Immunomodulatory glycan LNFP III alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways

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Parasitic worms express host-like glycans to attenuate the immune response of human hosts. The therapeutic potential of this immunomodulatory mechanism in controlling the metabolic dysfunction that is associated with chronic inflammation remains unexplored. We demonstrate here that administration of lacto-N-fucopentaose III (LNFP III), a LewisX-containing immunomodulatory glycan found in human milk and on parasitic helminths, improves glucose tolerance and insulin sensitivity in diet-induced obese mice. This effect is mediated partly through increased interleukin-10 (Il-10) production by LNFP III-activated macrophages and dendritic cells, which reduces white adipose tissue inflammation and sensitizes the insulin response of adipocytes. Concurrently, LNFP III treatment upregulates nuclear receptor subfamily 1, group H, member 4 (Fxr-α, also known as Nr1h4) to suppress lipogenesis in the liver, conferring protection against hepatosteatosis. At the signaling level, the extracellular signal-regulated kinase (Erk)-activator protein 1 (Ap1) pathway seems to mediate the effects of LNFP III on both inflammatory and metabolic pathways. Our results suggest that LNFP III may provide new therapeutic approaches to treat metabolic diseases.

The metabolic syndrome is often associated with obesity and is a major medical and economic concern worldwide. Nutrient surplus is one of the main factors that contribute to the obesity pandemic, as well as to increased metabolic burdens on the mitochondria and the endoplasmic reticulum, leading to subcellular organelle dysfunction². Chronic inflammation is often observed in states of obesity and is a common outcome of these metabolic stresses and a key contributor to the pathologies that are associated with metabolic diseases, such as insulin resistance, type 2 diabetes, atherosclerosis and nonalcoholic fatty liver diseases. Although the features of chronic metabolic-related inflammation (or meta-inflammation) differ from those of acute inflammatory responses to exogenous insults, studies have shown that several pathogen-sensing mechanisms of innate immunity are negative regulators of insulin sensitivity³. For example, pattern-recognition receptors and downstream effectors (such as Toll-like receptor-4 (Tlr4), IkB kinase β (Ikk-β), Ikk-ε, tumor necrosis factor α (Tnf-α) and double-stranded RNA-dependent protein kinase) have been shown to be activated by high-fat feeding and induce metabolic diseases²,⁴. In addition, activation of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (Nlrp3) inflammasome, possibly in response to lipid metabolites (for example, fatty acids or ceramides)⁵,⁶, results in the cleavage of pro-caspase-1 (pro-Casp-1) and release of mature Il-1β, which causes insulin resistance⁵,⁶.

Resident macrophages and lymphocytes in metabolic tissues, such as white adipose tissue (WAT) and liver, are believed to have important roles in meta-inflammation⁷–¹³. For example, obesity triggers the appearance of proinflammatory macrophages in the WAT, where they are histologically presented as crown-like structures (CLS)¹⁴,¹⁵. These so-called ‘classically activated’ (or M1) macrophages are partly responsible for the low-grade, chronic inflammation that is associated with metabolic dysregulation¹. In contrast, adipose-resident macrophages from lean individuals have an ‘alternatively activated’ (or M2) phenotype¹⁶, which functions to repair damage inflicted by pro-inflammatory M1 signals¹⁷. Studies have identified several sources of T helper type 2 (T H2) cytokines (for example, Il-4 and Il-13) within WAT that mediate alternative activation¹⁰,¹³,¹⁸. Depletion of T H2 cytokine-producing cells or the downstream mediators in macrophages leads to insulin resistance¹⁰,¹³,¹⁸. In contrast, increased T H2 cytokine production, as in helminth infection, improves glucose homeostasis¹⁸. Although it remains unclear how T H2-biased, anti-inflammatory immune responses improve metabolic homeostasis, overexpression of the anti-inflammatory cytokine Il-10 has been shown to improve insulin sensitivity²⁰.

