 4–5 mice).

(C) Body weights of Beta7 null mice after 14 weeks of HFD or HFD 5-ASA (n = 15 HFD Beta7 null, n = 11 HFD 5-ASA Beta7 null).

(D) Fasting glucose (left), GTT (middle), and ITT (right) of Beta7 null mice after 12 weeks of HFD or HFD 5-ASA (n = 9 HFD Beta7 null, n = 10 HFD 5-ASA Beta7 null).

(E) PPARγ mRNA expression of small bowel (SB) T cells compared to splenic T cells isolated from HFD-fed C57BL/6 mice (*p = 0.004, n = 3 mice).

(F) PPARγ transcription factor activity of SB T cells from HFD- or HFD 5-ASA-fed mice (*p = 0.04, n = 4 mice, normalized to total nuclear protein).

(G) Levels of secreted IFNγ cytokine from small bowel (SB) T cells (left) or splenic (SP) T cells (right) from HFD-fed WT mice compared to HFD-fed Lck-Cre+ PPARγ fl/fl mice treated with the indicated doses of 5-ASA in vitro (*p ≤ 0.02 at all doses of 5-ASA for small bowel T cells, n = 3 mice).

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increased Firmicutes, and increased Clostridiales (Figures S6A–S6E) (Andrews et al., 2011).

5-ASA Targets Adaptive Gut Immunity and PPARγ

To determine if the effects of 5-ASA were mediated through anti-inflammatory actions that require adaptive immune cells rather than direct effects on gut epithelium, we treated 6-week-old Rag1null mice with HFD 5-ASA. Preventative treatment of Rag1null mice with HFD 5-ASA had no effect on body weight, glucose tolerance, or IR (Figures 5A and 5B), suggesting that the beneficial effects of 5-ASA required components of the adaptive immune system. To further pinpoint the location of 5-ASA action on glucose tolerance, we fed Beta7null mice a HFD with 5-ASA. Interestingly, similar to the Rag1null mouse, treatment with 5-ASA had no major effects on glucose tolerance and IR (Figures 5C and 5D). Thus, the beneficial metabolic effects of 5-ASA require an “intact” gut immune system.

Since knock-out studies linked potential effects of 5-ASA on glucose metabolism to the gut immune system, and 5-ASA has been reported to possess PPARγ agonist properties (Rousseaux et al., 2005), we next determined if 5-ASA could be directly influencing intestinal immune cell function in HFD through targeting PPARγ. As the effects of 5-ASA were more robust with small bowel T cells than colonic T cells, we focused our studies on small bowel T cells. Indeed, we observed higher PPARγ gene expression in small bowel T cells compared to total splenic T cells in both HFD and NCD-fed mice (Figure 5E; Figure S5E). Mice fed HFD 5-ASA showed increased PPARγ functional activity in purified small bowel T cells compared to those fed with control HFD (Figure 5F). We next tested if 5-ASA can suppress IFNγ production in vitro. Indeed, similar to another PPARγ agonist, rosiglitazone, 5-ASA reduced IFNγ production by anti-CD3/CD28-activated small bowel but not splenic T cells (Figure S5F). Furthermore, loss of PPARγ in T cells (Lck-Cre PPARγfl/fl) abrogated the suppressive effects of 5-ASA, confirming that 5-ASA acts in a PPARγ-dependent manner (Figure 5G). In addition, 5-ASA indirectly reduced T cell IFNγ expression by modulating intestinal dendritic cell function, as shown by reduced IFNγ levels in antigen-specific co-culture systems using OT-II CD4+ T cells and 5-ASA-pre-treated small bowel but not splenic dendritic cells (Figure 5H).

5-ASA and Reduced Gut Inflammation Improve Intestinal Barrier Function during HFD

