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Immune modulation by Lacto-N-fucopentaose III in experimental autoimmune encephalomyelitis

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Abstract

Parasitic infections frequently lead to immune deviation or suppression. However, the application of specific parasitic molecules in regulating autoimmune responses remain to be explored. Here we report on the immune modulatory function of Lacto-N-fucopentaose III (LNFPIII), a schistosome glycan, in an animal model for multiple sclerosis. We found that LNFPIII treatment significantly reduced the severity of experimental autoimmune encephalomyelitis (EAE) and CNS inflammation, and skewed peripheral immune response to a Th2 dominant profile. Inflammatory monocytes (IMCs) purified from LNFPIII-treated mice had increased expression of nitric oxide synthase 2, and mediated T cell suppression. LNFPIII treatment also significantly increased expression of arginase-1, aldehyde dehydrogenase 1, indoleamine 2,3-dioxygenase and heme oxygenase 1 in splenic IMCs. Furthermore, LNFPIII treatment significantly reduced trafficking of dendritic cells across brain endothelium *in vitro*. In summary, our study demonstrates that LNFPIII glycan treatment suppresses EAE by modulating both innate and T cell immune response.

Keywords

lacto-N-fucopentaose; experimental autoimmune encephalomyelitis; autoimmune; immune modulation

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1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is induced by immunizing animals with myelin antigens in strong adjuvant, and it serves as a model for multiple sclerosis (MS) (1). EAE development is mediated by antigen-specific Th1 and Th17 cells, although the function of innate immune cells in this model is critical in both the promotion and regulation of immune response and inflammation (2, 3). In recent years, myeloid dendritic cells (DCs) in the CNS were shown to present endogenous antigens to infiltrating T cells, and promote Th17 response as well as epitope spreading in the CNS (4–6). In contrast, transfer of DCs modified by cytokines or expressing negative costimulatory molecules suppressed EAE disease. In addition, CD11b⁺Ly-6C^{hi} inflammatory monocytes (IMCs) are greatly increased in the bone marrow, blood and spleen after immunization, and accumulate in the central nervous system (CNS) during clinical disease (7–9). In the early stage of EAE, CNS infiltrating IMCs may differentiate into inflammatory dendritic cells, and promote autoimmune T cell function (10). On the other hand, resting IMCs may respond to signals from activated T cells, upregulate the expression of nitric oxide synthase 2 (NOS2), produce a high level of nitric oxide (NO), resulting in T cell apoptosis. At EAE peak, CNS CD11b⁺Ly-6C^{hi} cells have highly activated phenotype, and suppress T cell proliferation and Th1/Th17 differentiation in vitro, suggesting that T cell regulation by IMCs is dependent on their activation state (10).

Several reports have demonstrated the efficacy of schistosome infection or injection of schistosome antigens in modulating EAE (11–13). Intraperitoneal and subcutaneous injection of *S. mansoni* ova reduced EAE severity and CNS inflammation (12). This was associated with reduced IFN- γ and increased IL-4, TGF- β , and IL-10 in the periphery and CNS. EAE was also reduced in mice with pre-established *S. mansoni* infection (11). Furthermore, injection of soluble egg antigen (SEA) from *S. japonicum* before EAE induction and in the preclinical phase also reduced EAE severity, which was associated with reduced IFN- γ but increased IL-4 production in the spleen and CNS (13). So far, no specific molecules purified from schistosome have been reported to modulate EAE disease.