The ability to drive T H2-type and anti-inflammatory responses during helminth infection has been associated with decreased damage to host tissues, prolonging host survival²¹. During infection with Schistosoma mansoni, parasite eggs trapped in host tissues,
such as the liver, are the main stimuli for T\(_{H2}\) cytokine production in mice\(^{22}\). Injection of a saline-soluble homogenate of eggs (soluble egg antigen, SEA) is sufficient to induce T\(_{H2}\) biasing of the immune response\(^{23}\). Subsequently, it was shown that glycans and glycoproteins found in SEA, such as the Lewis\(^{2}\)-containing LNFPIII, GaINAc\(\beta_1\)-4GLcNAc (LacdiNAc), fucosylated LacdiNAc and omega-1 (a T2 RNase), are capable of mediating immunomodulatory activity\(^{24-27}\). Notably, LNFPIII is one of the major sugars found in postpartum human milk and is thought to have a similar protective immunomodulatory effect in the fetus\(^{28,29}\). These observations suggest that during coevolution with human hosts, \(S.\) \(mansioni\) parasites expressing immunomodulatory glycans were able to escape detection and had a developmental advantage. In fact, macrophages treated with LNFPIII show a T\(_{H2}\) cytokine-independent, M2-like phenotype characterized by the expression of the M2 markers arginase 1 (Arg1) and chitinase 3-like 3 (Ym1, also known as \(Cd301\)) expression, albeit to a lesser extent than IL-4, in macrophages (Fig. 1a). In contrast, LNFPIII was more effective than IL-4 in inducing IL10 expression and release into macrophage-conditioned media (Fig. 1a, b and Supplementary Fig. 1a). This effect was blunted when we treated macrophages with PD98059 to block the activity of Erk (Fig. 1b), which has been implicated in mediating LNFPIII signal transduction\(^{31,34,36}\). We also found that LNFPIII increased IL-10 production in dendritic cells (Supplementary Fig. 1b). The ability of LNFPIII to upregulate IL10 expression was independent of signal transducer and activator of transcription 6 (Stat6) and peroxisome proliferator activator receptor \(\delta\) (Ppar-\(\delta\)), which are known effectors of T\(_{H2}\) cytokines (Supplementary Fig. 1c). Taken together, LNFPIII induces an anti-inflammatory state that increases IL-10 production.

To determine whether the immunomodulatory activity of LNFPIII might be beneficial for treating metabolic diseases, we injected 8-week-old male mice (C57BL/6) with vehicle or LNFPIII (25 \(\mu\)g, twice a week) for 4–6 weeks after the onset of high-fat diet (HFD)-induced obesity and metabolic dysfunction. Consistent with the \textit{in vitro} study, LNFPIII-treated mice had higher circulating concentrations of IL-10 but the same concentrations of IL-4 compared to vehicle-treated mice (Fig. 1c). When subjected to glucose tolerance tests (GTTs), LNFPIII-treated mice showed significantly higher glucose-handling capability compared to the vehicle-treated control group (Fig. 1d; area under the curve (AUC), vehicle = 36,727.5 \(\pm\) 1,769.13 (mean \(\pm\) s.e.m.); LNFPIII = 29,244 \(\pm\) 1,119.7, \(P = 0.0159\)). LNFPIII treatment also led to higher insulin sensitivity compared to vehicle treatment, as shown by reductions in fasting serum insulin concentrations (vehicle, 1.54 \(\pm\) 0.07 ng ml\(^{-1}\); LNFPIII: 1.29 \(\pm\) 0.06 ng ml\(^{-1}\), \(P < 0.05\)), and improvements in both the results of insulin tolerance tests (ITTs) (AUC, vehicle = 123.58 \(\pm\) 4.79; LNFPIII = 98.23 \(\pm\) 3.30, \(P < 0.05\)).

**RESULTS**

**LNFPIII treatment improves insulin sensitivity**

LNFPIII has been shown to induce M2-like macrophage activation independent of T\(_{H2}\) cytokines\(^{30}\). We confirmed that LNFPIII treatment upregulated Arg1, Ym1 and macrophage galactose-type C-type lectin-1 (Mgl1, also known as \(Cd301\)) expression, albeit to a lesser extent than IL-4, in macrophages (Fig. 1a). In contrast, LNFPIII was more effective than IL-4 in inducing IL10 expression and release into macrophage-conditioned media (Fig. 1a, b and Supplementary Fig. 1a). This effect was blunted when we treated macrophages with PD98059 to block the activity of Erk (Fig. 1b), which has been implicated in mediating LNFPIII signal transduction\(^{31,34,36}\). We also found that LNFPIII increased IL-10 production in dendritic cells (Supplementary Fig. 1b). The ability of LNFPIII to upregulate IL10 expression was independent of signal transducer and activator of transcription 6 (Stat6) and peroxisome proliferator activator receptor \(\delta\) (Ppar-\(\delta\)), which are known effectors of T\(_{H2}\) cytokines (Supplementary Fig. 1c). Taken together, LNFPIII induces an anti-inflammatory state that increases IL-10 production.

