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Intestinal epithelial Toll-Like Receptor 4 Prevents Metabolic Syndrome by Regulating Interactions Between Microbes and Intestinal Epithelial Cells in Mice

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Abstract

Little is known about the pathogenesis of metabolic syndrome, although Toll-like receptor 4 (TLR4) has been implicated. We investigated whether TLR4 in the intestinal epithelium regulates metabolic syndrome by coordinating interactions between the luminal microbiota and host genes that regulate metabolism. Mice lacking TLR4 in the intestinal epithelium ($TLR4^{\Delta IEC}$), but not mice lacking TLR4 in myeloid cells nor mice lacking TLR4 globally, developed metabolic syndrome; these features were not observed in $TLR4^{\Delta IEC}$ mice given antibiotics. Metagenomic analysis of the fecal microbiota revealed differences between $TLR4^{\Delta IEC}$ and wild type mice, while meta-transcriptome analysis of the microbiota showed that intestinal TLR4 affected the expression of microbial genes involved in the metabolism of lipids, amino acids, and nucleotides. Genes regulated by peroxisome proliferator-activated receptors (PPARs) and the antimicrobial peptide lysozyme were significantly down-regulated in $TLR4^{\Delta IEC}$ mice, suggesting a mechanism by which intestinal TLR4 could exert its effects on the microbiota and metabolic syndrome. Supportingly, antibiotics prevented both downregulation of PPAR genes and the development of metabolic syndrome, while PPAR agonists prevented development of metabolic syndrome in $TLR4^{\Delta IEC}$ mice. Thus, intestinal epithelial TLR4 regulates metabolic syndrome through altered

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host-bacterial signaling, suggesting that microbial or PPAR based strategies might have therapeutic potential for this disease.

Introduction

Metabolic syndrome refers to a cluster of disorders including abdominal obesity, glucose intolerance and hepatic steatosis, and is an important cause of morbidity and mortality[1]. While the precise causes of metabolic syndrome remain incompletely understood, genetic[2], dietary[3] and microbial factors[4] have each been recognized to play a role in its pathogenesis. The importance of bacteria in the development of metabolic syndrome is supported by the striking observation that the administration of antibiotics prevents its development in mice [56], while the transfer of bacteria from obese mice or humans to lean mice induces metabolic syndrome in reciepient mice[78]. From the point of view of the host, polymorphisms in the receptor for Gram-negative bacterial endotoxin, namely Toll-like receptor 4 (TLR4), have been associated with an increased risk for the development of metabolic syndrome and obesity in humans[9], and patients with metabolic syndrome show increased TLR4 expression in monocytes[1011]. The endotoxin receptor complex consists of TLR4, CD14 and MD-2, and this complex signals in response to either the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway, which is critical for the production of several pro-inflammatory cytokines, or the MyD88-independent pathway, which depends on the TIR domain containing adaptor inducing interferon-beta (TRIF) signal adaptor protein and is crucial for type I interferon production[1213]. In mice receiving a high fat diet, TLR4 deficient mice show either reduced[14], unaffected[15], or increased[16] risk for the development of metabolic syndrome compared with wild type counterparts. The apparent discrepancy in the findings regarding the role for TLR4 in the development of metabolic syndrome has proven to be a source of significant controversy in the field, and point to a greater need to understand the impact of host-microbial interactions in its pathogenesis. One possible explanation for the varying results may lie in the fact that TLR4 signaling in various different cells – for example the myeloid cells versus the intestinal epithelial cells – could exert different, and perhaps even opposite, effects on the development of metabolic syndrome.

We now seek to address this controversy by testing the hypothesis that the expression of TLR4 in the intestinal epithelium as opposed to other cell types plays a critical role in the development of metabolic syndrome by coordinating the interaction between the luminal microbiota and genes that regulate metabolically important pathways in the host.

Results

Intestinal epithelial TLR4 expression regulates the development of metabolic syndrome in mice.

To evaluate the role of intestinal epithelial TLR4 in the development of metabolic syndrome, we first administered standard chow, containing 22% calories as fat, to mice harboring floxed alleles of TLR4 (wild type) or to age- and gender-matched mice in which TLR4 was selectively deleted from the intestinal epithelium ($TLR4^{\Delta IEC}$), from the ages of 3 to 24

weeks. We observed that despite being fed standard chow, when compared with floxed wild type mice, TLR4$^{\Delta IEC}$ mice developed a constellation of symptoms consistent with metabolic syndrome[1,17], which included significant weight gain (38.64±3.829 g vs 43.08±3.970 g, $p<0.05$ over 21 weeks) (**Figure 1A**), and increased weight of adipose tissue and liver (**Figure 1B**). The differences in weight gain between wild type and TLR4$^{\Delta IEC}$ mice strains became noticeable at approximately 12 weeks of age, and became significantly different from wild type mice at 24 weeks of age (**Figure 1Ai**). The increased body weight in TLR4$^{\Delta IEC}$ mice did not depend on food intake (**Supplemental Figure 1A)**, nor hormone levels that regulate appetite since both strains had similar serum leptin and ghrelin expression (**Supplemental Figure 1B**). Other markers of metabolic syndrome were also observed in the TLR4$^{\Delta IEC}$ but not wild type mice, including "hepatocellular ballooning" which indicates the accumulation of fat droplets within hepatocytes (**Figure 2A and 2B**), a trend towards increased liver triglycerides (**Supplemental Figure 1C**), the histologic presence of "crown-like structures" within the adipose tissue (**Figure 2C**) indicating the accumulation of macrophages[18], and the increased expression of macrophage markers (*F4/80* and *Cd68*) and the pro-inflammatory M1 phenotype macrophage markers (*Tnf* and *Cd11c*), but not the anti-inflammatory M2 phenotype macrophage marker (*Retnlb*), in the adipose tissue at the age of 24 weeks (**Figure 2D**). We noted that there was no difference observed in the size of the adipocytes between strains (**Supplemental Figure 1D**). Despite similar serum insulin (**Supplemental Figure 1E**), TLR4$^{\Delta IEC}$ mice but not wild type counterparts displayed significant insulin resistance, as measured by significant hyperglycemia after the administration of an oral glucose challenge at the age of 24 weeks (**Figure 2E**). There were no differences in serum cholesterol (**Supplemental Figure 1F**), serum triglycerides (**Supplemental Figure 1G**) and serum endotoxin content (**Supplemental Figure 1H**). Taken together, these findings illustrate that the lack of TLR4 on the intestinal epithelium leads to the development of metabolic syndrome in mice.

Given that many studies examining the development of metabolic syndrome in mice have utilized a high fat diet, whereas the above data were observed in the presence of a "standard" diet, we next administered a high fat diet in which 60% of the total calories are derived from fat to wild type and TLR4$^{\Delta IEC}$ mice from the age of 3 to 12 weeks. As shown in **Supplemental Figure 2A**, on a high fat diet, TLR4$^{\Delta IEC}$ mice still gained significantly more weight than wild type mice (40.96±5.061 g vs 35.57±3.423 g, $p<0.05$ over 9 weeks), and the weight gain was observed at much earlier time points as compared with mice fed which were fed standard chow (3 weeks for a high fat diet vs. 9 weeks for those fed standard chow). Further, although 12-week-old TLR4$^{\Delta IEC}$ mice fed standard chow started to show significantly increased body weight (**Figure 1Ai**), there was no difference between wild type and TLR4$^{\Delta IEC}$ mice in glucose tolerance (**Supplemental Figure 3**). On the contrary, 12-week-old TLR4$^{\Delta IEC}$ mice who were fed a high fat diet also showed impaired glucose tolerance as compared with age-matched wild type mice fed high fat diet (**Supplemental Figure 2B**). Moreover, 12-week-old TLR4$^{\Delta IEC}$ mice fed high fat diet showed an increased accumulation of adipose tissue (inguinal fat and mesenteric fat) and increased liver weight (**Supplemental Figure 2C**), elevated serum cholesterol (**Supplemental Figure 2D**), but similar serum triglycerides (**Supplemental Figure 2E**) as compared with wild type mice, supportive of our findings that the lack of TLR4 on the intestinal epithelium results in the

development of significant weight gain, insulin resistance and other features of metabolic syndrome regardless the fat content of the diet.