Lacto-N-fucopentaose III (LNFPIII) was identified using monoclonal antibodies reactive to *S. mansoni* SEA. LNFPIII contains the Lewis^X trisaccharide found on schistosome eggs, the surface of the organism and in parasite secretions (14, 15). LNFPIII/Lewis^X play a key role in inducing Th2 immune response by schistosome egg molecules, since altering the conformation of glycans by sodium metaperiodate abrogates Th2 cytokine induction (16). In addition, LNFPIII treatment induces the expression of arginase I and Ym-1, markers for alternatively activated macrophages (17). Signaling of LNFPIII in macrophages may be mediated by TLR4 (18), or c-type lectins such as DC-SIGN, mannose receptor and macrophage galactose lection-1 (19). Reduced JNK and p38 signaling as well as lack of sustained NF- κ B translocation into cell nuclei may contribute to functional changes in DCs and macrophages (18, 20). In fsn/fsn ‘flaky skin’ mice that spontaneously develop psoriatic lesions, LNFPIII treatment significantly reduced disease severity, as well as IFN- γ production and infiltration of macrophages and T cells in the lesions (21). Interestingly, LNFPIII is present in human milk, and has very low cytotoxicity (22).

In this study, we show that LNFPIII treatment significantly reduced EAE severity and CNS inflammation. Mechanistically, LNFPIII treatment markedly upregulated IL-10 and Th2 cytokine production, enhanced the expression of immune regulatory enzymes in IMCs, and reduced the migratory capacity of DCs across brain endothelium. These findings suggest a therapeutic potential for LNFPIII glycan in autoimmune diseases.

2. Materials and Methods

2.1. Animals and reagents

Female C57BL/6 mice were obtained from The Jackson Laboratory. MOG TCR transgenic 2D2 mice were originally provided by Dr. Vijay Kuchroo. All animals were housed according to local and National Institutes of Health guidelines, and used at 6–8 weeks of age. NOS2 inhibitor N6-(1-iminoethyl)-L-lysine (L-NIL) was obtained from Cayman Chemical. Lipopolysaccharides (*E. coli*) 055:B5 (LPS) was obtained from Sigma-Aldrich. Recombinant cytokines were obtained from R&D.

2.2. LNFPIII conjugates

LNFPIII was synthesized by Neose Technologies (Horsham, PA) and conjugated to a 40 kDa dextran (Sigma) or to bovine serum albumin. The dextran conjugates were prepared by Dr. Thomas Norberg. The average rate of substitution was 8–10 LNFPIII per dextran molecule. The BSA conjugates were prepared by Drs. Castegner and Seeberger, and on average had a substitution rate of 12–14 LNFPIII per BSA molecule. For carrier controls, 40 kDa Dextran, or BSA were used respectively.

2.3. EAE induction

Animals were subcutaneously immunized with 200 μ l of emulsion made of 75 μ g of MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK, New England Peptide) and complete Freund's adjuvant (CFA). Each animal also received 200 ng of pertussis toxin (PT) on day 0 and 2 post-immunization via intravenous injections. The EAE clinical score was determined as follows: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, complete hind limb and partial front limb paralysis; 5, moribund or dead animals.

2.4. Histology

Animals were sacrificed and perfused with PBS. The entire spinal cord was cut into nine segments, and twenty-micron frozen spinal cord cross-sections were prepared for all segments on a cryostat. Both H&E and immunohistochemistry (IHC) staining were performed. For IHC staining, the sections were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum and 1% bovine serum albumin, and then incubated with biotin-conjugated primary antibodies at 4°C overnight. After blocking endogenous peroxidase activity, the sections were incubated with avidin-biotin-peroxidase complex (Vector), and then visualized with DAB peroxidase substrate kit (Vector). The sections were counter-stained in Gill's hematoxylin (Sigma). For quantitation, inflammatory foci were identified as areas with an aggregation of 20 or more cells in H&E stained sections (23). The numbers of inflammatory foci were counted from nine levels of spinal cord sections, and immunostaining positive cells were counted from both sides of ventromedial areas of nine level spinal cord sections under 400x magnification. The average of inflammatory foci for a single-level tissue section and positively stained cells for a single view field were calculated. Images were acquired on a light microscope (Axioskop 2; Carl Zeiss, Inc.).

