



OPEN Immunomodulation of behavior impairment via spleen-specific targeting lipid nanoparticles in a MeCP2 transgenic mouse model

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This study investigates a novel approach using spleen-targeting lipid nanoparticles (LNPs) to immunomodulate behavioral impairment in a Methyl-CpG-binding protein 2 (*Mecp2*) transgenic mouse model. In a human *Mecp2* transgenic mouse model (B6.*Mecp2*^{Tg1}), spleen-targeting lipid nanoparticles encapsulated with antisense oligonucleotides (ASO) were employed as a mitigating agent. Splenic immune cells were analyzed using flow cytometry; autoantibody production was assessed using autoantigen protein microarrays, including a central nervous system (CNS) protein microarray. Furthermore, proinflammatory cytokines, MeCP2-mediated signaling pathways, and manifestations of behavior functions and kidney disease were examined. Our findings indicated a reduction in splenic MeCP2 levels following in vivo knockdown via ASO. Notably, splenic immune cells, including B and T cells, particularly plasma B cells, were significantly reduced after LNP-ASO treatment. Levels of autoantibodies against typical nuclear antigens such as Sm, U1-snRNP B/B, and nucleosome antigens, as well as brain antigens including OLIG2, GAD2, GJA1, and YWHAЕ, were significantly alleviated. Proinflammatory molecules such as IL-5, TNF- α , CCL20 and TGF- β 1 showed significant reduction. Additionally, after the in vivo knockdown of MeCP2, levels of MeCP2 protein, phospho-CREB, and phospho-mTOR were reduced compared to the placebo group. A reduced IgG deposition in the brain of the B6.*Mecp2*^{Tg1} mice were observed following treatment. Interestingly, behavioral functions and defects of the blood-brain barrier (BBB) were attenuated after spleen-targeted LNP-ASO treatment. Tissue-specific knockdown of a disease-promoting gene, MeCP2, via spleen-targeting LNPs effectively modulated the peripheral immune system resulting in subsequent amelioration of autoantibody production, inflammation, and behavioral deficits. These preclinical findings suggest that immunomodulation may be a promising therapeutic strategy for neuropsychiatric diseases.

Keywords Neuropsychiatric disease, Drug targeting, MeCP2, Lipid nanoparticles (LNPs), Immunomodulation

The Methyl-CpG-binding protein 2 (MeCP2), which binds to methyl-CpG dinucleotides, is believed to recruit co-repressor and histone deacetylase complexes to repress gene expression through chromatin modification^{1,2}. Previous research has extensively examined the neurological roles of MeCP2, underscoring its pivotal function in brain development and its links to various neurodevelopmental and neurological disorders, including MeCP2 duplication syndrome, a disease caused by duplications of the *MECP2* locus^{3–5}. Gene therapy targeting MeCP2 has been advanced as a treatment for MeCP2 duplication syndrome^{6–9}. As for MeCP2's function in systemic immunology, genetic polymorphisms in the MeCP2/IRAK1 locus have been linked to a higher risk of several autoimmune disorders^{10,11}, including systemic lupus erythematosus (SLE)^{12–14}. Recently, by studying a transgenic murine model (B6.*Mecp2*^{Tg1}), which was created through the insertion of a human *Mecp2* gene, resulted in the overexpression of the MeCP2 protein^{15,16}, our group demonstrated that this mouse model manifests a wide range of autoimmune phenotypes including increased CD3⁺CD4⁺ T cells, activated germinal center B cells and activated CD11b⁺F4/80⁺ macrophages, enhanced autoantibody production and neuropsychiatric deficits¹⁷, and can serve as a mouse model to study SLE and neuropsychiatric lupus (NPSLE). Other studies have also

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indicated that MeCP2 is expressed in immune cells, and the changes in MeCP2 expression levels could affect immune function^{18,19}. However, whether the overexpression of MeCP2 contributes to neuropsychiatric deficits through over-activation of the immune system and inflammation machinery is not fully understood. Hence, the investigation into whether MeCP2 can serve as a target for immune-mediated activation of inflammation-related signaling cascades and neuropsychiatric diseases represents a novel avenue.