To confirm the role of intestinal epithelial TLR4 expression on metabolism, we next studied two additional TLR4 transgenic mouse strains, namely $TLR4^{IEC\text{-}only}$ mice in which TLR4 is expressed *only* in the intestinal epithelial cells, and $TLR4^{-/-}$ mice that are globally TLR4 deficient. As shown in **Figure 1A and 1B,** the administration of standard chow containing 22% calories from fat to both $TLR4^{-/-}$ and $TLR4^{IEC\text{-}only}$ resulted in similar total body weight, as well as the weight of adipose tissue and liver, compared with wild type mice at 24 weeks of age. Similarly, $TLR4^{-/-}$ and $TLR4^{IEC\text{-}only}$ mice displayed equivalent glycemic curves after a bolus of glucose compared with wild type mice (**Figure 2E**). Of note, although the inguinal and mesenteric fat content is higher in the $TLR4^{\Delta IEC}$ mice compared with the wild type and the $TLR4^{-/-}$ mice, there are no differences in the other parameters among all the other strains, and in particular there are no differences in fat depositions between the $TLR4^{\Delta IEC}$ and $TLR4^{IEC\text{-}only}$ mice.

Given that $TLR4^{\Delta IEC}$ mice showed increased weight gain and the development of metabolic syndrome, we next investigated further the observation that $TLR4^{-/-}$ mice did not develop metabolic syndrome, suggesting that the lack of TLR4 on some cells may have opposing effects in the development of metabolic syndrome than its expression on other cells. We therefore next hypothesized that TLR4 expression on myeloid cells plays an opposite role to that on the intestinal epithelium in the regulation of host metabolism. To test this possibility directly, we subjected mice in which TLR4 was selectively deleted from myeloid cells ($TLR4^{\Delta Myeloid}$) mice to the same standard diet above, and observed that at 24 weeks, $TLR4^{\Delta Myeloid}$ mice did not develop significant weight gain (**Figure 1A and 1B**), and had a similar glycemic curve to $TLR4^{-/-}$ mice and wild type mice after a glucose bolus (**Figure 2E**). These findings indicate that deletion of TLR4 from the intestinal epithelium as opposed to other cell types is required for the development of metabolic syndrome in mice, and we next sought to explore the mechanisms involved by focusing on the intestinal microbiota.

The administration of broad spectrum oral antibiotics or co-housing with wild type mice prevented the development of metabolic syndrome in $TLR4^{\Delta IEC}$ mice.

Given that TLR4 is a receptor for bacterial endotoxin, we next hypothesized that the effects of intestinal epithelial TLR4 on the regulation of host metabolism could be mediated in part by an effect on the intestinal microbiota. To test this possibility directly, we orally administered broad spectrum antibiotics to either wild type or $TLR4^{\Delta IEC}$ mice which were fed standard chow. The antibiotic treatment was determined to significantly reduce the bacterial load within the intestinal tract as shown in **Supplemental Figure 4A**, and prevented the previously observed excess weight gain observed in the $TLR4^{\Delta IEC}$ strains, which were now found at similar body weights throughout the 24-week period to wild type mice (**Figure 3A**) and did not show excess weight gain of the adipose tissue and liver (**Figure 3B**). The administration of antibiotics also prevented the previously observed hepatocellular ballooning (**Figure 3C and 3D**) and the presence of crown-like structures in the adipose tissue (**Figure 3E**), and abrogated changes in expression of the macrophage genes in the adipose tissues (**Figure 3F**). Most strikingly, the administration of oral broad

spectrum antibiotics prevented the previously observed glucose intolerance in the in the TLR4$^{\Delta IEC}$ mice seen after a glucose bolus (**Figure 3G**). These findings point to an important role for the microbiota in the development of metabolic syndrome in the TLR4$^{\Delta IEC}$ mice. To further evaluate the hypothesis that the effects of intestinal epithelial TLR4 on the regulation of host metabolism could be mediated through effects on the intestinal microbiota, we co-housed TLR4$^{\Delta IEC}$ mice and wild type mice together after weaning, and supplied standard chow until 24 weeks of age. Importantly, co-housing resulted in similar body weight gain (**Supplemental Figure 4B**), similar weight of adipose tissue and liver (**Supplemental Figure 4C**), and similar glucose tolerance (**Supplemental Figure 4D**) between TLR4$^{\Delta IEC}$ mice and wild type mice, and completey reversed the previously observed metabolic syndrome phenotype. Taken together, these findings support a role for the microbiota in the pathogenesis of metabolic syndrome in the TLR4$^{\Delta IEC}$ mice, leading us to next evaluate the effects of TLR4 on the composition and function of the intestinal microbiota in greater detail.

Intestinal epithelial TLR4 influences the composition and function of the intestinal microbiota in mice.

To further explore the role of intestinal epithelial TLR4 on the development of metabolic syndrome, we next subjected stool samples from TLR4$^{\Delta IEC}$ and wild type mice to 16S pyrosequencing followed by UniFrac clustering analysis[19], and then performed a detailed analysis of the bacterial meta-transcriptome derived from both mouse strains. The composition of the 20 most abundant operational taxonomic units (OTUs) in wild type and TLR4$^{\Delta IEC}$ mice are shown in **Table 1** and the taxonomic domains are shown in **Figure 4A**. At the phyla level, the intestinal microbiota of both TLR4$^{\Delta IEC}$ and wild type mice were dominated by *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Although there was an apparent difference in the stool *Verrucomicrobia* between strains, this was not statistically significant (p=0.09). However, the intestinal microbiota in TLR4$^{\Delta IEC}$ mice were revealed to cluster differently from those in wild type mice (**Figure 4B**), and displayed a significantly lower diversity (**Figure 4C**). To determine the functional consequences of the loss of TLR4 within the intestinal epithelium on microbial function, we next performed a detailed metatranscriptomic analysis of the stool samples from wild type and TLR4$^{\Delta IEC}$ mice. After removing the sequences from mouse origin and thus confining the analysis to bacterial products, 1,001,228 bacterial sequences were identified from the metatranscriptome which were converted to 4,067 unique clusters of orthologous groups (COGs) by running against the COGs protein database. This analysis determined that 246 COGs were found to be differentially expressed between TLR4$^{\Delta IEC}$ and wild type mice which were then grouped into the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. This investigation revealed a significant alteration in several functional categories that may play a role in the development of metabolic syndrome, including the metabolism of lipid, amino acids, and nucleotides (**Table 2**), in the bacteria from TLR4$^{\Delta IEC}$ as compared with wild type mice, providing insights into the mechanisms by which altered bacterial signaling in response to TLR4 deficiency could lead to the development of metabolic syndrome.

In seeking to investigate the potential mechanisms by which TLR4 expression in the intestinal epithelium could influence the composition of the microbiota in the first place, we

observed that mRNA expression of the anti-microbial peptide lysozyme – which has an established role in regulating the composition of the intestinal microbiota and maintaining intestinal homeostasis[20] – was significantly reduced in the small intestine of TLR4$^{\Delta IEC}$ mice as compared with wild type mice (**Supplemental Figure 5**). In addition, the administration of antibiotics did not restore lysozyme expression to wild type levels (**Supplemental Figure 5**), suggesting a predominant role for intestinal TLR4 in its regulation. These findings led us to next investigate how TLR4mediated host-microbial interactions could lead to the development of metabolic syndrome in mice.

TLR4 expression within the intestinal epithelium regulates the expression of metabolic and inflammatory genes in mice.