2.5. Splenocyte proliferation and cytokine assays

On day 10 and day 20 post-immunization, splenocytes were purified from individual dextran or LNFPIII treated mice, and cultured at 4×10^5 cells/well in triplicates in a 96 well plate. MOG35–55 was added at 0, 1, 5, and 25 μ g/ml. DMEM medium containing 10% fetal bovine serum (FBS), glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acid, and antibiotics (BioWhittaker) was used for culture. After 48 h, culture supernatants were collected, then 1 μ Ci [3 H]-thymidine was added into each well. Cells were harvested

16 h later for the proliferation assay. Cytokine concentrations in the culture supernatants were examined with the Milliplex cytokine/chemokine immunoassay kit (Millipore).

2.6. Nitrate/nitrite assay

This assay was performed using the nitrate/nitrite colorimetric assay kit from Cayman Chemical Company. After converting nitrate in the culture supernatant to nitrite with nitrate reductase, Griess Reagents were added to convert nitrite into a deep purple compound. The absorbance at 550 nm was read using a microplate reader (Bio-Rad). The concentration of nitrate/nitrite was determined by comparison with the standard curve.

2.7. Inflammatory monocyte and dendritic cell isolation

On day 9 post-immunization, CD11b⁺ cells were purified from splenocytes of dextran- or LNFPIII-treated mice, using CD11b microbeads (Miltenyi). CD11b⁺Ly-6C^{hi}Ly-6G⁻ IMCs were purified by FACS sorting after staining with anti-Ly-6C-FITC (clone AL-21) and anti-Ly-6G-PE (clone 1A8) Abs. For *in vitro* treatment experiments, IMCs were purified from B6 mice on day 9 after CFA/PT immunization.

To generate dendritic cells (DCs), bone marrow obtained from femurs and tibias was washed and plated in 24-well plates in RPMI 1640 supplemented with 5% FBS, antibiotics, and 20ng/mL GM-CSF. GM-CSF was titrated from the supernatants of the GM-CSF secreting X63 cell line (gift from Dr. A. Erdei, Eotvos, University of Budapest, Hungary). On day 7, the nonadherent and loosely adherent cells were removed and replaced in the absence of GM-CSF. Following overnight incubation, non-adherent cells were collected and used in migration assays and analyzed by flow cytometry.

2.8. Flow cytometry

For surface staining, isolated cells were blocked with 10 µg/ml Mouse Fc Block (BD Biosciences) for 5 min at 4°C, and then labeled with various fluorochrome-conjugated antibodies and 7-aminoactinomycin D (7-AAD), including proper isotype controls, for 15 min at 4°C. Anti-CD11c (N418), anti-CD40 (HM40-3), anti-CD80 (16-01A1), and anti-CD86 (GL1) Abs were purchased from BD. After washing, cells were analyzed on the FACS Calibur (BD Biosciences). Data analysis was performed by gating on 7-AAD⁻ alive cells.

2.9. Brain endothelium and DC transendothelial migration

Mouse brain capillary endothelial cells (BCEC) were isolated as described (24). Briefly, cerebra of 4- to 6-weeks old mice were mechanically homogenized and microvessels were isolated by two rounds of collagenase digestion and density centrifugation. Capillary fragments were directly seeded onto collagen type IV/fibronectin (10µg/mL, Sigma) Falcon inserts (3-µm pore, Fischer Scientific) in DMEM medium supplemented with 10% FBS. Endothelial cells were cultured until they reached confluence and then cultured for another two days in serum-free medium supplemented with hydrocortisone (550nM, Sigma) to maintain strong blood-brain barrier permeability function.

DC migration across *in vitro* BCEC monolayers was analyzed using the QCM 24-well invasion assay (Chemicon Int.). A total of 2.5×10^5 DC were added in serum-free medium to the top of filter insert and allowed to migrate over 24 h. Different concentrations of LNFPIII or dextran as a control were added to the basolateral side of the filter inserts. DCs were removed by Cell Detachment Solution from the lower chambers and incubated with CyQuant GR nucleic acid dye. Fluorescent intensity was proportional to the number of migrated DCs and was measured at 480/520nm. Migration index was calculated as a percentage of migrated DCs from the whole population. Experiments were repeated three

times using three separate migration chambers and data are presented as the geometric mean $\pm$ SEM.