Nucleic acid-based treatments, such as siRNA, mRNA, and antisense oligonucleotides (ASO), are increasingly recognized for their potential to precisely target the genetic basis of diseases, significantly expanding the possibilities for medical intervention²⁰. However, the delivery of nucleic acids to their functional sites within cells presents difficulties due to their limited in vivo stability and swift host clearance. Moreover, the effective uptake of nucleic acids through cell membranes is hindered by their negative charge, substantial molecular size, and hydrophilicity²¹. Lipid nanoparticles (LNPs) are increasingly recognized as a promising delivery vehicle for nucleic acids^{21–23}. This is due to their excellent biocompatibility, biodegradability, and ability to efficiently encapsulate these molecules^{24–27}. Remarkably, two FDA-approved vaccines²⁸ for coronavirus disease 2019 (COVID-19), which are mRNA-1273²⁹ and BNT162b2³⁰, utilize lipid nanoparticles for successful and safe delivery of antigen mRNA into the human body. Additionally, a wide range of lipid nanoparticle–mRNA (LNP–mRNA) formulations have been undergoing thorough research for the prevention and treatment of viral infections^{31,32}, different types of cancers^{33–35}, and genetic disorders^{35–37}. A notable example is Onpattro³⁸ (Patisiran), which, in 2018, became the first approved therapy using LNPs to deliver double-stranded small interfering RNA (siRNA). However, there has been ongoing concerted effort to explore extrahepatic delivery of LNPs to other organs, as a major bottleneck in expanding the clinical applications of LNP-based RNA therapeutics is their preferential accumulation in the liver³⁹. Based on the structure of the four-component traditional LNP, a new approach modifies LNPs for targeted delivery to specific organs by adding a fifth lipid^{40,41}. This versatile platform offers a modular approach for targeting different organs in nucleic acid delivery, providing new insights into the potential of gene therapy. The targeted organs include the lungs^{42,43}, spleens⁴⁴, kidneys⁴⁵, and skeletal muscles⁴⁶, with additional targets currently under investigation for various applications.

Spleen is the largest secondary lymphoid organ in human body, and its crucial role in the immunological processes has been proved^{47,48}. Splenectomy is considered a second-line therapy for certain autoimmune diseases, particularly those affecting the blood, such as immune thrombocytopenia⁴⁹ and SLE patients with autoimmune hemolytic anemia^{50,51}, highlighting the role of abnormal function of spleen in the progression of autoimmune diseases. However, splenectomy eliminates the spleen's function rather than restoring it, leading to the known risks associated with surgery and the post-splenectomy condition⁵². Beyond blood-related autoimmune diseases, targeting the spleen is also considered promising for conditions with out-of-spleen phenotypes. The brain–spleen crosstalk has been investigated for its role in neurological diseases, and several clinical drug studies targeting this axis are currently underway for the treatment of brain-related disorders⁵³. In our study, the hypothesis is that spleen-specific targeting LNPs encapsulated with MeCP2-specific targeting ASO, which could bind to MeCP2 mRNA and inhibit its further expression⁵⁴ will mitigate the autoimmune and neuropsychiatric phenotypes in the B6.*Mecp2*^{Tg1} mouse model. Herein, the desired nanoparticles were prepared, and the treatment effects were evaluated by a comprehensive study on immune cell populations, autoantibody levels, cytokine levels, as well as inflammation-related protein expression, IgG deposition in the brain, BBB defects, and neuropsychiatric deficits. The objective is to explore the possibility of treating neuropsychiatric disorders by targeting the peripheral immune system.

Results

Selective organ targeting (SORT) lipid nanoparticles can be specifically delivered to the spleen

To achieve spleen-specific knockdown of MeCP2, we prepared SORT LNPs that encapsulate ASOs targeting the human copy of MeCP2 in the B6.*Mecp2*^{Tg1} mouse model. These LNPs are based on the FDA-approved Onpattro formulation, which consists of Dlin-MC3-DMA, DSPC, cholesterol, and DMG-PEG2000. A fifth component, 18PA, a negatively charged lipid, was added with a molar percentage of 20%, following methodologies described in prior research⁵⁵. The composition and characterization of these nanoparticles are detailed in Supplementary Figure S1A, with the preparation protocol provided in Fig. 1A. The structures of the lipids used in the synthesis are shown in Fig. 1B.