While permeability-related gut-derived endotoxin alone may contribute to VAT inflammation and potentially IR (Caesar et al., 2012), it is thought that this trigger works alongside other gut-associated antigens to activate antigen-specific T cells in VAT, thereby influencing glucose homeostasis (Wang et al., 2010). Thus, to further understand how a gut-specific anti-inflammatory agent may contribute to reduced inflammation in VAT, we examined the effects of 5-ASA on oral immune tolerance to a gut-derived antigen. NCD-, HFD-, or HFD 5-ASA-fed C57BL/6 mice were administered oral ovalbumin (OVA) antigen for 1 week prior to immunization with OVA-CFA. Interestingly, HFD 5-ASA-fed mice showed a stronger oral tolerance response to OVA antigen systemically, as reflected by an increased OVA-specific IgG1/IgG2c ratio (indicative of reduced Th1 inflammatory responses), and a nearly 3-fold increase in OVA-specific IgA (Figure 6D). Moreover, draining lymph nodes in mice fed HFD 5-ASA demonstrated a reduction in OVA-specific T cell-derived IL-2 and IFNγ, which is also consistent with the improved oral tolerance and reduced antigen-specific inflammation to gut antigen (Figure 6E). Finally, 5-ASA treatment induced a nearly 4-fold increase in antigen-specific Tregs to OVA in VAT as (Figure 6E). Collectively, the data suggest that reducing low-grade inflammation in the gut during HFD feeding can impact multiple pathways associated with IR, including gut barrier function, tolerance to

[1] Levels of secreted IFNγ cytokine from OT-II T cells stimulated with 5-ASA-treated (0.1 or 1.0 mM) or untreated splenic (left) or small bowel (right) dendritic cells presenting the indicated concentrations of OVA323-339 peptide (*p ≤ 0.03, n = 3 samples, 3 spleens; n = 2 samples, 4 pooled bowels). Data in bar graphs represent mean ± SEM.
Figure 6. 5-ASA and Reduced Gut Inflammation Improve Intestinal Barrier Function and Oral Tolerance during HFD Feeding

(A) Plasma FD4 concentration of age-matched NCD WT, HFD WT, HFD 5-ASA WT, and HFD Beta7null mice after 12–16 weeks of diet following oral gavage as a measure of intestinal permeability (*p = 0.02 for NCD Control versus HFD Control, *p = 0.04 for HFD Control versus HFD 5-ASA, and *p = 0.04 for HFD Control versus HFD Beta7null; n = 10 for NCD Control, n = 10 for HFD Control, n = 8 for HFD 5-ASA, and n = 6 for HFD Beta7 null mice).

(B) Far left: serum anti-LPS IgG levels of age-matched NCD WT, HFD WT, HFD 5-ASA WT, and HFD Beta7 null mice after 14 weeks HFD (*p < 0.03, n = 5–8). Middle and far right: serum endotoxin levels (middle) and VAT endotoxin levels (right) of age-matched NCD WT, HFD WT, and HFD 5-ASA WT after 14 weeks HFD (*p < 0.05 for serum endotoxin, p = 0.19 for VAT endotoxin; n = 3–4 for serum endotoxin, n = 5 for VAT endotoxin).

(C) Left: plasma FD4 concentrations, following oral gavage, of age-matched IFNγ null mice after 10 weeks of HFD (*p = 0.01, n = 4 mice). Right: ZO-1 mRNA expression relative to housekeeping gene expression in MODE-K intestinal cells treated with indicated amounts of IFNγ in vitro (*p = 0.006, n = 3 in each treatment).

(D) Ratio of OVA-specific IgG1/IgG2c (left) and OVA-specific IgA (right) in age-matched NCD WT, HFD WT, and HFD 5-ASA WT mice 2 weeks after immunization with OVA-CFA (*p = 0.03 for IgG1/IgG2c, *p = 0.03 for oral NCD versus oral HFD IgA, *p = 0.02 for oral HFD versus oral HFD 5-ASA IgA; n = 4–6 mice).

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gut-derived antigen, and antigen-specific immunity to gut-derived antigen in VAT. Taken together, these results suggest that anti-inflammatory targeting of gut immune cells is a novel approach to treat obesity-related IR.