**Figure 1** LNFPIII increases IL-10 production and improves insulin sensitivity. (a) Real-time quantitative PCR (qPCR) examining the expression of M2 (alternative activation) genes in bone marrow–derived macrophages treated with IL-4 (20 ng ml\(^{-1}\)) or LNFPIII (20 \(\mu\)g ml\(^{-1}\)) for 24 h. (b) IL-10 concentrations in conditioned medium from LNFPIII- or vehicle-treated macrophages determined by ELISA, PD98059 (10 \(\mu\)M) is an Erk inhibitor. (c) Serum IL-10 and IL-4 concentrations in vehicle- or LNFPIII-treated mice (\(n = 5–7\) mice per treatment). (d–e) GTT (d) andITT (e) in vehicle- and LNFPIII-treated mice. The inset in e shows the HOMA-IR. (f) Western blot analyses examining insulin-stimulated Akt phosphorylation in WAT from vehicle- and LNFPIII-treated mice (from four individual mice per treatment). pAkt, phosphorylated Akt; tAkt, total Akt. (g) The relative signal of pAkt to tAkt in WAT with or without insulin injection quantified by densitometry using the western signals in f or by ELISA-based assays. All values are expressed as means \(\pm\) s.e.m. For \textit{in vitro} assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. In vivo studies were reproduced in three mouse cohorts (\(n = 5–7\) mice per treatment). Insulin signaling was examined in two of the three cohorts. *\(P < 0.05\) (LNFPIII or IL-4 compared to vehicle control).
**Figure 2** Reduced inflammation and enhanced insulin signaling in WAT of LNFPIII-treated mice. (a) WAT histology showing CLS (left). The arrows point to CLS, and the images on the right are enlargements of the boxed areas in the images on the left. Scale bar: left, 200 μm; right, 50 μm. CLS quantified in 90 fields from 30 sections (three fields per section) for each individual mouse (right). n = 4 mice per group. The values on the y axis indicate the percentage of fields that contain a certain percentage of CLS. The values on the x axis indicate the percentage of CLS-containing adipocytes in a given field. ND, not detected. (b) Real-time qPCR analyses of M1 and M2 gene expression in WAT of vehicle- and LNFPIII-treated mice (n = 5 mice per treatment). (c) Metabolic gene expression in WAT determined by real-time qPCR. FA, fatty acid. (d) Western blotting showing C/ebp-α and Irs2 protein abundances in WAT (top) and relative C/ebp-α and Irs2 signals normalized to tubulin (bottom). All values are expressed as means ± s.e.m. Metabolic studies were reproduced in three mouse cohorts (n = 5–7 mice per treatment). CLS and expression analyses were examined in one and three of the three cohorts, respectively. *P < 0.05 (LNFPIII compared to vehicle).

**Figure 3** LNFPIII-primed macrophage conditioned medium improves insulin sensitivity in 3T3-L1 adipocytes in an II10–dependent manner. (a) Western blotting showing protein abundances of tAkt and insulin-stimulated pAkt in 3T3-L1 adipocytes treated with vehicle, LNFPIII or rll-10 (top; representative samples from three experiments are shown, each with three biological replicates). Insulin-stimulated glucose uptake determined using radioactive 2-[14C]deoxy-glucose (bottom left) and cellular triglyceride contents measured at day 6 of 3T3-L1 adipocyte differentiation (bottom right). The vehicle controls for LNFPIII (20 μg ml⁻¹) and II10 (10 ng ml⁻¹) were dextran and PBS, respectively. (b) Western blotting showing protein abundances of tAkt and insulin-stimulated pAkt in adipocytes treated with control and conditioned medium (CM) from LNFPIII-primed WT and II10–/– macrophages (top) and insulin-stimulated glucose uptake in adipocytes (bottom). (c) Gene expression in 3T3-L1 adipocytes determined by real-time qPCR. (d) ITT in vehicle- and LNFPIII-treated II10–/– mice (n = 6 mice per treatment per genotype). AUC, area under the curve of ITT (mg dl⁻¹ min). (e) Ex vivo glucose uptake assay performed in adipose tissue slices collected before and after portal vein injection of 5 U kg⁻¹ insulin. All values are expressed as means ± s.e.m. For in vitro assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. Studies in II10–/– and control mice were conducted in one cohort (n = 6 mice). *P < 0.05 (treatment compared to control).
and Il1b were downregulated by LNFPIII treatment (Fig. 2b). Consistent with the higher insulin sensitivity, the expression of insulin receptor β (Insrb), insulin receptor substrate 2 (Irs2), CCAAT/enhancer binding protein α (C/ebpa), glucose transporter 4 (Glut4, also known as Slc2a4) and lipogenic genes was higher in WAT from LNFPIII-treated mice than from vehicle-treated control mice (Fig. 2c). C/ebp-α and Irs2 protein abundances were also higher in these mice than in controls (Fig. 2d). We obtained similar results in SEA-treated cohorts of mice (Supplementary Fig. 2). Of note, SEA induced Arg1 expression in WAT to a greater extent than did LNFPIII, reflecting the fact that SEA treatment increased serum Il-4 concentrations (Supplementary Fig. 3a). We found that expression of the Lxr-α (also known as Nr1h4) and its target nuclear receptor subfamily 0, group B, member 2 (Shp, also known as Nr0b2) 38. The Fxr-α (also known as Nr1h3) 37 and the negative regulators Fxr-α3 and Fxr-α4 (or Fxr-1/2, respectively) were upregulated in the livers of LNFPIII-treated mice compared to vehicle-treated control mice (Fig. 2b). The only difference between Fxr-α1 and Fxr-α2 (or Fxr-α3 and Fxr-α4) is a four–amino-acid insertion in Fxr-α1 (or Fxr-α3). We found that expression of the Fxr-α3/α4 isoforms, as well as several known Fxr target genes 39, including Shp, organic anion-transporting polypeptides (Oatp, also known as Scl0a1), phospholipid transfer protein (Pltp) and bile salt efflux pump (Bsep, also known as Abcb11), were upregulated in the livers of LNFPIII-treated mice compared to vehicle-treated control mice (Supplementary Fig. 3c). The expression of the Lxr-α gene was the same in LNFPIII-treated and vehicle-treated mice. We obtained similar effects with SEA treatment, except that SEA upregulated both Fxr-α1/2 and Fxr-α3/α4 (Supplementary Table 1). Taken together, the results suggest that Il10 is required for LNFPIII-mediated improvement in WAT insulin signaling and systemic glucose homeostasis.