2.10. Real-time (TaqMan) PCR

Total RNA was extracted using Absolutely RNA Miniprep Kit (Agilent), and complementary DNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *Nos2*, Arginase-1 (*Arg1*), indoleamine 2,3-dioxygenase 1 (*Ido1*), heme oxygenase 1 (*Hmox1*), and β -actin mRNA expression were examined using TaqMan gene expression assays. The expression of Aldehyde dehydrogenase 1, subfamily A2 (*Aldh1a2*) mRNA was examined by QuantiFast probe assay (Qiagen). Triplicate samples were examined in each condition. A comparative threshold cycle (C_T) value was normalized for each sample using formula: $\Delta C_T = C_{T(\text{gene of interest})} - C_{T(\beta\text{-actin})}$, and the relative expression was then calculated using the formula $2^{-\Delta C_T}$.

2.11 Data analysis

The data in the text represent the mean $\pm$ SEM, and the error bars in figures also represent SEM. Unpaired two-tailed *t* tests and Mann-Whitney U tests were used to analyze the statistical significance between two groups. One-way ANOVA analysis followed by Bonferroni's post hoc test was used to analyze the statistical significance among multiple groups. EAE disease data were also analyzed by linear regression. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LNFPIII treatment suppresses EAE disease and CNS inflammation

C57BL/6 mice were immunized with MOG35–55, and treated with LNFPIII conjugates or control dextran twice a week, starting from the day of immunization. We found that LNFPIII treatment significantly reduced the severity of EAE (Fig. 1A, $p < 0.0001$ by linear regression analysis, (25)). The mean maximal disease score was 3.5 ± 0.4 in control treated group, and 2.3 ± 0.2 in the LNFPIII conjugate treated group ($p = 0.016$ by Mann-Whitney U test), although disease onset was not significantly different. On day 20 post-immunization, we collected spinal cord tissues from dextran- or LNFPIII-treated mice, for histology (Fig. 1B & C). H&E staining showed significantly less inflammatory foci and infiltrating inflammatory cells in LNFPIII-treated animal tissues. Immunohistochemistry showed significantly reduced CNS infiltration of $CD4^+$, $F4/80^+$ and $CD11c^+$ cells.

3.2. LNFPIII treatment preferentially increases Th2 cytokine production

We examined cytokine production in a splenocyte recall assay performed on day 20 post-immunization. We found that splenocytes from the LNFPIII-treated group had increased production of IFN- γ , IL-4, IL-5, IL-10, IL-13, but not IL-17 (Fig. 2A). The ratios of IFN- γ :IL-4, IFN- γ :IL-10, IL-17:IL-4, and IL-17:IL-10 were markedly reduced in response to ex vivo MOG re-stimulation (Fig. 2B, similar changes were noted for IL-5 and IL-13). These data suggest that LNFPIII treatment preferentially increases the production of Th2 cytokines, and changes the balance of pro-inflammatory and regulatory cytokines in EAE model.

3.3. LNFPIII treatment induces NO production in inflammatory monocytes

When splenocytes from LNFPIII- and control-treated groups were isolated on day 10 post-immunization and stimulated with MOG peptide in vitro, proliferation was not significantly different between the two groups (Fig. 3A). However, when NOS2 was inhibited by L-NIL, proliferation was significantly increased in the LNFPIII-treated group (Fig. 3A), suggesting