**DISCUSSION**

We have identified the gut immune system as an active orchestrator and therapeutic target in obesity-related IR. Previous work has shown that HFD increases ileal TNF-α mRNA, induces expression of TLR4 and NF-κB in small bowels (Ding et al., 2010; Wang et al., 2013), and increases IL-1β, IL-12p40, NF-κB, and TLR4 in colons of DIO mice (Kim et al., 2012; Li et al., 2008). Consistently, we show that DIO promotes a pro-inflammatory shift in gut immune cell populations, characterized by reduced LP Foxp3+ Tregs cells, increased IFNγ-producing Th1 and CD8+ T cells, as well as increased IL-17-producing γδ T cells. Similar to the changes in mice, altered ratios of T-bet+ cells—Foxp3+ Tregs cells, as well as changes in CD8+ T cells—were found in both small and large bowels of obese humans, though these studies involved the use of negative margin specimens from patients with tumors and thus need more rigorous follow-up in additional cohorts of patients, including bariatric patients. A recent report has also demonstrated reduction in IL-22 in the gut of obese mice post-immune challenge (Wagner et al., 2014). Consistently, we saw reduced percentages of NKP46+ CD4− ILCs, which are important producers of IL-22. Moreover, the pro-inflammatory shift in immune cell populations observed in the gut was not associated with obvious inflammatory histological changes, and so we classify this pro-inflammatory shift as a sub-histological change, or “low-grade subclinical inflammation.”

We next investigated if the gut immune system as a whole could exert systemic effects on glucose homeostasis. In this model, we used Beta7null mice, which have marked hypoplasia of the gut lymphoid system. We noted improved metabolic parameters in the Beta7null mice despite similar body weights. These mice showed reduced immune cell infiltrates in the gut during HFD, including reductions in IFNγ-producing CD4+ and CD8+ T cells, consistent with a potential pathogenic role for some intestinal immune cells in DIO. However, additional work is needed to rule out whether other off-target effects of this molecule, such as potential traffic to other tissues, exist in the setting of DIO that might also contribute to the phenotype. Furthermore, Beta7null mice are susceptible to bacterial overgrowth (Wagner et al., 1996), which can cause changes in the microbiome and contribute to the observed phenotype. This phenotype may be similar to lymphotxin-deficient mice that show hypoplasia of Peyer’s patches and improved glucose tolerance due to altered gut colonization of segmented filamentous bacteria (SFB) and reduced energy-harvesting bacteria (Upadhyay et al., 2012). Nonetheless, taking together the phenotypic data of both models, it appears that active gut inflammation contributes to downstream pathways, ultimately leading to obesity or related IR. Potential pathways include modulation of the gut flora with effects on energy-harvesting bacteria (Upadhyay et al., 2012), bile acid and short-chain fatty acid release (Brown et al., 2013), modulation of the gut epithelial barrier (Pastorelli et al., 2013), control of gut hormone release such as GLP-1 leading to hyperinsulinemia (Kahlès et al., 2014), and/or a role in dictating inflammatory responses to gut-derived antigen and endotoxin (Caesar et al., 2012; Wang et al., 2010).

In HFD Beta7null mice, we observed overall improvements in gut barrier function, characterized by reduced FD4 and anti-LPS response. These findings are potentially linked to reduced infiltrates of IFNγ-producing cells in the bowel, as IFNγ has direct pathological effects on disrupting barrier function (Beaurepaire et al., 2009). Consistently, HFD IFNγnull mice have improved barrier function compared to HFD WT mice, implicating local intestinal IFNγ production as one critical pathogenic mediator of intestinal permeability in DIO.

The overall HFD-induced phenotype of intestinal immune cells observed in WT mice is consistent with changes described in other diseases characterized by breech of intestinal barrier, dysbiosis, and subsequent anti-bacterial immune response (Brown et al., 2013; Petnicki-Ocieja et al., 2009). LP CD4+ Foxp3+ Tregs, in particular, are critical in maintaining a tolerant response to gut microbiota and are reduced in the presence of intestinal barrier defects. In healthy hosts, Tregs maintain the intestinal barrier through promotion of TGF-β-dependent microbiota-specific IgA responses (Cong et al., 2009). Upon breech of the barrier, Tregs suppress Th1 responses via IL-10 and TGF-β (Cong et al., 2009). Our observed HFD-associated reductions in LP Tregs and increases in pro-inflammatory IFNγ-secreting Th1 and CD8+ cells, as well as IL-17-producing γδ T cells, are thus consistent immunologically with intestinal barrier breech. Several studies have shown that DIO is associated with a breech in the intestinal barrier, leading to increases in circulating levels of gut-derived microbial products, such as LPS (Cani et al., 2007, 2008). In addition to direct leakage, gut-derived LPS can be transported along with chylomicrons into circulation (Ghoshal et al., 2009). While we described one mechanism of immune cell IFNγ-mediated effect on the intestinal barrier during DIO, it is possible that changes in IL-10, associated with reduced Tregs, or altered inflammatory status of the intestinal epithelial cells may also contribute to decreased barrier function. Indeed, IL-10 has been shown to promote intestinal barrier mucin production (Hasnain et al., 2013), while a recent study showed improvements in gut barrier function in HFD mice lacking the pro-inflammatory molecule, MyD88, only in intestinal epithelial cells (Everard et al., 2014). In the latter study, knockdown of intestinal epithelial MyD88 also improved glucose homeostasis, in agreement with our data showing an overall pathogenic role for intestinal inflammation in DIO-related metabolic disease. Thus, a combination of cues from both cells of the