To investigate further how TLR4 expression within the intestinal epithelium could lead to the development of metabolic syndrome, we next performed an unbiased RNA sequencing analysis on intestinal mucosal samples obtained from the terminal ileum of wild type and TLR4$^{\Delta IEC}$ mice. This study revealed that key genes within the metabolically relevant PPAR signaling pathway were significantly down-regulated in TLR4$^{\Delta IEC}$ mice compared to wild type mice (**Table 3 and Supplemental Figure 6A**), although the PPAR genes themselves were unchanged (**Supplemental Figure 6B**). Specific PPAR-regulated genes that were down-regulated included fasting induced adipose factor (*Fiaf*), fatty acid transport protein 1 (*Fatp1*), 3-hydroxy-3methylglutaryl-CoA synthase 2 (*Hmgcs2*) and fatty acid-binding protein 1 (*Fabp1*) (**Figure 5A**).

In control experiments, the expression of those PPAR-regulated genes were similar between wild type and TLR4$^{\Delta Myeloid}$ mice (**Supplemental Figure 6C**), consistent with the lack of metabolic syndrome observed in the TLR4$^{\Delta Myeloid}$ mice. Given that we had identified that the administration of antibiotics prevented the development of metabolic syndrome in TLR4$^{\Delta IEC}$ mice, we next sought to assess the effects of antibiotic administration of the expression of these key PPARregulated genes. As shown in **Figure 5B**, the administration of broad spectrum antibiotics prevented the previously observed differences in expression of *Fiaf*, *Fatp1*, *Hmgcs2*, and *Fabp1*, in both wild type and TLR4$^{\Delta IEC}$ mice after treatment with broad spectrum antibiotics. We next evlauted whether the reduced expression of these key PPAR-regulated metabolic genes in response to TLR4 deletion was required for the development of metabolic syndrome. To do so, we next administrated pharmacologic agonists of the PPAR signaling pathway to wild type and TLR4$^{\Delta IEC}$ mice and assessed the consequences on glucose metabolism. Importantly, the oral administration of bezafibrate (which activates all three PPAR subtypes), rosiglitazone (which selectively activates PPAR gamma), or WY-14643 (which activates PPAR alpha, gamma and delta) completely reversed the impaired glucose tolerance in TLR4$^{\Delta IEC}$ mice (**Figure 5C**), and restored the expression of these PPAR-regulated genes, namely *Fiaf*, *Fatp1*, *Hmgcs2*, and *Fabp1* (**Figure 5D**). It is noteworthy that TLR4 deletion did not only regulate PPAR-regulated metabolic genes, as the gene expression profile of TLR4$^{\Delta IEC}$ mice revealed a pro-inflammatory phenotype, characterized by up-regulated expression of macrophage markers (*F4/80*, *Cd68* and *Mcp1*, **Supplemental Figure 6D**), neutrophil markers (*Mpo* and *Elane*, **Supplemental Figure 6E**), a trend towards increased expression of the pro-inflammatory cytokine interleukin 6 (*Il6*) in the ileum (**Supplemental Figure 6F**). These findings are consistent with a potential degree

of subtle inflammation in the small intestine of TLR4$^{\Delta IEC}$ mice, which could contribute to the development of metabolic syndrome. Taken together, these findings suggest that TLR4 regulates both host and bacterial genes that have a key role in energy metabolism, which acts in concert to govern the development of metabolic syndrome and obesity in mice.

Discussion

We now show that TLR4 expression in the intestinal epithelium plays a previously unrecognized role in the development of metabolic syndrome in mice through the regulation of host-bacterial interactions. The conclusion that TLR4 deficiency from the intestinal epithelium leads to the development of metabolic syndrome was reached by studying five different strains of mice, including TLR4$^{\Delta IEC}$ mice which developed metabolic syndrome, as well as mice lacking TLR4 on myeloid cells, mice globally deficient in TLR4, mice expressing TLR4 only on the intestinal epithelium and wild type mice which each did not. In terms of understanding how TLR4 signaling in the intestinal epithelial cells regulates body energy metabolism, we now describe a key role for TLR4 in the regulation of the intestinal microbiota, as the administration of antibiotics or co-housing of TLR4$^{\Delta IEC}$ mice with wild type mice reversed the induction of metabolic syndrome. We further showed that TLR4 deletion from the intestinal epithelium led to a downregulation of key genes in the regulation of host metabolism, namely PPAR-regulated genes, and that PPAR activation reversed the development of metabolic syndrome. Taken together, these findings illustrate important roles for TLR4 in the regulation of bacteriaepithelium interactions in the development of metabolic syndrome, and suggest the possibility that modulating either the microbiota or host PPAR-regulated genes could offer novel preventive of therapeutic approaches for this disease.

The current study adds to a body of work that has examined the roles of TLR4 in the pathogenesis of metabolic syndrome, around which there has been significant controversy. Researchers have determined that TLR4 inhibition either reduced[14,21,22], increased[16], or had no effect[15] on diet-induced obesity in mice, and attributed these opposing findings to differences in food consumption[16], or energy expenditure[22] between the knockout and wild type animals. We now report that the lack of TLR4 on the intestinal epithelium as opposed to other cell types is responsible for the development of metabolic syndrome, and that TLR4-dependent differences in the genetic pathways that drive host metabolism play an important mechanistic role. The cellspecific effect of TLR4 on host metabolism in the current study is supported by earlier findings showing that deleting TLR4 from hepatic cells improved insulin resistance[23], while deleting TLR4 from myeloid cells either improved[21] or had no effect on insulin resistance[23]. It is reassuring to us that the impaired glucose tolerance in the TLR4$^{\Delta IEC}$ mice was consistently observed over a four year period, and was seen in two different animal colonies at two separate institutions, namely at the University of Pittsburgh and Johns Hopkins University.

One of the most intriguing findings of the current work is the observation that the expression of TLR4 on the intestinal epithelium plays a key role not only in the composition but also in the meta-transcriptomic profile of the intestinal microbiota. Explaining, rather than speculating, the role of specific microbial groups would require a more focused

investigation, including work with gnotobiotic mice with defined gut microbial communities. The meta-transcriptome analysis of the bacteria in wild type and TLR4$^{\Delta IEC}$ mice intestines identified key differences which were primarily linked to carbohydrate metabolism (11 pathways) and energy metabolism (5 pathways) between strains. The observed changes in the microbiota gene expression in the TLR4$^{\Delta IEC}$ mice may have effects on host metabolism, as suggested by prior investigators. Specifically, Musso *et al* have shown that changes in gene expression in gut microbiota metabolism may lead to increased nutrient absorption and bile acid reabsorption leading to metabolic syndrome[24], and this topic has been reviewed in recent years[25–28]. Given that the mammalian intestine has a limited ability to hydrolyze complex polysaccharides, it is noteworthy that we found that 11 genes for carbohydrate metabolism pathways were altered in the microbiota of the TLR4$^{\Delta IEC}$ mice which developed metabolic syndrome. These findings are reminiscent of the observations of prior studies showing that the abundance of *Firmicutes* versus *Bacteroidetes* has been linked to the development of obesity[7] through the differential ability of these strains to break down complex carbohydrates, and thus vary the degree of carbohydrate delivery and absorption by the host[29].

While the current findings reveal that TLR4 expression in the intestinal epithelium can impact the composition of the intestinal microbiota, a precise explanation of how this occurs remains lacking. We have previously shown that the deletion of intestinal epithelial TLR4 leads to increased expression of mucin MUC2[30], which is secreated by goblet cells and forms a mucus barrier which can influence to growth and composition of commensal bacteria in the small intestine[31]. Further, we now demonstrate that TLR4$^{\Delta IEC}$ mice reveal significantly reduced expression of the antimicrobial pepetide lysozyme (**Supplemental Figure 5**), which is secreated by Paneth cells and plays important roles in maintaining the composition of the intestinal microbiota[32]. It is possible that the host expression of TLR4 could alter the composition of the microbiota through either effects on goblet cells or lysozyme secretion, and do so in a manner that influences metabolic genes, for reasons that are not obvious. Of note, TRIF$^{-/-}$ mice were shown to also exhibit increased globle cells[30], reduced lysozyme-positive Paneth cells[33], and more interestingly, impaired glucose tolerance[34], supporting this concept, and indicating a potential role for TLR4-TRIF signaling in the development of metabolic syndrome in TLR4$^{\Delta IEC}$ mice.