LNFPIII protects against diet-induced hepatic steatosis

In addition to improving WAT function, histological and triglyceride content analyses showed a strong protective effect of treatment with LNFPIII against HFD-induced hepatic lipid accumulation compared to treatment with vehicle (Fig. 4a). Furthermore, overall liver function, determined by circulating concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), was substantially better in LNFPIII-treated mice than in vehicle-treated control mice (Fig. 4b). In line with lower hepatic steatosis, the expression of lipogenic genes, including fatty acid synthase (Fas), acetyl-CoA carboxylases 1 and 2 (Acc1 and Acc2, also known as Acaca and Acacb), steraryl-CoA desaturase 1 (Scd1) and sterol regulatory element-binding protein 1c (Srebp1c, also known as Srebf1), were lower in LNFPIII-treated livers than in vehicle-treated livers (Fig. 4c).

Srebp-1c is a master lipogenic transcription factor whose expression and transcriptional activity are controlled by a network of nuclear receptor signaling pathways, notably, the positive regulator nuclear receptor subfamily 1, group H, member 3 (Lxr-α, also known as Nr1h3) 37 and the negative regulators Fxr-α (also known as Nr1h4) and its target nuclear receptor subfamily 0, group B, member 2 (Shp, also known as Nr0b2) 38. The Fxr-α gene consists of two major 5′ regulatory regions, with the upstream and downstream promoters driving the expression of two isoforms of the gene, referred to here as Fxr-α1 and Fxr-α2, and Fxr-α3 and Fxr-α4, respectively 39. The only difference between Fxr-α1 and Fxr-α2 (or Fxr-α3 and Fxr-α4) is a four–amino-acid insertion in Fxr-α1 (or Fxr-α3). We found that expression of the Fxr-α3/α4 isoforms, as well as several known Fxr target genes 39, including Shp, organic anion-transporting polypeptides (Oatp, also known as Scl0a1), phospholipid transfer protein (Pltp) and bile salt efflux pump (Bsep, also known as Abcb11), were upregulated in the livers of LNFPIII-treated mice compared to vehicle-treated control mice (Fig. 4c). The expression of the Lxr-α gene was the same in LNFPIII-treated and vehicle-treated mice. We obtained similar effects with SEA treatment, except that SEA upregulated both Fxr-α1/2 and Fxr-α3/α4 (Supplementary Table 1). Taken together, the results suggest that Il10 is required for LNFPIII-mediated improvement in WAT insulin signaling and systemic glucose homeostasis.

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ectopic fat accumulation in the liver and regulates the expression of transcriptional factors that are crucial for de novo lipogenesis.

To assess the role of LNFPIII or LNFPIII-primed macrophages in hepatic lipid homeostasis, we conducted lipogenic assays in isolated primary hepatocytes. LNFPIII, but not conditioned medium from LNFPIII-treated macrophages, suppressed lipogenesis and caused higher fatty acid β-oxidation compared to vehicle treatment (Fig. 5a). LNFPIII also induced expression of Fxr-α3/α4 and Shp and suppressed the expression of Srebplc and Actl (Fig. 5b). As LNFPIII did not affect the expression of β-oxidation genes (Figs. 4c and 5b), the increased fat burning resulting from LNFPIII treatment was probably secondary to the decreased fatty acid synthesis. These results suggest that, unlike in WAT, the effect of LNFPIII in the liver was not mediated by II-10. Consistent with this notion, II-10 treatment did not affect hepatic de novo lipogenesis in vitro or in vivo (Supplementary Fig. 5ac), and the protective effect of LNFPIII was preserved in II10−/− liver (Supplementary Fig. 5d.e).

**LNFPIII suppresses hepatic de novo lipogenesis through Fxr**

The upregulation of the Fxr-α-dependent transcription program by LNFPIII suggested that LNFPIII may induce Fxr-α to inhibit fat synthesis. In fact, in hepatocytes with ablation of the Fxr-α gene

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**Figure 5** LNFPIII suppresses lipid synthesis through Fxr-α. (a) De novo lipogenesis (left) and fatty acid β-oxidation (right) assays in primary hepatocytes treated with vehicle, LNFPIII (20 μg ml−1) or conditioned medium (CM) from LNFPIII-primed WT macrophages. (b) Gene expression in hepatocytes treated with vehicle or LNFPIII determined by real-time qPCR. (c) Lipogenic (left) and β-oxidation (right) assays in hepatocytes isolated from WT or Nr1h4−/− mice treated with or without LNFPIII and with or without PD98059. PD + LNFPIII, treated with both PD98059 and LNFPIII. (d,e) Hepatic triglyceride content and serum AST and ALT concentrations in WT and Nr1h4−/− mice with or without LNFPIII treatment (n = 6 mice per treatment). All values are expressed as means ± s.e.m. For in vitro assays, the mean and s.e.m. were determined from 3–4 biological replicates from one of three repeats. Hepatic pErk was determined from one of three metabolic study cohorts (n = 5–7 mice per cohort, showing three representative samples). *P < 0.05 (LNFPIII compared to vehicle control).