that enhanced NO production induced by LNFPIII conjugates may play a role in T cell regulation *in vivo*. We then isolated splenic CD11b⁺Ly-6C^{hi}Ly-6G⁻ IMCs from LNFPIII- and control-treated mice on day 9 post-immunization, and found that IMCs from LNFPIII-treated mice had significantly increased *Nos2* mRNA expression (Fig. 3B). To compare the T cell regulatory function of these IMCs, we stimulated MOG TCR transgenic 2D2 CD4⁺ T cells with MOG35–55 and splenic CD90⁻ APCs from naïve B6 mice in the presence of *ex vivo* purified IMCs. This antigen-specific T cell proliferation was not significantly affected by IMCs from control-treated mice, but was suppressed by IMCs from LNFPIII-treated mice (Fig. 3C). Treatment with L-NIL, fully reversed T cell suppression, suggesting that LNFPIII-induced NO production in IMCs is T cell suppressive (Fig. 3C).

To investigate whether LNFPIII could directly induce NO production in IMCs *in vitro*, we treated isolated IMCs with dextran or LNFPIII alone or in combination with IFN- γ and GM-CSF. LPS was used as a positive control (Fig. 4). LNFPIII treatment alone did not induce NO production by IMCs, but when combined with IFN- γ or IFN- γ /GM-CSF, NO production was strongly induced to levels comparable to LPS treatment. Dextran treatment, either alone or in combination with IFN- γ and/or GM-CSF, did not induce significant levels of NO production.

3.4. LNFPIII treatment induces immune regulatory enzymes and phenotypic changes but not pro-inflammatory factors in inflammatory monocytes

Recently, several enzymes have been shown to play key roles in immune regulation by innate immune cells, such as tumor infiltrating macrophages and regulatory dendritic cells (26–29). We examined the expression of these enzymes in IMCs after *in vitro* LNFPIII treatment (Fig. 5). Indeed, we found that LNFPIII treatment significantly increased mRNA expression of arginase I (*Arg1*), aldehyde dehydrogenase 1, subfamily A2 (*Aldh1a2*), indoleamine 2,3-dioxygenase 1 (*Ido1*), and heme oxygenase 1 (*Hmox1*).

FACS analysis of the phenotype of IMCs showed that LNFPIII treatment significantly increased expression of MHC class II (I-A), CD86 and Jagged-1, but not CD80 or Jagged-2 expression (Fig. 6). Increased expression of these molecules was shown in both the frequency and the mean fluorescence intensity (MFI).

Interestingly, while LPS-treated IMCs produced several pro-inflammatory cytokines or chemokines, including IL-1 α , IL-1 β , IL-6, IL-12 p70, and MCP-1 (Fig. 7), LNFPIII conjugate treated IMCs produced only marginal levels of these mediators similar to the dextran control (Fig. 7).

3.5. LNFPIII treatment reduces migration of dendritic cells across brain endothelium

CNS-infiltrating dendritic cells have been shown to be crucial modulators in the initiation and persistence of immune responses within CNS during EAE (30). Among other features, their unique property is due to the ability to migrate between periphery and target organ. Therefore, we tested whether LNFPIII treatment could potentially influence the ability of DCs to cross the brain endothelium in an *in vitro* model of blood-brain barrier. We have previously used bone marrow-derived DCs, which express DC markers and are fully functional in antigen presentation, for dissecting the role of these cells in the CNS autoimmunity (31). When bone marrow-derived dendritic cells were treated with different concentrations of LNFPIII, DC migration across *in vitro* mouse brain endothelium was significantly reduced (Fig. 8).

4. Discussion

In this study, we found that LNFPIII conjugate treatment significantly reduced EAE severity as well as CNS inflammation, compared to the vehicle (dextran) treated control. Splenocytes from LNFPIII treated mice had significant increases in IL-4, IL-5, IL-10 and IL-13 production relative to IFN- γ or IL-17 production. LNFPIII treatment also induced NO production and several key immune regulatory enzymes in IMCs, but little pro-inflammatory cytokines or chemokines. In addition, LNFPIII treatment inhibited DC migration across brain endothelium *in vitro*.