A major finding of the current study centered around the observation that TLR4 expression within the terminal ileum plays a previously unrecognized role in the regulation of key metabolic and inflammatory genes within the intestine of the host, in particular those within the PPAR family[3536]. It is noteworthy that in inflammatory diseases, PPAR signaling can decrease the extent of TLR4 signaling in myeloid cells[3738], adipocytes [39], and the intestinal epithelium[40], linking the PPAR signaling with altered TLR4 responsiveness. We now extend these prior findings by showing that the reverse can also occur, namely that TLR4 deficiency also reduces PPAR expression. Prior authors have shown that high fat diet-induced obesity is related to an increase in circulating free fatty acids, which can act as endogenous ligands of TLR4[41] and PPAR[42], thus providing a potential explanation as to why TLR4-mutant mice may be protected from obesity and metabolic syndrome[41]. However, in the current study, in which we show that TLR4$^{\Delta IEC}$ mice are at greater risk for metabolic syndrome and obesity,

we now posit that in the presence of a non-high-fat diet, in which circulating free fatty acids are not expected to be increased, the genetic consequences of intestinal epithelial TLR4 deficiency predominate, namely a lack of PPAR, which can negatively regulate metabolism in mice[4344]. In support of this concept, agonists of PPAR profoundly reversed the effects of TLR4 deficiency on the development of metabolic syndrome in the TLR4$^{\Delta IEC}$ mice (**Figure 5C**).

It is noteworthy that the current findings may allow us to postulate a general signaling mechanism by which bacterial recognition by microbial receptors in the intestinal epithelium leads to alterations in the metabolic profile of the host. Specifically, the work of Gewirtz has revealed that mice lacking the receptor for certain Gram-negative species including *Salmonella,* namely Toll-like receptor 5, either globally (TLR5$^{-/-}$)[5] or in the intestinal epithelium (TLR5$^{\Delta IEC}$)[45], or mice lacking the conserved TLR adaptor proteins namely MyD88 (MyD88$^{-/-}$)[46] or TRIF (TRIF$^{-/-}$)[34], each develop both low-grade intestinal inflammation and metabolic syndrome, supporting the concept that bacterial signaling influences metabolism in the host through MyD88 and/or TRIF. Further studies revealed that mice lacking TLR5 from dendritic cells (TLR5$^{\Delta DC}$)[45], or mice lacking MyD88 from dendritic cells (MyD88$^{\Delta DC}$)[47] or other myeloid cells (MyD88$^{\Delta Myeloid}$)[48] do not show any signs of metabolic syndrome. By contrast, additional studies reveal that the deletion of MyD88 from myeloid cells (MyD88$^{\Delta Myeloid}$) or endothelial cells (MyD88$^{\Delta Endo}$) protected mice from high fat diet-induced metabolic syndrome[49]. These intriguing studies provide further evidence for the cell-specific nature of TLR signaling in the development of metabolic syndrome, as we now reveal to be the case for TLR4.

In summary, we have now shown that TLR4 signaling in the intestinal epithelium plays a key role in the regulation of metabolic syndrome through the regulation of microbial and host metabolic pathways, and that these effects can be reversed by the administration of PPAR agonists. Our findings suggest a novel link between host metabolism, host genetics and the intestinal microbiota, and indicate that by exploring this relationship in greater detail, we can potentially reverse this complex and highly morbid disease.

Materials and Methods

Animal strains and experimentation

All mice experiments were approved by the Animal Care and Use Committee of the University of Pittsburgh and by the Johns Hopkins University Animal Care and Use Committee. Mice harboring floxed alleles of TLR4 (wild type), mice in which TLR4 was selectively deleted from the intestinal epithelium (TLR4$^{\Delta IEC}$), mice that are globally TLR4 deficient (TLR4$^{-/-}$) and mice in which TLR4 was expressed *only* in the intestinal epithelium (TLR4$^{IEC\text{-}only}$) were generated in our laboratory as recently described[30,50]. The breeding scheme for the generation of mice in which TLR4 was selectively deleted from the myeloid cells (TLR4$^{\Delta Myeloid}$), and confirmation of the lack of TLR4 in the macrophages, are shown in Supplemental Methods and **Supplemental Figure 7**. All animals were housed in a specific pathogen free environment on a 12-hour-light/12-hourdark cycle with free access to acidified tap water and standard rodent chow (PicoLab mouse diet 20, 22% kcal% fat) from weaning until 24 weeks. Only male mice were included for analysis, and up to 5 male

littermates of each strain were housed in one cage after weaning. For cohousing experiments, up to total 5 male mice of mixed TLR4$^{\Delta IEC}$ mice and wild type mice were housed in one cage after weaning. Where indicated, mice were administered a high fat diet in which 60% of the total calories were derived from fat[51]. In further experiments, mice were administered broad spectrum antibiotics in the drinking water (1 g/L neomycin, 0.5 g/L vancomycin, 1 g/L ampicillin and 1 g/L metronidazole) as the only source of water from the age of 3 to 24 weeks. PPAR agonist-treated mice received 285mg/kg/day bezafibrate, 25 mg/kg/day rosiglitazone, or 140 mg/kg/day WY-14643 orally from the age of 23 to 24 weeks. The total abdominal fat content was determined by micro-MRI, in which isoflurane anesthetized mice were secured to a micro-MRI cradle and advanced into the magnet of a 7Tesla micro-MRI system (Bruker Bio Spin Corporation, Billerica, MA, USA). A rapid acquisition with relaxation enhancement with fat saturation T1 protocol was applied on the abdomen[52].

Histologic preparation and oil red-O staining

Adipose tissue and liver were freshly harvested, paraformaldehyde-fixed, and embedded in paraffin. Histological staining was carried out on 4-μm-thick sections which were stained with heamatoxylin and eosin (H&E). Oil red-O staining was performed in fresh liver cryosections which were fixed with 4 % (w/v) paraformaldehyde, and after washing with 60% isopropanol, the cryosections were counterstained with hematoxylin.

RNA isolation, cDNA synthesis, and quantitative PCR (qPCR)

Total RNA was isolated from the epididymal white adipose tissue and ileum of mice of 24-weekold mice using the RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Complementary DNA was synthesized from 0.5 μg RNA using M-MLV reverse transcriptase. The qPCR analysis was performed with the Bio-Rad CFX96 Real-Time System (Biorad, Hercules, CA) using the primers listed in **Table 4**. The relative mRNA expression levels were normalized against the expression of housekeeping gene ribosomal protein lateral stalk subunit P0 (*Rplp0*) relative to wild type mice.

Oral glucose tolerance test (OGTT)

Glucose levels were determined via glucometer (Bayer, Whippany, NJ, USA). Oral glucose tolerance test was performed in overnight-fasted 24-week-old mice administered 2g of glucose per kg body weight by oral gavage, and the blood glucose levels were measured via tail puncture at 1 min before and 15, 30, 60, 90 and 120 min after the oral gavage[53].