**Figure 6** Induction of Fxr-α activity by LNFPIII is mediated by Erk-Ap1 signaling. (a) Genomic structure showing alternative promoter usage by human Fxr-α3/α4 (promoter 1) and Fxr-α3/α4 (promoter 2) (top). Relative activities (RLU) of luciferase reporters driven by human Fxr-α promoter 1 (~2 kb) or human Fxr-α promoter 2 (~0.13 kb) in HepG2 cells with or without LNFPIII (20 μg ml−1) or SEA (2 μg ml−1) treatment (bottom left) and western blotting showing Fxr-α protein abundances in livers from vehicle- or LNFPIII-treated mice (bottom right). (b) Diagram showing Fxr-α promoter 2 (p2–0.13kb) or mutant (p2–0.13kb–M) reporter constructs (top). The two overlapping AP1 binding sites and the mutation are shown. Relative luciferase activities of p2–0.13kb and p2–0.13kb–M with or without LNFPIII and with or without PD98059 treatment (bottom). (c) Erk phosphorylation (pErk) in livers of vehicle- and LNFPIII-treated mice (top) and quantification of the western signal (bottom). (d) Western blot analyses showing Erk phosphorylation in hepatocytes with or without LNFPIII (L) and with or without PD98059 (PD) treatment (top; representative samples from three experiments, each with three biological replicates) and quantification of the western signal (bottom). All values are expressed as means ± s.e.m. For in vitro assays, the mean and s.e.m. were determined from 3–4 biological replicates from one of three repeats. Hepatic pErk was determined from one of three metabolic study cohorts (n = 5–7 mice per cohort, showing three representative samples). *P < 0.05 (LNFPIII or SEA compared to vehicle control). (e) Model for direct and indirect regulation of metabolic pathways by LNFPIII. DC, dendritic cells; FA, fatty acid.
or inhibition of Erk by PD98059, LNFPIII was unable to inhibit de novo lipogenesis and increase fat oxidation (Fig. 5c). Furthermore, the ability of LNFPII to reduce hepatic triglyceride accumulation, improve liver function and suppress lipogenic gene expression was lost in Nrl1h4−/− mice (Fig. 5d,e and Supplementary Fig. 5f), whereas the insulin-sensitizing activity of LNFPIII was unaffected (Supplementary Fig. 5g–i and Supplementary Table 1). Together, these results suggest that LNFPIII inhibits hepatic lipogenesis in a Fxr–α-dependent manner.

We next sought to determine whether Fxr–α is a molecular target of LNFPIII. As mentioned above, both the human and mouse Fxr–α genes contain two major promoters (Fig. 6a). The 5′ proximal regulatory sequences are highly conserved between the mouse, rat and human Fxr–α genes (Supplementary Fig. 6a). We examined the activities of reporters driven by regulatory regions of promoter 1 or promoter 2 in the Fxr–α gene in HepG2 cells (human hepatoma cells). In accordance with the regulation of the Fxr–α gene in the liver or isolated hepatocytes, LNFPIII activated human promoter 2, whereas SEA induced the activity of both promoters (Fig. 6a). In vivo, Fxr–α protein abundance was higher in liver lysates from LNFPIII-treated compared to vehicle-treated mice (Fig. 6a). Through serial deletion of promoter 2, we defined a minimal LNFPIII-responsive region (approximately 130 bp upstream of the transcriptional start site) that contained consensus binding sites for C/EBP and AP1 (Supplementary Fig. 6a). Site-directed mutagenesis experiments further identified two overlapping AP1 sites (located between −57 bp and −47 bp) that were required for the induction of promoter 2 by LNFPIII (Fig. 6b and Supplementary Fig. 6b). Similarly, inhibition of Erk activation, which is upstream of AP1, by PD98059 abolished the LNFPIII-induced activity of promoter 2. In concert, LNFPIII treatment increased the amounts of phosphorylated Erk and total Erk in the liver (Fig. 6c) and hepatocytes (Fig. 6d). We obtained similar results in SEA-treated liver cells (Supplementary Fig. 6c,d). Collectively, these findings suggest that LNFPIII regulates hepatic lipogenesis through the Erk–Ap1–Fxr–α axis.