We analyzed the physical characteristics of the 20%PA LNPs and control LNPs (the original Onpattro formulation, 0%PA), focusing on their size, polydispersity index (PDI), zeta potential and encapsulation efficiency as depicted in Fig. 1C and Supplementary Figure S1B–E. The uniformity of nanoparticles was confirmed by their small PDI and the intensity figures of their size distribution (Figure S1B). Moreover, a high encapsulation efficiency was achieved. The 20%PA LNP formulation demonstrated a slightly negative zeta potential, consistent with prior studies⁵⁵. To examine functional biodistribution, we encapsulated firefly luciferase mRNA in the LNPs and measured mRNA expression through bioluminescence using in vivo imaging system (IVIS) six hours after injecting the mice with a dose of 0.25 mg/kg (mRNA weight/mice body weight). As illustrated in Fig. 1D, no significant bioluminescence was detected following the injection of naked mRNA. However, the 20%PA group displayed remarkable spleen-specific bioluminescence, unlike the control group (0%PA), where bioluminescence was primarily observed in the liver. This pattern was further confirmed by ex vivo imaging, as shown in Fig. 1E, reinforcing the spleen-specific targeting of the 20%PA LNPs. Additionally, we labeled these LNPs with a near-infrared fluorescent dye (DIR) and performed further ex vivo imaging six hours post-injection. This imaging revealed increased fluorescence in the spleen compared to the liver, as shown in Fig. 1F. In contrast, mice injected with PBS only showed no notable signals, confirming the successful preparation of spleen-targeted LNPs.