Statistical analysis

Data were analyzed for statistical significance by Mann-Whitney test using GraphPad Prism (GraphPad, La Jolla, CA, USA). A p value of less than 0.05 was considered statistically significant, and data are presented as mean ± SEM as indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

TLR4	Toll like receptor 4
TLR4$^{\Delta IEC}$ mice	mice lacking TLR4 in the intestinal epithelium
TLR4$^{-/-}$ mice	TLR4 deficient mice, TLR4$^{IEC\text{-}only}$ mice, mice in which TLR4 is expressed *only* in the intestinal epithelial cells
TLR4$^{\Delta Myeloid}$ mice	mice lacking TLR4 in leukocytes
OTUs	operational taxonomic units
COGs	clusters of orthologous groups
KEGG	Kyoto Encyclopedia of Genes and Genomes
Tnf	tumor necrosis factor
PPAR	peroxisome proliferator-activated receptor
Fiaf	fasting induced adipose factor
Fatp1	fatty acid transport protein 1
Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2
Fabp1	fatty acid-binding protein 1
MyD88	myeloid differentiation primary response gene 88
TRIF	TIR domain containing adaptor inducing interferon-beta
Retnlb	Resistin like beta
Mcp1	monocyte chemotactic protein 1
Mpo	myeloperoxidase
Elane	elastase
Il6	interleukin 6
Rplp0	ribosomal protein lateral stalk subunit P0
TLR5	Toll-like receptor 5
LysM	lysozyme 2
H&E	heamatoxylin and eosin

qPCR	quantitative PCR
OGTT	oral glucose tolerance test

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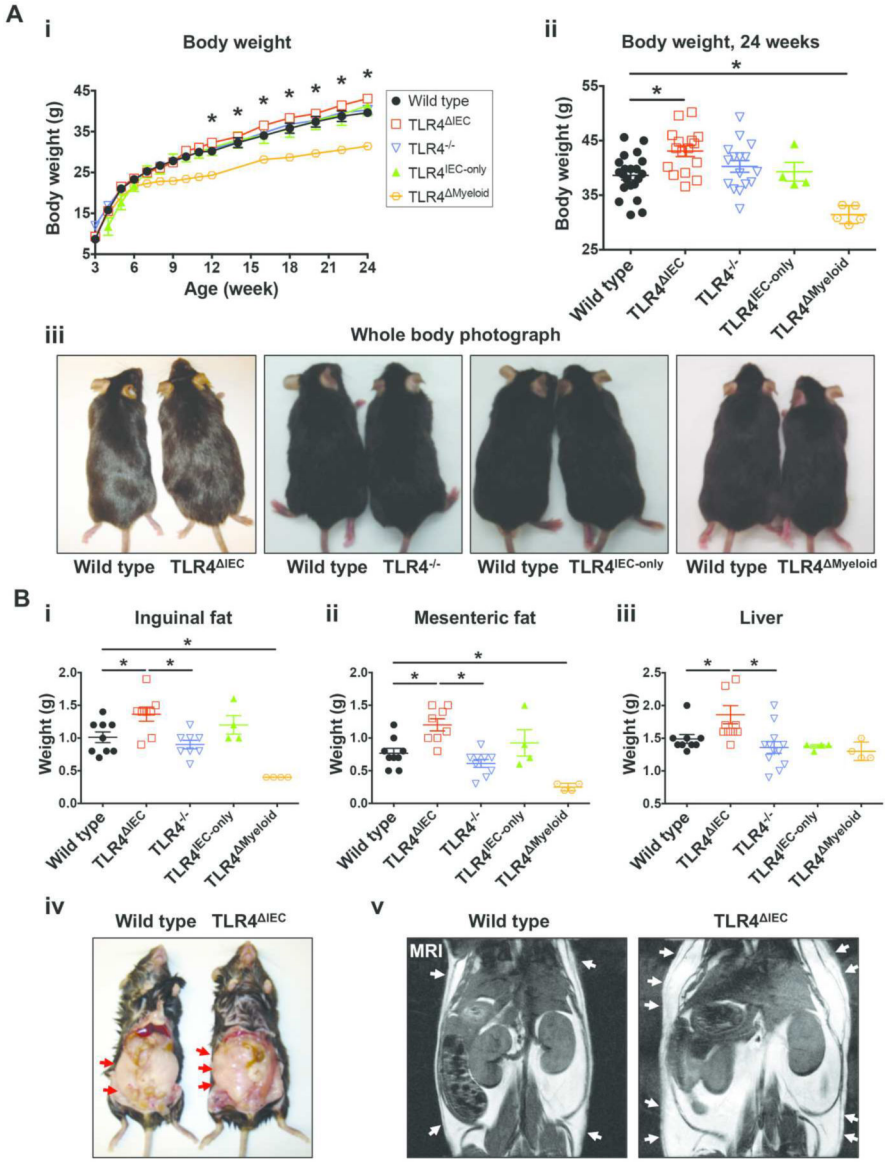

Figure 1. Deletion of the lipopolysaccharide receptor TLR4 from intestinal epithelial cells leads to obesity in mice.

A: i: Determination of body weight between 3 and 24 weeks of wild type (n=33), TLR4$^{\Delta IEC}$ (n=31), TLR4$^{-/-}$ (n=31), TLR4$^{IEC\text{-}only}$ (n=6) and TLR4$^{\Delta Myeloid}$ (n=8) mice who were fed standard chow; **ii**: body weight at 24 weeks for the wild type (n=21), TLR4$^{\Delta IEC}$ (n=16), TLR4$^{-/-}$ (n=16), TLR4$^{IEC\text{-}only}$ (n=4) and TLR4$^{\Delta Myeloid}$ (n=5) mice is shown. Data are represented as mean ± SEM; *p<0.05 wild type vs TLR4$^{\Delta IEC}$ mice at the indicated time point; each symbol represents a separate mouse; **iii**: representative photographs of wild type, TLR4$^{\Delta IEC}$, TLR4$^{-/-}$, TLR4$^{IEC\text{-}only}$ and TLR4$^{\Delta Myeloid}$ at 24 weeks of age is shown revealing the significantly larger size of the TLR4$^{\Delta IEC}$ mice compared with wild type mice. **B**: Weight of inguinal fat (**i**), mesenteric fat (**ii**) and liver (**iii**) of wild type (n=9), TLR4$^{\Delta IEC}$ (n=8 to 10 as indicated), TLR4$^{-/-}$ (n=8 to 12 as indicated), TLR4$^{IEC\text{-}only}$ (n=4) and TLR4$^{\Delta Myeloid}$ (n=4) mice fed standard chow at the age of 24 weeks. Data are represented as mean ± SEM;

*p<0.05 for the indicated comparison; each symbol represents a separate mouse; **iv-v**: representative photograph (**iv**) and micro-MRI (**v**) revealing increased abdominal fat in the $TLR4^{\Delta IEC}$ as compared with wild type mice at 24 weeks of age; arrows show the deposition of adipose tissue in the subcutaneous tissue.

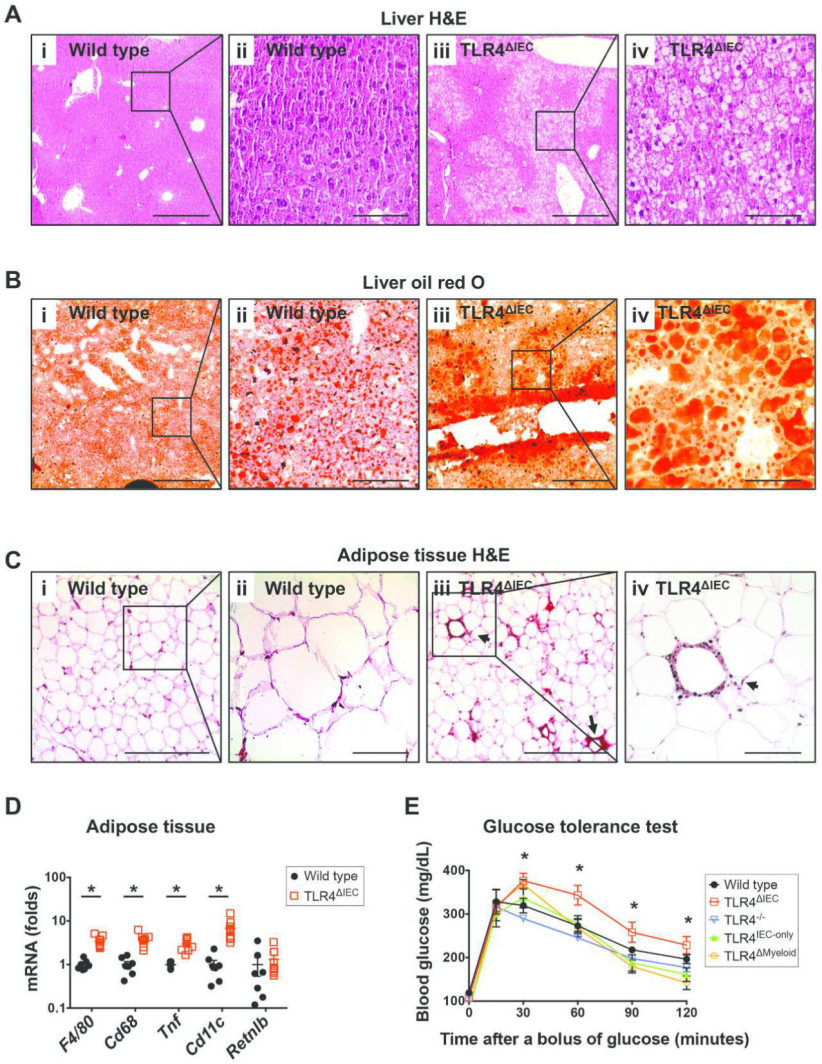

Figure 2. Determinants of metabolic syndrome in mice lacking TLR4 from the intestinal epithelium.