DISCUSSION

In this study, we demonstrate that LNFPIII (and SEA) alleviates the pathologies that are associated with HFD-induced obesity. LNFPIII treatment shifts the immune profile to an anti-inflammatory state and decreases macrophage infiltration in metabolic tissues of mice that have already become obese. This effect is, in part, driven by IL-10, which inhibits inflammation and enhances the insulin response in WAT. In the liver, LNFPIII upregulates the bile acid sensing nuclear receptor Fxr–α and its downstream transcriptional targets. Activated Fxr–α signaling suppresses lipogenesis and protects against hepatic steatosis. Taken together, our results demonstrate a therapeutic potential for LNFPIII in treating components of metabolic syndrome through its ability to directly modulate both immune and metabolic pathways.

The hygiene hypothesis attributes the increased incidence of autoimmune diseases and allergic responses in developed countries to reduced human contact with pathogens46. A special emphasis has been on the interaction between parasitic worms and humans40,41; in addition to the Th2-biasing immune phenotype, helminth infections induce the proliferation of regulatory T cells and production of the anti-inflammatory cytokine IL-10 (ref. 42). Our data show that LNFPIII treatment is sufficient to increase IL-10 production in macrophages and dendritic cells. Regulatory T cells are probably another major source of IL-10 (ref. 42). These cells are enriched in fat tissues from lean individuals and have a role in maintaining WAT function9. Anti-inflammatory pathways are thought to improve metabolic homeostasis primarily through attenuating the action of proinflammatory signaling. Overexpression of IL-10 in the muscle was previously shown to improve systemic insulin resistance through suppression of inflammation20. We found that conditioned medium from LNFPIII-treated WT macrophages directly enhances insulin responses in adipocytes by upregulation of InsR and Irs2 and increases insulin-mediated glucose uptake and lipogenesis in an IL-10–dependent manner. Acute rIL-10 injection in HFD-fed mice also improved glucose homeostasis but does not affect WAT macrophage infiltration (data not shown), suggesting that the insulin-sensitizing effect of IL-10 can be separated from its anti-inflammatory activity.

Several studies have identified IL-10 polymorphisms that are associated with insulin resistance and type 2 diabetes in humans43,44. Of note, a previous study has demonstrated that deficiency in hematopoietic cell–derived IL-10 in mice through transplantation of Il10−/− bone marrow does not affect HFD-induced tissue inflammation and insulin resistance45. However, IL10 mRNA and protein abundances were several fold higher in WAT and liver in these mice than in mice that received WT bone marrow. It is unclear whether the compensatory increase in IL-10 production is from nonhematopoietic cells or residual WT bone marrow–derived cells. The result seems to suggest that increased IL-10 concentrations within metabolic tissues are sufficient to maintain metabolic homeostasis.

Although adipose tissue is known to produce adipokines, such as leptin, adiponectin and resistin, that modulate systemic glucose and lipid metabolism, this tissue, unlike the muscle, is not a major tissue for glucose uptake under physiological conditions. LNFPIII treatment did not affect the expression of these adipokines in WAT (Supplementary Table 1 and data not shown). How does increased insulin sensitivity in WAT after LNFPIII treatment lead to improvement in systemic glucose homeostasis? It has been shown that GTT and ITT values in musclespecific insulin receptor knockout (MIRKO) mice are indistinguishable from those in control mice, even though insulin-stimulated glucose uptake is substantially lower in the muscle of the knockout mice46. Subsequent studies attribute the normoglycemic phenotype of MIRKO mice to a threefold higher glucose deposition to the WAT compared to control mice47, suggesting that, at least in mice, increased glucose uptake by adipocytes is able to sustain glucose homeostasis. Consistent with this notion, overexpression of the glucose transporter Glut4 in adipose tissues improves glucose tolerance48. Recently, increased glucose flux in adipocytes has been shown to upregulate fatty acid synthesis through a new isoform of carbohydrate-responsive element-binding protein (Chrebp-β, also known as Mxipl)49. In addition, the expression of adipose Mlxipl and lipogenic genes positively correlates with insulin sensitivity in humans49. Our data demonstrate that LNFPIII treatment increases WAT glucose uptake and lipogenic gene expression, and Mxipl was also induced (data not shown).

Although the mechanism through which fat synthesis in adipose tissues contributes to whole-body homeostasis is not completely understood, a lipogenic product of adipocytes has been linked to improved systemic metabolism50. The beneficial effect of LNFPIII could also be mediated by central regulation, in which reduced inflammation may lead to the improved central insulin sensitivity that is known to regulate hepatic glucose production51. However, LNFPIII does not seem to affect glucoseogenesis, as determined by pyruvate tolerance tests and the expression of gluconeogenic genes in the liver (data not shown).