EAE is an autoimmune model for human multiple sclerosis. Previous studies have shown that MOG-CFA immunization of animals with established schistosome infection or injection of schistosome ova or soluble egg antigens (SEA) suppressed development of EAE (11–13). These observations suggested the potential of using schistosome antigens to regulate autoimmune responses, but so far no single molecule from schistosome parasites has been shown to be effective. Here we show that treatment with LNFPIII conjugates significantly suppresses clinical EAE and decreases CNS inflammation. Harn's lab reported that a similar treatment protocol with LNFPIII prevented the development of psoriasis-like skin lesions in the flaky skin (fsn)/fsn mutant mice (21). While fsn/fsn mouse model mainly involves pathogenic B cells and neutrophils (32, 33), EAE model has clearly defined autoantigens as well as pathogenic Th1/Th17 responses (2). Therefore, further dissecting the immune regulatory mechanism of LNFPIII in EAE model is important in both mechanistic and clinical aspects.

CD11b⁺Ly-6C^{hi} IMCs are greatly expanded after EAE immunization, and efficiently migrate into the CNS after disease onset (7–9). They constitute about 30% of CNS inflammatory cells at the peak of disease (9). Our recent data suggest that the immune functions of IMCs and more differentiated inflammatory dendritic cells (IDCs) are plastic and depend on their activation status: While resting IMCs and IDCs function as antigen presenting cells, activated IMCs/IDCs are potent T cell suppressors through NO production (10). In this study, we found that *in vivo* LNFPIII treatment increased NO production in IMCs, which suppressed T cell proliferation *in vitro*, suggesting that LNFPIII treatment induced IMC activation. *In vitro*, we found that strong induction of NO production in IMCs required the treatment with LNFPIII along with IFN- γ and GM-CSF. This is similar to LPS treatment, and is consistent with previous finding that LNFPIII could signal through TLR4 on innate immune cells (18). Upregulation of MHC II and CD86 further supports the activation of IMCs by LNFPIII. While NOS2 inhibition revealed NO mediated T cell suppression in splenocyte recall assay, similar levels of splenocyte proliferation in cultures without NOS2 inhibition would suggest a balance between enhanced antigen presentation and NO mediated T cell suppression by IMCs. It is intriguing that LNFPIII treatment did not induce production of pro-inflammatory cytokines, which is a feature of LPS treatment. The molecular signaling mechanism remains to be determined.

Our data show that *in vivo* LNFPIII treatment strongly upregulated production of IL-10 and Th2 cytokines. These cytokines were produced at very low level in the control EAE group, but LNFPIII treatment enhanced their production by several hundred to a thousand fold. Although IFN- γ production was also moderately increased by LNFPIII treatment, the ratio of IFN- γ to Th2 cytokines was markedly reduced after treatment. Interestingly, IL-17 production was unchanged by *in vivo* LNFPIII treatment, and this is consistent with the minimal induction of IL-6 production in IMCs after *in vitro* LNFPIII treatment. LNFPIII treated IMCs increased expression of Jagged-1, which plays a role in promoting Th2 differentiation (34). Since NO production in IMCs is mainly induced by IFN- γ from Th1 cells, increased NO production in LNFPIII-treated IMCs may preferentially suppress Th1

cells in a negative feedback fashion. We found that upregulation of Th2 cytokine production is more evident at day 20 post-immunization compared with day 10. This may be due to the slower kinetics of Th2 response development, or the initial suppression of Th2 response by strong Th1 response after EAE immunization. On the other hand, Th2 cytokines were produced at high levels even without MOG antigen stimulation in splenocytes from LNFPIII treated mice. Whether these Th2 cytokines could be derived from cells other than antigen specific T cells, e.g., mast cells or basophils remains to be determined. Although expansion of regulatory T cells has been reported in several parasite infection models (35, 36), the frequency of splenic FoxP3⁺ CD4 Tregs was not significantly changed in LNFPIII treated mice (data not shown).