A: Representative photomicrographs showing liver sections of wild type mice (i and ii) and TLR4$^{\Delta IEC}$ mice (iii and iv) fed standard chow at the age of 24 weeks, which were stained with haematoxylin and eosin. Scale bars represent 400-μm and 100-μm in the 10× (i and iii) and 40× (ii and iv) panels, respectively. **B**: Representative photomicrographs showing liver sections of wild type mice (i and ii) and TLR4$^{\Delta IEC}$ mice (iii and iv) fed standard chow at the age of 24 weeks, which were stained with oil red O. Scale bars represent 400-μm and 100-μm in the 10× (i and iii) and 40× (ii and iv) panels, respectively. **C**: Representative photomicrographs showing white adipose tissue sections of wild type mice (i and ii) and TLR4$^{\Delta IEC}$ mice (iii and iv) fed standard chow at the age of 24 weeks, which were stained with haematoxylin and eosin. Arrows point to crown-like structure in the white adipose tissue of TLR4$^{\Delta IEC}$ mice, and scale bars represent 400-μm and 100-μm in the 10× (i and iii) and 40× (ii and iv) panels, respectively. **D**: qPCR showing the expression of the indicated macrophage markers in the white adipose tissues of wild type (n=7) and TLR4$^{\Delta IEC}$ (n=9) mice fed standard chow at the age of 24 weeks. All data were normalized to the mRNA

expression of *Rplp0*, and the mRNA expression in wild type mice was set to 1. Data are represented as mean ± SEM; *p<0.05 between groups shown; each symbol represents a separate mouse. **E**: Oral glucose tolerance test of wild type (n=12), $TLR4^{\Delta IEC}$ (n=9), $TLR4^{-/-}$ (n=5), $TLR4^{IEC\text{-}only}$ (n=4) and $TLR4^{\Delta Myeloid}$ (n=4) mice fed standard chow at the age of 24 weeks. Data are represented as mean ± SEM; *p<0.05 wild type vs. $TLR4^{\Delta IEC}$ mice.

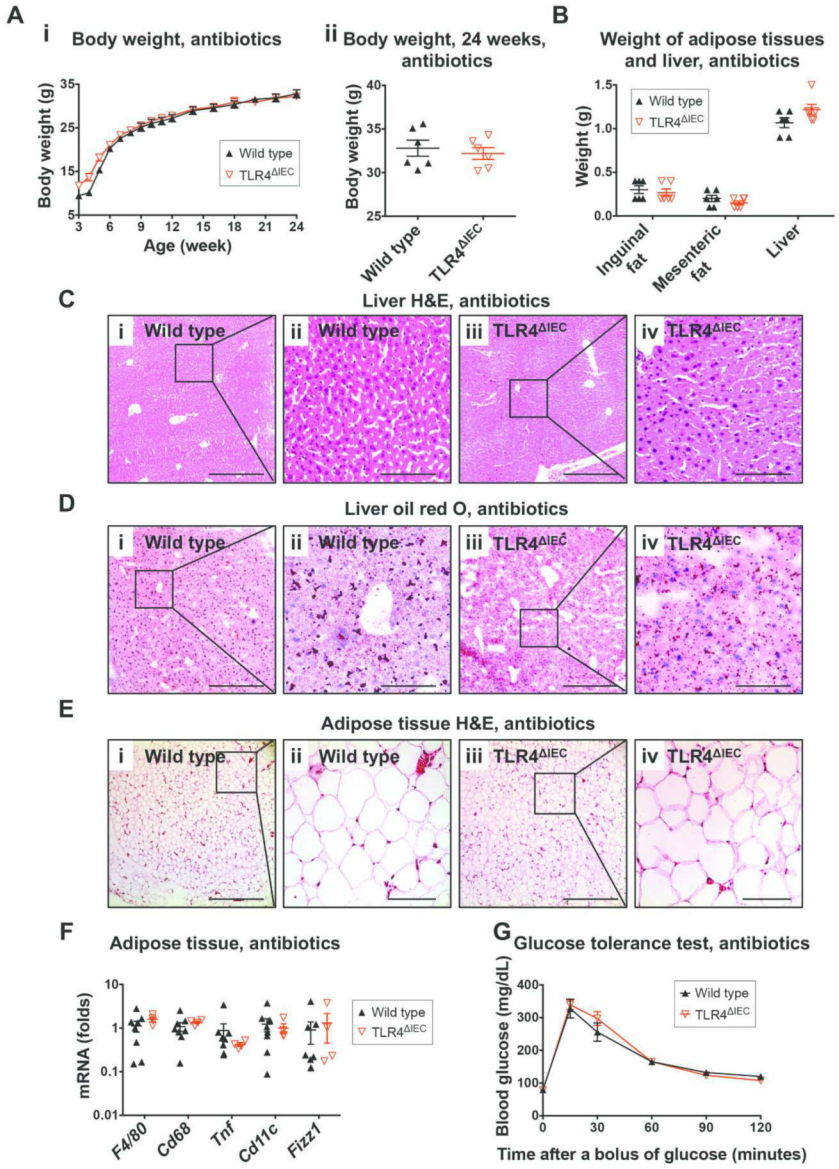

Figure 3. The administration of broad spectrum antibiotics prevented the development of obesity and metabolic syndrome in TLR4$^{\Delta IEC}$ mice.

A: The body weight of wild type (n=6) and TLR4$^{\Delta IEC}$ (n=6) mice treated with antibiotics from the age of 3 to 24 weeks (**i**) and at the age of 24 weeks (**ii**). Data are mean ± SEM; each symbol represents a separate mouse. **B**: The weight of the inguinal fat, mesenteric fat and liver of wild type (n=6) and TLR4$^{\Delta IEC}$ (n=6) mice treated with antibiotics at the age of 24. Data are represented as mean ± SEM; each symbol represents a separate mouse. **C**: Representative photomicrographs showing liver sections of wild type mice (i and ii) and TLR4$^{\Delta IEC}$ mice (iii and iv) treated with antibiotics at the age of 24 weeks, which were stained with haematoxylin and eosin. Scale bars represent 400-μm and 100-μm in the 10× (i and iii) and 40× (ii and iv) panels, respectively. **D**: Representative photomicrographs showing liver sections of wild type mice (i and ii) and TLR4$^{\Delta IEC}$ mice (iii and iv) treated with antibiotics at the age of 24 weeks, which were stained with oil red O. Scale bars

represent 400-μm and 100-μm in the 10× (i and iii) and 40× (ii and iv) panels, respectively. **E**: Representative photomicrographs showing white adipose tissue sections of wild type mice (i and ii) and TLR4$^{\Delta IEC}$ mice (iii and iv) treated with antibiotics at the age of 24 weeks, which were stained with haematoxylin and eosin. Scale bars represent 400-μm and 100-μm in the 10× (i and iii) and 40× (ii and iv) panels, respectively. **F**: qPCR showing the expression of macrophage markers (*F4/80* and *Cd68*), M1 macrophage markers (*Tnf* and *Cd11c*) and M2 macrophage marker (*Retnlb*) in the white adipose tissues of wild type (n=6) and TLR4$^{\Delta IEC}$ (n=6) mice treated with antibiotics at the age of 24 weeks. All data were normalized to the mRNA expression of *Rplp0*, and the mRNA expression in wild type mice was set to 1. Data are represented as mean ± SEM; each symbol represents a separate mouse. **G**: The oral glucose tolerance test (OGTT) of wild type (n=6) and TLR4$^{\Delta IEC}$ (n=6) mice treated with antibiotics at the age of 24 weeks is shown. Data are represented as mean ± SEM; *$p<0.05$ wild type vs. TLR4$^{\Delta IEC}$ mice.