After entering the body, S. mansoni settles in the hepatic portal system, where male and female worms mate and produce eggs22.
Egg deposition in the liver is crucial for the induction of a systemic T<sub>2</sub> response<sup>22</sup>. It is therefore not surprising that the liver is also a target of LNPPIII. At first glance, it seems unexpected that LNPPIII directly controls Fxr-α signaling to regulate hepatic lipid metabolism. Fxr-α senses endogenous bile acids and controls bile acid homeostasis by inhibiting production while increasing the recycling of bile acids in the enterohepatic system<sup>52</sup>. Fxr-α activation also suppresses lipogenesis through Shp-mediated inhibition of Srebp-1c in the liver<sup>38</sup>. This is not unexpected, as SEA is known to contain multi-

Mgl1 and mannose receptor. In addition, Erk is proposed to be C-type lectin receptors, including DC-SIGN (also known as Cd209), α<sub>v</sub> the Fxr-αtors, including DC-SIGN (data not shown), and LNFPIII is able to We find that primary hepatocytes also express C-type lectin recep-

tors, including DC-SIGN (also known as Cd209), Mgl1 and mannose receptor. In addition, Erk is proposed to be C-type lectin receptors, including DC-SIGN (data not shown), and LNFPIII is able to activate Erk in the liver, which mediates transcriptional regulation of the Fxr-α gene and the subsequent suppression of lipogenesis. The gene products of the two promoters (Fxr-α1 and Fxr-α2 compared to Fxr-α3 and Fxr-α4) differ by several amino acids located at the N terminus. Functional differences between the isoforms have not been reported<sup>59</sup>. Our data suggests that the induction of Fxr-α3 and Fxr-α4 by LNPPIII is sufficient to suppress lipogenesis. Whereas LNPPIII only activates the downstream promoter, SEA induces the activities of both promoters. This is not unexpected, as SEA is known to contain multiple bioactive glycans that could signal through different pathways<sup>56</sup>. Together, our data show that helminths may utilize the Erk-Ap1 signaling pathway to modulate host metabolism and immune responses (Fig. 6e). Our study suggests that the M2-like, anti-inflammatory activity of LNPPIII, which is probably a product of the survival strat-

gy of helminths, may be used to treat metabolic disorders such as insulin resistance and nonalcoholic fatty liver diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.B. was involved in experimental design and execution, data analyses and manuscript preparation. K.I.S., M.R.G. and C.L. conducted GTTs and ITTs with P.B. D.J. and L.D. provided technical assistance. S.L. assisted in hepatocyte isolations. C.L. provided LNPPIII and SEA preparations. D.A.H. helped with experimental design, data interpretation and manuscript writing. C.-H.L. directed the project, participated in data analyses and interpretation and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal experiments. We placed male C57BL/6 mice at 8–10 weeks of age (obtained from The Jackson Laboratory) on a high-fat, high-carbohydrate diet (F3282, Bio-Serv) for the duration of the experiments. Six weeks after high-fat feeding, we injected mice (intrapitoneally, i.p.) twice per week with 25 µg of dextran (40 kDa; vehicle) or LFNPPII conjugated to dextran (∼8–10 LFNPPII per dextran or 0.168–0.210 µg LFNPPII per µg of dextran). Experiments were reproduced in three independent mouse cohorts (n = 5–7 mice per treatment). We conducted similar metabolic experiments in three additional cohorts (n = 4–6 male mice per treatment), which were given 0.9% NaCl (vehicle) or SEA dissolved in 0.9% NaCl (25 µg, twice a week). LFNPPII and SEA were prepared as previously described10,57. Metabolic studies started 4 weeks after LNFPIII or SEA treatment and were conducted after 6 h of fasting. We euthanized the mice after six weeks of treatment for serum and tissue collection. For rIl-10 experiments, mice were treated with PBS (vehicle) or rll-10 (Peprotech; 1 µg i.p. every other day for a total of three doses) after 4 weeks of high-fat diet. Body weight and food intake were monitored weekly. We performed GTTs by injecting 1 g glucose per kg body weight into the mouse peritoneum and measuring blood glucose concentrations before and after injection at the time points indicated using a OneTouch glucose monitoring system (LifeScan). ITTs were conducted similarly by injecting 1 U per kg body weight of insulin. In vivo insulin signaling was determined by injecting 3 U per kg body weight of insulin through the portal vein. We collected pieces of liver, epididymal fat and muscle before and 10 min after insulin injection and rapidly stored the tissues in liquid nitrogen. Additional adipose tissue slices were immediately incubated with 2-µCi [3H]-deoxy-d-glucose to determine insulin-stimulated glucose uptake ex vivo. To estimate crown-like structures, we divided one piece of epidymal fat pad into three sections and embedded them in a single paraffin block. We collected sequential sagittal sections every 20 µm for H&E staining. HOMA-IR was calculated as previously described58. Serum and tissue lipids, ALT and AST were measured using commercial kits as described previously59. We determined the concentrations of pAkt, Akt, insulin, IL-10 and IL-4 using ELISA-based plates from Meso Scale Discovery and Peprotech. The Dana-Farber/Harvard Cancer Center Research Pathology Core provided all histological services and preliminary assessments by a pathologist. We obtained 1101/− and Nr1h4/−/− mice (male, n = 6 per genotype per treatment, C57BL/6J background) from the Jackson Laboratory. Metabolic studies and treatments in these mice were similar to those in WT C57BL/6 mice. The Harvard Medical Area Standing Committee on Animals approved all the animal protocols.