In addition to NOS2, we observed that LNFPIII treatment also increased gene expression of other immune regulatory enzymes, such as arginase 1, RALDH2, IDO and HO-1, in IMCs. Arginase 1 and IDO over-expression in macrophages and dendritic cells may reduce the availability of arginine and tryptophan to T cells respectively, and thus suppress T cell function (26, 29) as well as EAE (27, 37–39). IFN- γ and IL-4 preferentially induce iNOS and arginase expression respectively, but activation of IMCs or macrophages through TLRs such as by LPS may induce the expression of both genes ((40, 41) and our own observations). We believe that LNFPIII does not induce typical M1 or M2 like cells, but may induce IMCs to express both enzymes through TLR activation. RALDH2 catalyzes the synthesis of retinoic acid, a key molecule in suppressing Th17 differentiation and inducing regulatory T cells (28, 42). HO-1 enhances defense against free radicals, reduces MHC class II molecule expression on neighboring APCs, and suppresses T cell function (27, 38, 43). Further investigation of the role of these enzymes in EAE suppression is important to understand the immune regulatory mechanisms driven following LNFPIII treatment.

Furthermore, *in vitro* LNFPIII treatment inhibited DC migration across brain endothelial monolayers. CNS infiltrating T cells require *in situ* activation within the CNS to initiate autoimmune inflammation (44). Myeloid DCs are able to migrate into the CNS, and promote pathogenic functions of infiltrating T cells, suggesting that DC migration across the blood-brain barrier is a critical step in EAE development (4–6). Therefore, the reduction of the number of CD11c⁺ DCs in the CNS, following LNFPIII treatment, as supported by histology evidence, may also contribute to the beneficial effects of this compound.

In summary, our study suggests that LNFPIII treatment may suppress the development of CNS autoimmunity through modulating Th1/Th2 balance, T cell regulation by IMCs, and DC migration. Earlier reports have described that LNFPIII may signal through TLR2 and C-type lectins, such as DC-SIGN, the mannose receptor and macrophage galactose lectin-1, to mainly activate Erk rather than JNK or NF- κ B (19, 45, 46). These events may mediate the development of anti-inflammatory APCs. Future work will need to link these signaling events to the immune regulatory functions in IMCs. Further study of LNFPIII function may reveal its therapeutic potential for autoimmune diseases.

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Highlights

- Lacto-N-fucopentaose III (LNFPIII) is a schistosome glycan.
- LNFPIII treatment reduced the severity of experimental autoimmune encephalomyelitis
- LNFPIII treatment skewed peripheral immune response to a Th2 dominant profile.
- LNFPIII treatment induced the expression of immune regulatory enzymes in inflammatory monocytes.
- LNFPIII treatment reduced trafficking of dendritic cells across brain endothelium.

Fig. 1. LNFPIII treatment suppresses EAE and CNS inflammation

(A) C57BL/6 mice were immunized with MOG35–55 on day 0, and injected with 50 μ g of control dextran or LNFPIII conjugate twice a week, starting from the day of immunization. Linear regression analysis of the clinical data is shown on the right. (B) Spinal cord tissues were collected from dextran- or LNFPIII-treated mice on day 20. Typical H&E staining, as well as CD4, F4/80 and CD11c immunostaining (in brown) plus hematoxylin counterstaining (in blue) are shown. (C) CNS inflammatory foci and infiltrating inflammatory cells were quantified. Data are representative of 3 independent experiments. #, $p < 0.01$; *, $p < 0.05$.

Fig. 2. LNFPIII treatment preferentially induces IL-10 and Th2 cytokine production

(A) Splenocytes harvested on day 20 from individual animals treated with dextran or LNFPIII (3 mice per group) were stimulated with MOG35-55 from 0–25 μ g/ml in triplicates for 48 h. Cytokine concentrations in the culture supernatants were examined. #, $p < 0.01$; *, $p < 0.05$. (B) The average concentration ratio of IFN- γ or IL-17 to IL-4 and IL-10 was calculated for dextran- or LNFPIII-treated groups. Data are representative for 2 independent experiments.