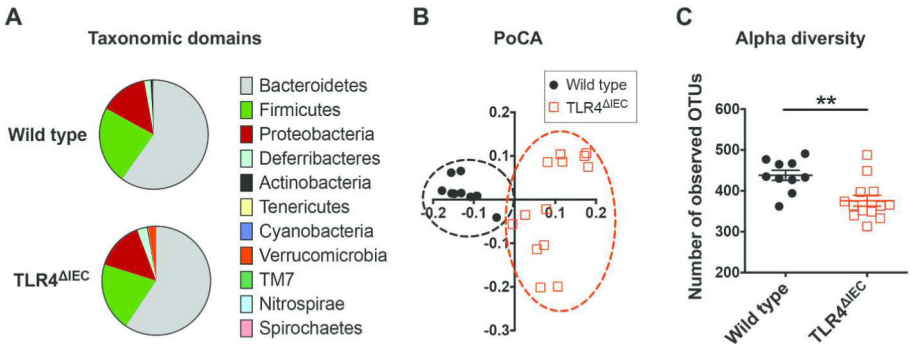

Figure 4. The effects of intestinal epithelial TLR4 on the luminal microbiota in mice.

A: The major taxonomic domains of intestinal microbiota of wild type and TLR4$^{\Delta IEC}$ mice fed standard chow at the age of 24 weeks. **B**: UNIFRAG clustering analysis of the intestinal microbiota of wild type (n=10) and TLR4$^{\Delta IEC}$ (n=13) mice fed standard chow at the age of 24 weeks. **C**: The alpha-diversity of the intestinal microbiota of wild type (n=10) and TLR4$^{\Delta IEC}$ (n=13) mice fed standard chow at the age of 24 weeks. Data are represented as mean ± SEM; *p<0.05 between wild type and TLR4$^{\Delta IEC}$ mice; each symbol represents a separate mouse.

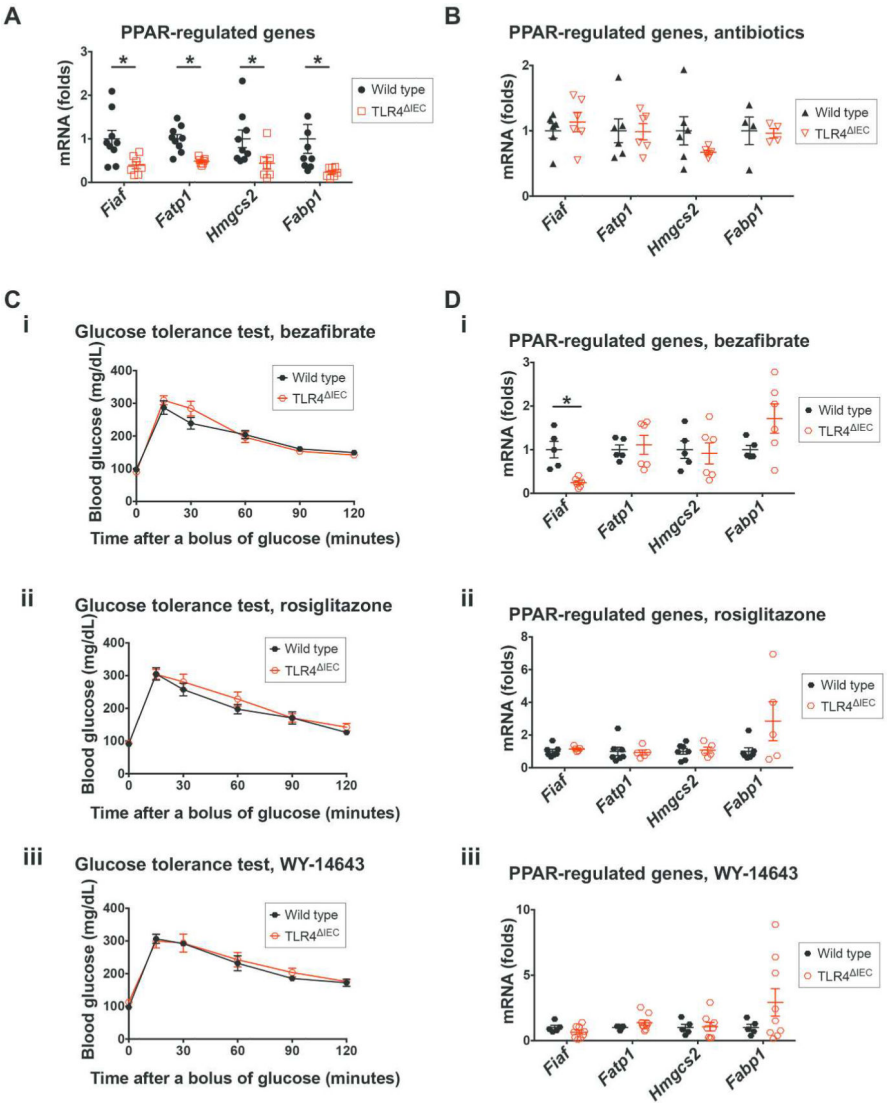

Figure 5. Intestinal epithelial TLR4 regulates the expression of PPAR-related genes in the gut while PPAR agonists reversed the glucose intolerance in TLR4$^{\Delta IEC}$ mice.

A-B: qPCR showing the expression of PPAR family genes *Fiaf*, *Fatp1*, *Hmgcs2* and *Fabp1* in the ileum of wild type (n=9 and 6, respectively) and TLR4$^{\Delta IEC}$ (n=7 and 6, respectively) mice fed standard chow at the age of 24 weeks in the absence (**A**) or presence (**B**) of oral antibiotics. All data were normalized to the mRNA expression of *Rplp0*, and the mRNA expression in wild type mice was set to 1. Data are represented as mean ± SEM; *p<0.05 wild type vs TLR4$^{\Delta IEC}$ mice; each symbol represents a separate mouse. **C**: Blood glucose concentrations during oral glucose tolerance test of wild type (n=5, 7 and 5, respectivley) and TLR4$^{\Delta IEC}$ (n=6, 6 and 9, respectively) mice treated with PPAR agonists bezafibrate (**i**), rosiglitazone (**ii**) and WY-14643 (**iii**) at the age of 24 weeks are shown. Data are represented as mean ± SEM, *p<0.05 wild type vs TLR4$^{\Delta IEC}$ mice. **D**: qPCR showing mRNA expression of *Fiaf*, *Fatp1*, *Hmgcs2* and *Fabp1* in the ileum of wild type (n=5, 7 and 5, respectively) and TLR4$^{\Delta IEC}$ (n=6, 5 and 9, respectively) mice treatment with PPAR agonists bezafibrate (**i**), rosiglitazone (**ii**) and WY-14643 (**iii**) at the age of 24 weeks were analyzed

using qPCR. All data were normalized to the mRNA expression of *Rplp0*, and the mRNA expression in wild type mice was set to 1. Data are represented as mean ± SEM; each symbol represents a separate mouse. *$p<0.05$ wild type vs $TLR4^{\Delta IEC}$ mice.

Table 1.