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**E** OVA-specific recall IL-2 and IFNγ responses in age-matched HFD WT and HFD 5-ASA WT mice from axillary lymph nodes, 2 weeks after immunization with OVA-CFA (p = 0.01 for IL-2; p = 0.006 for IFNγ; n = 4–5 mice in duplicates).

**F** OVA tetramer-stained Treg cells from VAT of age-matched oral or non-oral challenged HFD WT and HFD 5-ASA WT mice 2 weeks after immunization with OVA-CFA (p = 0.03; n = 2 experiments, 6 pooled mice). Data in bar graphs represent mean ± SEM.
intestinal immune system, as we have described, and innate pathways within the intestinal epithelium collaborates to regulate intestinal barrier function and downstream glucose homeostasis during DIO.

We further show that inhibition of low-grade gut inflammation with a gut-specific anti-inflammatory agent, 5-ASA, during HFD feeding can alter systemic glucose metabolism. Treatment with gut anti-inflammatory agents, including 5-ASA and Balsalazide, has beneficial effects on the intactness of the gut epithelial barrier in IBD models (Di Paolo et al., 1996; Liu et al., 2009). We observed similar beneficial effects of 5-ASA on gut barrier function during HFD feeding, which are linked to reduced levels of inflammatory cytokines, such as TNF-α and IFNγ, that can directly worsen barrier leakage of gut bacteria (Barreau et al., 2010; Beaurepaire et al., 2009). Accordingly, similar alterations in intestinal IFNγ-producing cells contribute to gut barrier defects in the setting of DIO. In addition to its well-known role as a COX-2 inhibitor, 5-ASA has PPARγ agonistic effects, which also contribute to our observed anti-inflammatory phenotype (Rousseaux et al., 2005). We noted increased PPARγ activity from bowel T cells of HFD 5-ASA-fed mice and that PPARγ contributes to 5-ASA inhibitory effects on IFNγ production by intestinal T cells in vitro. Interestingly, PPARγ induction in T cells can also bolster Treg function and numbers in other tissues, including VAT (Cipolletta et al., 2012). However, systemic effects of PPARγ agonism in fat or liver are unlikely in our study due to minimal metabolic effects seen in HFD 5-ASA-fed Rag1null mice and Beta7null mice, the lack of changes in our study due to minimal metabolic effects seen in HFD gut inflammation from bowel T cells of HFD 5-ASA-fed mice and that induction by intestinal T cells in vitro. Interestingly, PPARγ induction in T cells can also bolster Treg function and numbers in other tissues, including VAT (Cipolletta et al., 2012). However, systemic effects of PPARγ agonism in fat or liver are unlikely in our study due to minimal metabolic effects seen in HFD 5-ASA-fed Rag1null mice and Beta7null mice, the lack of changes in adipogenesis gene expression in VAT and SAT, and the lack of detectable compound in VAT of HFD 5-ASA-fed mice. Thus, intestinal immune cell PPARγ may be another potential target of action for immune modulatory drugs with PPARγ agonistic effects.

Consistent with other reports (Andrews et al., 2011), we noted that 5-ASA could elicit changes in gut bacteria, including increased bacterial diversity, and increased abundances of Firmicutes, Clostridiales, and Ruminococcaceae. While these changes could reflect primary effects of the drug, they could also be secondary to reduced inflammation (Andrews et al., 2011; Sartor, 2010). Reduced bacterial diversity and decreased abundance of certain Clostridial groups and Ruminococcaceae have been linked to increased inflammation in IBD (Sartor, 2010). Ruminococcaceae are prominent producers of short-chain fatty acids, including butyrate, which have protective activity in the intestine (Sartor, 2010). Thus, an interesting future direction will be to tease out specific effects of 5-ASA-associated microbial influences on improving metabolic syndrome.