Fig. 3. Inflammatory monocytes from LNFPIII-treated mice have increased *Nos2* expression and suppress T cells by NO production

(A) Splenocytes were harvested on day 10 from animals treated with dextran or LNFPIII, and stimulated with MOG35–55 from 0–25 $\mu\text{g/ml}$, with or without L-NIL. Proliferation assay was performed 48 h later. (B) Splenic IMCs were purified from dextran- or LNFPIII-treated mice on day 9 post-immunization, and the expression of *Nos2* mRNA was examined by real time PCR. (C) MOG TCR transgenic CD4⁺ T cells were cultured with splenic APCs and MOG35–55 peptide. IMCs were purified from dextran- or LNFPIII-treated mice on day 9 post-immunization, and cultured with T cells and APCs. T cells, APCs and IMCs were all seeded at 1×10^5 cells/well. 0.5 mM L-NIL was added as indicated to block NOS2 activity. Proliferation assay was performed 48 h later. Data are representative of 3 independent experiments. #, $p < 0.01$; *, $p < 0.05$.

Fig. 4. In vitro LNFPIII treatment induces the production of nitric oxide in inflammatory monocytes

CD11b⁺Ly-6C^{hi} inflammatory monocytes (IMCs) from CFA/PT immunized B6 mice were loaded 1×10^5 cells/well, and treated with 50 μg/ml dextran, 50 μg/ml glycan, 20 ng/ml IFN-γ, 20 ng/ml GM-CSF, and/or 100 ng/ml LPS as indicated for 48 h. Nitrite/nitrate concentrations in the culture supernatants were examined. Data were analyzed by one-way ANOVA analysis. Selected Bonferroni's test results within the same color groups are shown. *, $p < 0.05$. Data are representative for 3 independent experiments.

Fig. 5. LNFPIII treatment induces immune regulatory enzymes in inflammatory monocytes
 CD11b⁺Ly-6C^{hi} inflammatory monocytes (IMCs) were loaded 1×10^5 cells/well, and treated with 20 μ g/ml GM-CSF, together with 50 μ g/ml dextran or LNFPIII glycan in triplicates for 48 h. The expression of *Arg1*, *Aldh1a2*, *Ido1* and *Homx1* was examined by real time PCR. Data are representative for 2 independent experiments. * $p < 0.05$.

Fig. 6. Phenotypic changes in IMCs induced by LNFPIII treatment

CD11b⁺Ly-6C^{hi} inflammatory monocytes (IMCs) were loaded 1×10^5 cells/well, and treated with 20 $\mu\text{g/ml}$ GM-CSF, together with 50 $\mu\text{g/ml}$ dextran or LNFPIII glycan for 48 h. (A) FACS analysis was performed to compare the cell phenotype after dextran or glycan treatment. (B) Mean fluorescence intensity (MFI) was calculated from triplicates of treated samples. Data are representative for 2 independent experiments. # $p < 0.01$.

Fig. 7. LNFPIII treatment induces little production of pro-inflammatory factors
 CD11b⁺Ly-6C^{hi} inflammatory monocytes (IMCs) from CFA/PT immunized B6 mice were loaded 1×10^5 cells/well, and treated with 20 ng/ml GM-CSF, together with 50 μ g/ml dextran, 50 μ g/ml LNFPIII, or 100 ng/ml LPS as indicated for 48 h. Cytokine/chemokine concentrations in the supernatants were examined. Data are representative for 2 independent experiments.

Fig. 8. LNFPIII treatment reduces DC trans-migration across brain endothelial cells
 Bone marrow derived DCs were seeded on top of brain endothelial cell monolayers, and treated with either LNFPIII or control dextran. After 24 h, migrating DCs were collected and quantified using CyQuant GR nucleic acid dye. Each data point was run in triplicate, and these data are representative for 3 independent experiments. # $p < 0.01$; * $p < 0.05$.