Twenty most abundant OTUs in the stools of wild type and TLR4$^{\Delta IEC}$ mice

OTU	Test-Statistic	P	FDR P	Bonferroni P	TLR4$^{\Delta IEC}$ mean	WT mean	taxonomy
250270	18.356813	0.000018	0.010953	0.042384	0.8	28.4	*Firmicutes, Clostridia, Clostridiales*
214919	17.787644	0.000025	0.010953	0.057152	0.6	18.7	*Firmicutes, Bacilli, Turicibacterales, Turicibacteraceae, Turicibacter*
449353	17.487012	0.000029	0.010953	0.066939	0.5	12.2	*Firmicutes, Clostridia, Clostridiales, Dehalobacteriaceae, Dehalobacterium*
296045	17.274160	0.000032	0.010953	0.074871	47.1	0.1	*Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae, Bacteroides*
193680	17.033824	0.000037	0.010953	0.084970	0.0	6.1	*Firmicutes, Clostridia, Clostridiales, Lachnospiraceae*
263518	16.988636	0.000038	0.010953	0.087016	10.7	0.2	*Proteobacteria, Deltaproteobacteria, Desulfovibrionales, Desulfovibrionaceae*
4466511	16.979866	0.000038	0.010953	0.087419	27.5	0.2	*Bacteroidetes, Bacteroidia, Bacteroidales, Odoribacteraceae, Odoribacter*
1105589	16.962352	0.000038	0.010953	0.088229	75.3	0.2	*Bacteroidetes, Bacteroidia, Bacteroidales, S24–7*
262752	16.627907	0.000045	0.010953	0.105234	2.0	241.8	*Bacteroidetes, Bacteroidia, Bacteroidales, S24–7*
312322	16.379482	0.000052	0.010953	0.119966	2.4	56.6	*Bacteroidetes, Bacteroidia, Bacteroidales, S24–7*
2937207	16.371329	0.000052	0.010953	0.120483	284.5	1.1	*Deferribacteres, Deferribacteres, Deferribacterales, Deferribacteraceae, Mucispirillum, schaedleri*
544996	15.889564	0.000067	0.012439	0.155380	0.6	10.4	*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, Oscillospira*
259820	15.775570	0.000071	0.012439	0.165029	0.1	34.3	*Firmicutes, Clostridia, Clostridiales*
176868	15.595223	0.000078	0.012439	0.181539	8.0	0.0	*Firmicutes, Clostridia, Clostridiales, Dehalobacteriaceae, Dehalobacterium*
835900	15.543325	0.000081	0.012439	0.186591	112.4	0.0	*Bacteroidetes, Bacteroidia, Bacteroidales, Odoribacteraceae, Odoribacter*
176952	14.922099	0.000112	0.016204	0.259267	0.1	2.0	*Firmicutes, Clostridia, Clostridiales*
259859	14.227922	0.000162	0.021115	0.374751	1.2	32.8	*Bacteroidetes, Bacteroidia, Bacteroidales, S24–7*
186358	14.201435	0.000164	0.021115	0.380063	3.5	0.1	*Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae, Bacteroides*
175485	13.881567	0.000195	0.023712	0.450533	3.5	0.1	*Bacteroidetes, Bacteroidia, Bacteroidales, Bacteroidaceae, Bacteroides*
4449524	13.549172	0.000232	0.026888	0.537759	10.6	0.0	*Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, Prevotella*

Table 2.

Altered KEGG metabolic pathways of the intestinal microbiota between wild type and TLR4$^{\Delta IEC}$ mice

Class of metabolism pathways	Metabolic pathway name
Energy metabolism	Carbon fixation in photosynthetic organisms
	Carbon fixation pathways in prokaryotes
	Methane metabolism
	Nitrogen metabolism
	Oxidative phosphorylation
Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
	Butanoate metabolism
	C5-Branched dibasic acid metabolism
	Citrate cycle (TCA cycle)
	Glycolysis / Gluconeogenesis
	Glyoxylate and dicarboxylate metabolism
	Inositol phosphate metabolism
	Pentose phosphate pathway
	Propanoate metabolism
	Pyruvate metabolism
	Starch and sucrose metabolism
Lipid metabolism	Ether lipid metabolism
	Glycerophospholipid metabolism
Amino acid metabolism	Alanine, aspartate and glutamate metabolism
	Arginine and proline metabolism
	Histidine metabolism
	Lysine biosynthesis
	Valine, leucine and isoleucine biosynthesis
Metabolism of other amino acids	Cyanoamino acid metabolism
	D-Glutamine and D-glutamate metabolism
	Glutathione metabolism
	Phosphonate and phosphinate metabolism
Amino acid metabolism	Valine, leucine and isoleucine degradation
	Purine metabolism
	Pyrimidine metabolism
Metabolism of cofactors and vitamins	Biotin metabolism
	Nicotinate and nicotinamide metabolism
	Pantothenate and CoA biosynthesis
	Porphyrin and chlorophyll metabolism
	Ubiquinone and other terpenoid-quinone biosynthesis
Xenobiotics biodegradation and metabolism	Benzoate degradation
	Chlorocyclohexane and chlorobenzene degradation
	Fluorobenzoate degradation
	Nitrotoluene degradation

Class of metabolism pathways	Metabolic pathway name
	Polycyclic aromatic hydrocarbon degradation
	Toluene degradation
Biosynthesis of other secondary metabolites	Penicillin and cephalosporin biosynthesis
Glycan biosynthesis and metabolism	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis

Table 3.

Pathways significantly altered in ileum of wild type and TLR4$^{\Delta IEC}$ mice

Pathway Name	*p*-value
WebGestalt analysis	
DNA replication	0.0087
PPAR signaling pathway	0.0087
Fructose and mannose metabolism	0.0391
Vitamin digestion and absorption	0.0391
Pathway Express analysis	
Circadian rhythm	0.00008
DNA replication	0.00081
PPAR signaling pathway	0.00156
Base excision repair	0.02190
Ingenuity Pathway analysis	
Cell Cycle Control of Chromosomal Replication	0.00004
LPS/IL-1 Mediated Inhibition of RXR Function	0.00417
Retinol Biosynthesis	0.00912
Antigen Presentation Pathway	0.01202
Bupropion Degradation	0.02042
Estrogen Biosynthesis	0.02399
Glutathione-mediated Detoxification	0.02399
PPARα/RXRα Activation	0.02692
Acetone Degradation I (to Methylglyoxal)	0.02692
Renal Cell Carcinoma Signaling	0.03090
Adenosine Nucleotides Degradation II	0.03981
Glucocorticoid Biosynthesis	0.03981
Mineralocorticoid Biosynthesis	0.03981
Interferon Signaling	0.04266
RAR Activation	0.04571
Maturity Onset Diabetes of Young (MODY) Signaling	0.04786

Table 4.

PCR primers

Gene	Forward primer (5’ - 3’)	Reverse primer (5’ - 3’)
Rplp0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
F4/80	GCTCCTGGGTGCTGGGCATT	TCCCGTACCTGACGGTTGAGCA
Cd68	CTTAAAGAGGGCTTGGGGCA	ACTCGGGCTCTGATGTAGGT
Il6	CCAATTTCCAATGCTCTCCT	ACCACAGTGAGGAATGTCCA
Tnf	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
Cd11c	CAAAATCTCCAACCCATGCT	CACCACCAGGGTCTTCAAGT
Retnlb	CGTGGAGAATAAGGTCAAGGAAC	CACACCCAGTAGCAGTCATC
Fiaf	CTCCGTGGGGACCTTAACTG	AGAGGATAGTAGCGGCCCTT
Fatp1	GTGCCACCAACAAGAAGATTG	CTGCGGTCACGGAAATACA
Hmgcs2	CAAGCTGGAAACAACCAGCC	TCAACCGAGCCAGGGATTTC
Fabp1	GGAAGGACATCAAGGGGGTG	TCACCTTCCAGCTTGACGAC
Mcp1	ATGCAGTTAACGCCCCACTC	CCCATTCCTTCTTGGGGTCA
Mpo	GACAGTGTCAGAGATGAAGCTACT	TTGATGCTTTCTCTCCGCTCC
Elane	CAGAGGCGTGGAGGTCATTT	GAAGATCCGCTGCACAGAGA
Pparα	AAGAACCTGAGGAAGCCGTTCTGTG	GCAGCCACAAACAGGGAAATGTCA
Pparg	AAGAACCATCCGATTGAAGC	CCAACAGCTTCTCCTTCTCG
Lysozyme	AAGCTGGCTGACTGGGTGTGTTTA	CACTGCAATTGATCCCACAGGCAT
LysM	GCAAAACCCCAAGAGCTGTG	CGGTTTTGACAGTGTGCTCG