We were able to obtain beneficial effects on glucose tolerance using a 5-ASA dose range of 150 mg/kg/day up to 1,500 mg/kg/day in mice, which using body surface area calculations (Reagan-Shaw et al., 2008) equates to approximate equivalent doses of 730 mg/day up to 7 g/day in a 60 kg human. Typical daily maintenance dosing of 5-ASA for mild to moderate IBD varies but often ranges between 1.5 and 4.8 g/day (Burger and Travis, 2011). Thus, our work highlights novel uses of such drugs in obesity-related IR, which may require dosing not unlike existing protocols for IBD, though controlled human clinical studies are needed to better validate the effects and safety of different formulations of 5-ASA on IR.

Improvements in systemic glucose tolerance with 5-ASA treatment were found to be dependent on adaptive and gut immune systems. The observed direct effect of 5-ASA in vitro on purified intestinal dendritic cells modulating antigen-specific T cell responses and IFNγ production highlights potential crosstalks between intestinal adaptive and innate immune cells in mediating the effects of 5-ASA. The improvements in intestinal and VAT inflammation in 5-ASA-treated or Beta7null HFD-fed mice, with no effect on the inflammatory status of systemic hematolymphoid organs such as the spleen, suggest a possible linked circuit between adipose tissue and bowel inflammation. Other studies have also suggested that bowel inflammation may directly contribute to VAT inflammation (Li et al., 2008; Teixeira et al., 2011). For instance, induction of colitis during HFD leads to marked increases in VAT macropages, lymphocytes, and neutrophils (Teixeira et al., 2011). Such results raise the possibility of downstream trafficking between immune cells of the bowel and VAT or the possibility that tolerance to leaked gut-soluble antigens in VAT is dependent on mechanisms governed by the gut immune system. Additional studies are needed to determine whether bowel immune cells routinely traffic to VAT and whether trafficking of gut-derived anti-inflammatory immune cells (or reduced trafficking of gut inflammatory cells) to VAT represents another mechanism of action of 5-ASA.

Another contributing role of the gut immune system during HFD may be in dictating downstream systemic inflammation to soluble gut-derived antigens, including in metabolic tissues like VAT, where inflammation directly impacts systemic disease. Improved oral tolerance may also manifest as reduced inflammatory responses, including IgG against gut-derived endotoxin. Oral tolerance to gut-derived antigens has been previously linked to reduced inflammation in VAT and improvements in IR, though the mechanisms were unknown (Wang et al., 2010). We show that aberrant handling of gut antigen is likely due to the gut inflammatory environment during HFD, which is reversible with gut anti-inflammatory medication. HFD-induced low-grade gut inflammation may be a key trigger for antigen-specific T cell responses in VAT, linking the inflammatory phenotype we describe in the bowel to downstream responses in VAT.

Overall, our work shows that low-grade inflammation in gut immune cells is a functional alteration induced by HFD with implications in IR (see Figure 7 for summary). Reducing low-grade gut inflammation leads to reduced VAT inflammation and improvements in metabolic homeostasis. These effects are dependent on the adaptive immune system and gut immunity. Thus, compounds that locally reduce gut inflammation may represent a novel approach in the control of obesity-related IR.

EXPERIMENTAL PROCEDURES

Mice
We purchased C57BL/6, Beta7null, Rag1null, and IFNγnull mice from Jackson Laboratory. Lck-Cre+ PPARγfl/fl were generated in-house by breeding Lck-Cre+ and PPARγfl/fl transgenic mice from Jackson Laboratory. Mice were maintained in a pathogen-free, temperature-controlled environment on a 12 hr light and dark cycle at the Toronto Medical Discovery Tower facility. All mice used in comparative studies were male, age-matched, and littermates where possible. Mice were fed either NCD (15 kcal% fat), HFD (Research Diets, 60 kcal% fat, irradiated), or HFD 5-ASA starting at 6 weeks of age.
All studies were performed under the approval of Animal User Protocols by the Animal Care Committee at the University Health Network. See Supplemental Experimental Procedures.

Compounds and Treatment Diets
5-aminosalicylic acid powder (Sigma-Aldrich) was incorporated directly into the HFD at two doses (150 mg/kg/day and 1,500 mg/kg/day) by Research Diets Inc. 5-ASA was mixed into NCD at 1,500 mg/kg/day by Harlan Laboratories.