Immunoblotting experiments. We performed western blot analyses using antibodies to detect the following proteins: pAkt (Cell Signaling, 9271, 1:1,000); tAkt (Cell Signaling, 9272, 1:1,000); Fxr-α (Santa Cruz, sc-61, 1:500); actin (Cell Signaling, 4970, 13E5, 1:1,000); tubulin (Cell Signaling, 2128, 9F3, 1:1,000); pErk (Cell Signaling, 9101, 1:1,000); and eIF2α (Cell Signaling, 4965, 1:1,000).

Primary cells, adipocyte differentiation and functional assays. We differentiated bone marrow–derived macrophages in L929 conditioned medium as previously described10. Dendritic cells were differentiated in the presence of macrophage colony-stimulating factor (3 ng ml−1) and IL-4 (5 ng ml−1). For the conditioned medium, we treated cells with dextran (control) or LFNPPII (20 µg ml−1) overnight. After treatment, cells were washed and cultured in DMEM alone. We collected media 8 h later and added 10% FBS, which constituted the conditioned medium. For M2 skewing experiments, we treated macrophages with dextran, LFNPPII (20 µg ml−1) or IL-4 (20 ng ml−1) overnight. Where indicated, cells were pretreated for 1 h with PD98059 (10 µM; Cell Signaling) before treatments. PD98059 is a MEK1 (also called mitogen-activated protein kinase 1 (MAPKK) or Erk kinase) inhibitor used to block Erk activation. We differentiated 3T3-L1 cells in a cocktail containing insulin, isobutylmethylxanthine and dexamethasone. For differentiation experiments, 3T3-L1 cells were given the various treatments indicated together with the differentiation cocktail. At day 2, the cocktail was removed, and differentiation continued in the presence of the treatment and insulin. We performed triglyceride analysis after 6 d of differentiation. Cellular lipids were extracted with a 2:1 (v/v) chloroform:methanol solution. For insulin signaling experiments, we treated fully differentiated 3T3-L1 adipocytes (at day 6) with the indicated treatments for 48 h without insulin. Cells were washed, serum starved for 2 h and stimulated with insulin (100 nM) for 60 min. We conducted glucose uptake using 2-[3H]deoxy-d-glucose with a 20-min insulin pre-stimulation. Cellular radioactivity was determined and normalized to protein content. Primary hepatocytes were isolated as previously described59,60. Hepatocytes were allowed to attach overnight in William’s E medium and 5% FBS, followed by treatments for 24 h. For de novo lipogenesis, we labeled cells with 14C-acetate and extracted lipids with a 2:1 (v/v) chloroform:methanol mixture 6 h later. β-oxidation was conducted as described previously60. For LFNPPII signaling, attached hepatocytes were pretreated with Erk inhibitor where indicated for 1 h, followed by a 30-min incubation with dextran or LFNPPII.

Expression analyses. For gene expression analyses, we determined relative expression levels using SYBR Green–based real-time qPCR reactions. We used 36B4 expression levels for normalization. For western blot analyses, we prepared tissue and cells lysates in the presence of protease and phosphatase inhibitors. The four Nr1h4 (Fxr-α) isoforms were based on previous reports, which were originally designated as Fxr-α1, Fxr-α2, Fxr-β1 and Fxr-β2. The latter two were renamed as Fxr-α3 and Fxr-α4 to avoid confusion with the rodent Nr1h5 (Fxr-β)39. For reporter assays, we cloned the upstream promoter 1 and downstream promoter 2 regions of the human Nr1h4 (Fxr-α) gene into the pGL3-basic luciferase reporter vector and conducted transient transfection in HepG2 cells in a 96-well format. We used a β-galactosidase reporter construct for normalization.

Statistical analyses. Statistical analyses comparing two parameters (between treatments or genotypes) in the cell-based work were conducted using a two-tailed Student’s t test. Two-parameter analyses for samples from the in vivo studies (non-Gaussian distribution) were determined using the Mann-Whitney test (Figs. 1c, g, 2, 3e, 4, 5a, 6c, and 6d; Supplementary Figs. 1g, 2b, 3a, 3b, f, h, 4a–d and 5d, e and Supplementary Table 1). Statistics for multiparameter analyses were determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests (Figs. 1a, 3a–c, 5a, and 6a, d, e and Supplementary Figs. 1a, 5a and 6d). Two-way ANOVA was used to determine statistical significance for GTTs and ITTs (Figs. 1d, e and 3d and Supplementary Figs. 1f, 3c–e and 5f). Values are presented as the mean ± s.e.m. For the in vitro assays, the mean and s.e.m. were determined from 3–4 biological replicates for one representative experiment. Experiments were repeated at least three times. P < 0.05 was considered significant.

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