All studies were performed under the approval of Animal User Protocols by the Animal Care Committee at the University Health Network. See Supplemental Experimental Procedures.

MODE-K Cell Line
The murine intestinal epithelial cell line derived from C3H/He mice, MODE-K, was a kind gift from Dr. K. Croitoru (University of Toronto). These cells were propagated under standard protocol using DMEM (GIBCO) containing 10% FBS, 10 mM HEPES, 50 μM 2-mercaptoethanol, 50 mg/ml streptomycin, and 50 U/ml penicillin. In MODE-K in vitro studies, we split cells at 70%–80% confluency and seeded at a density of 3 × 10^4/well for treatment with recombinant mouse IFNγ (BioLegend) (5 or 10 ng/ml) for 24 hr. We mechanically detached the cells for RNA isolation (QiAGEN).

Figure 7. Summary of Obese versus Lean Gut Milieu and the Impact of 5-ASA
In DIO, pro-inflammatory changes in immune cells contribute to increased gut permeability. These changes affect the gut locally but also have systemic manifestations due to leaked bacterial and dietary components. The net result is systemic inflammation that fuels insulin resistance. 5-ASA, a gut-specific anti-inflammatory drug, reverses this cascade of obesity-associated adverse events by restoring tolerance in the gut immune system, preserving gut barrier function, and improving immune regulation.
Metabolic Cage Studies
We placed mice in automated metabolic cages (Oxymax Systems, Columbus Instruments) for 48 hr with airflow held constant at 0.5 l/min (Revelo et al., 2015). See Supplemental Experimental Procedures.

Metabolic Studies
We measured body weights, GTTs, serum insulin, and fat cell diameter as previously described (Winer et al., 2009a). All GTTs were performed with a 1.5 g/kg glucose i.p. injection unless indicated otherwise.

Histology
We fixed VAT, colons, and small bowel ileums from mice for 48 hr in 10% buffered formalin before processing and H&E staining. We enumerated crown-like structures (CLSs) in VAT by counting the number of adipocytes completely surrounded by immune cells identified on H&E staining per 100 x low power field. Analysis of histochemical stains was performed in a blinded fashion by two certified pathologists (S.W. and D.A.W.).

Isolation of VAT and Bowel-Associated Immune Cells
We isolated VAT-associated immune cells as previously described (Winer et al., 2009a). For isolation of small intestine lamina propria immune cells, we used the protocol previously described (Fritz et al., 2012) and processed ~10 cm from the distal end of the small bowel (jejunum and ileum). For the isolation of colonic lamina propria immune cells, we used the protocol previously described (Geddes et al., 2011). Following lamina propria isolations, we passed immune cells through 70 μm strainers and used them for flow cytometry.

Flow Cytometry
We stained single-cell suspensions for 30 min on ice with commercial antibodies listed in Supplemental Experimental Procedures. We gated ILCs as described (Kirchberger et al., 2013).

Human Bowel Samples and Immunohistochemistry
We obtained colon and small bowel samples from histologically normal margins of surgical resection specimens for patients with sporadic colon cancer at the Toronto General Hospital. All human specimens were obtained with study approval by the Research Ethics Board for Human Subjects at the University Health Network. See Supplemental Experimental Procedures and Table S1.

Western Blotting
We injected mice i.p. with insulin (1.5 U/kg) or PBS and harvested and snap-froze tissues after 10 min. To make tissue lysates, we mechanically homogenized VAT, liver, and muscle tissues in ice-cold lysis buffer (Santa Cruz) and centrifuged them at 14,000 g for 10 min at 4°C. Supernatants were collected, separated by SDS-PAGE, and probed for phospho-Akt (S473), total Akt, and GAPDH (Cell Signaling Technology).

PPARγ Activity Assay
We measured PPARγ functional activity in T cell nuclear extracts using a PPARγ transcription factor binding assay following vendor’s instructions (Thermo Scientific and Cayman Chemical Company). See Supplemental Experimental Procedures.

In Vitro Co-Culture Studies
We purified bulk dendritic cells and T cells from murine small bowels and spleens using a negative-selection DC or T cell isolation kit (StemCell Technologies). We plated T cells at 5 x 10^5 cells/well with plate-bound anti-CD3/CD28 (1 μg/ml, BioLegend). We dissolved 5-ASA in culturing media (pH 7.3) and sterile-filtered the solution. Dissolved 5-ASA was added to designated wells at concentrations of 0, 0.01, 0.1, and 1 mM. We collected supernatants for cytokine measurements after 72 hr incubation. For DC-T cell co-culture experiments, we pre-treated DCs with 0, 0.1, or 1.0 mM 5-ASA. After 24 hr, we washed the cells with PBS and co-cultured them (1 x 10^5 cells/well) with OVA323-339 peptide and OT-II splenic or small bowel T cells (5 x 10^5 cells/well) for 48 hr. Supernatants were collected at 48 hr for cytokine measurement.

Gut Permeability Assays
We performed FD4 permeability assays by orally gavaging overnight-fasted mice with 0.4 mg/g body weight of FITC-conjugated dextran (Sigma) and collecting plasma after 4 hr. We prepared standards and measured fluorescence at 485/528 nm as previously described (Dong et al., 2014).

Endotoxin Measurements
We measured endotoxin levels in serum and adipose tissue using Pyrogen Recombinant C endotoxin detection fluorescence kit (Lonza). We measured mouse serum anti-LPS IgG antibody levels with a commercially available kit (Chondrex).

Cytokine Measurements
We measured serum cytokines by Luminescence Multiplex cytokine assay (Millipore, run by the UHN Microarray Center) and quantified IL-10 and IFNγ in supernatants of anti-CD3/CD28-stimulated splenocytes or bowel immune cells (Winer et al., 2009a) by ELISA (BioLegend).

qPcr
We extracted total RNA from isolated or cultured cells using a RNasy Mini Kit (QIAgen), and for subcutaneous adipose tissue (SAT) and VAT we used a RNasy Lipid Extraction kit (QIAgen). We reverse-transcribed the RNA by random primers with M-MLV (Invitrogen). We performed qPCR with a 7900HT PCR system (Applied Biosystems) using SYBR Green Master mix reagent (Applied Biosystems). Each sample was run in triplicate and normalized to housekeeping genes, 18s or GAPDH. We calculated relative fold changes in gene expression normalized to 18s or GAPDH by the ∆∆CT method using the equation 2^−∆∆CT. The results are shown as fold changes compared to the control group. See Supplemental Experimental Procedures for primer sequences.

Oral Tolerance Studies
We administered OVA in the form of drinking water (1 mg/ml) for 7 days to NCD-, HFD-, or HFD 5-ASA-fed mice to induce oral tolerance. Average consumption was similar across different groups, at 5 ml per day. Following oral tolerance induction, we immunized animals with or without oral tolerance induction subcutaneously with OVA/IFA (50 μg per side) on both sides of the chest. Axillary draining lymph nodes and VAT were collected 7 days following immunization and subjected to immunological analysis, including OVA323-339/I-Aα tetramer staining and OVA-induced cytokine production and proliferation in vitro. See Supplemental Experimental Procedures.

To measure OVA-specific Tregs, we stained VAT immune cells with a 1:200 dilution of PE-conjugated OVA323-339/I-Aα or control hCLIP/I-Aβ tetramers (NIH Tetramer Core Facility) in FBS-supplemented RPM-I for 4 hr at 37°C, followed by antibody staining for other surface antigens and Foxp3.

HPLC and Gut Microbiome Sequencing
See Supplemental Experimental Procedures.

Statistical Analyses
Statistical significance between two means was assessed with an unpaired, two-sided t test. In figure legends, where specified, the number of biological experiments is listed as the n value, followed by number of pooled mouse samples. All data represent mean ± SEM. Statistical significance was set at p < 0.05.

SUPPLEMENTAL INFORMATION
See Supplemental Experimental Procedures.

AUTHOR CONTRIBUTIONS
S.W. and D.A.W. conceived and supervised the study, designed experiments, and wrote the manuscript. D.A.W., S.T., and H. Luck designed and performed the majority of the experiments, interpreted results, generated figures and tables, and wrote the manuscript. C.T.L., S.Y.S., J.C., X.C.-C., M.G., X.S.R.,
J.A., D.P., B.A.R., M.H.Y.C., and H. Lei performed experiments. D.S.G., A.S., and J.K.C. performed and/or analyzed results from gut microbiome experiments. All other authors discussed results and/or reviewed the manuscript.

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