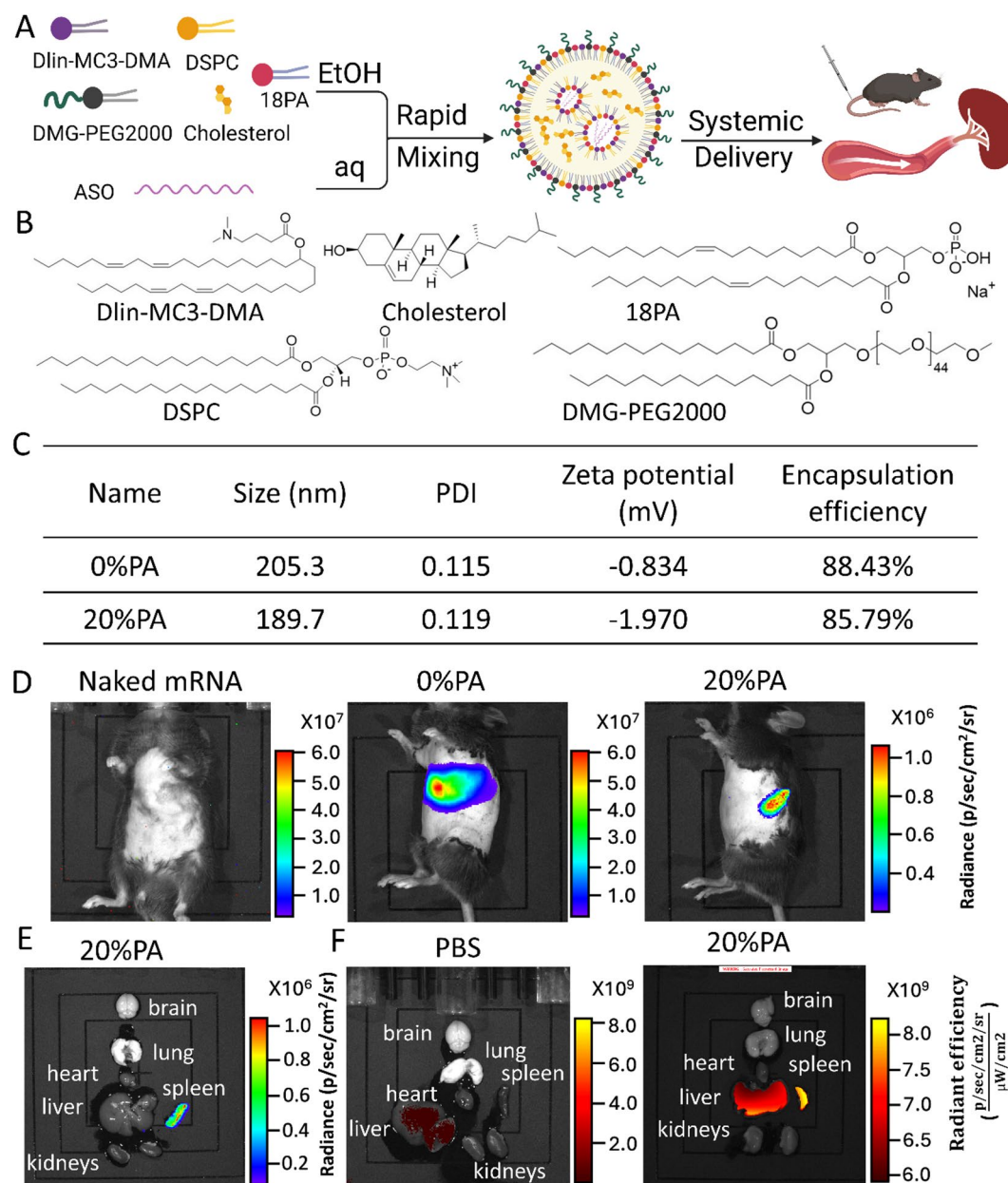


Fig. 1. Preparation of spleen-specific targeting LNPs. **A** Preparation of SORT lipid nanoparticles for spleen-specific knockdown of MeCP2 (created on Biorender.com). **B** Chemical structures of the five lipids participating in the synthesis of targeted lipid nanoparticles. **C** Characterization of nanoparticle size, PDI, zeta potential and encapsulation efficiency of traditional lipid nanoparticles (0%PA) and spleen-targeted lipid nanoparticles (20%PA). **D** IVIS imaging, showing the biodistribution of i.v. injected naked luciferase mRNA (left), luciferase mRNA encapsulated in 0%PA LNP (middle) and luciferase mRNA encapsulated in 20%PA LNP (right). **E** Ex vivo imaging of functional biodistribution six hours following the injection of 20%PA LNPs. **F** Ex vivo imaging six hours following the i.v. injection of PBS (left) and DIR-stained 20%PA LNPs (right).

The cytotoxicity of 20%PA LNPs was assessed through both in vivo and in vitro studies. In vitro, cytotoxicity was evaluated using the Jurkat cell line, using commercially available Lipofectamine 3000 as the benchmark transfection agent for comparison (Figure S1F). Our 20%PA LNPs demonstrated superior safety profiles at concentrations above 50nM. Notably, in our cell transfection system, the manufacturer recommended a concentration of Lipofectamine 3000, which is approximately 150nM. To further examine the in vivo cytotoxicity, we tracked changes in animal body weight over a month-long treatment period, with a final weight measurement taken four weeks post the last administration. This study showed no significant differences in weight between the two genotypes following administration of either PBS or 20%PA LNP (Figure S1G). Such findings highlight the biocompatibility and safety of the 20%PA LNP system for both in vitro and in vivo applications.

Spleen-targeted MeCP2 knockdown of B6.*MeCP2*^{Tg1}

Upon preparing the desired lipid nanoparticles (LNPs), it was essential to assess the efficiency of MeCP2 knockdown in the spleen. In this study, we used exclusively female mice because they exhibit more pronounced autoimmune phenotypes compared to males, especially in autoantibody secretion levels¹⁷; littermates lacking the human MeCP2 gene copy are designated as wildtype controls. Mice were randomly assigned into four groups for this purpose: wildtype mice treated with LNP-ASO, labeled as WT(ASO); wildtype mice administered with PBS, labeled as WT; transgenic mice treated with LNP-ASO, labeled as TG(ASO); and transgenic mice given PBS, labeled as TG. The timeline and strategy for the treatment are illustrated in Fig. 2A. Specifically, treatment commenced when mice were between 9 and 10 weeks old, with a dosage of 1.25 mg/kg (ASO/body weight) administered once a week for four weeks. Four weeks following the last treatment, all mice were euthanized for organ collection.

To verify the efficiency of the MeCP2 knockdown, we conducted western blot analysis. As depicted in Fig. 2B and quantified in Fig. 2C, MeCP2 expression was significantly reduced in the treated group. Further knockdown efficiency was evaluated using flow cytometry on the harvested spleens. The intracellular staining of MeCP2, as shown in Fig. 2D, was distinctly distinguished from the isotype control staining. Figure 2E illustrates the gating strategy used in flow cytometry. It compares the TG and TG(ASO) groups, showing a noticeable left shift in MeCP2 fluorescence intensity for the TG(ASO) group. This reduction in MeCP2 expression was further substantiated by analyzing the number of MeCP2⁺ cells and the mean fluorescence intensity of MeCP2 in the total splenocytes (Fig. 2F). The knockdown efficiency in specific cell types, including CD3⁺ cells and CD19⁺B220⁺ cells, was confirmed in Fig. 2G. These findings prove that MeCP2 expression was effectively suppressed in the spleen following treatment with LNP-encapsulated ASOs.

Immune cell subsets

Flow cytometry was utilized to dissect the phenotypes of immune cells within splenocytes, analyzing variations among T cells, B cells, and myeloid cells. The gating strategy for identifying different cell subsets is illustrated in Fig. 3A. The details of the alterations are illustrated in Fig. 3B, highlighting a notable reduction in the total count of CD3⁺CD4⁺CD8⁺ T cells post-treatment compared to disease controls. This decrease also extended to CD3⁺CD8⁺ T cells (Fig. 3B). However, total CD3⁺ cells, CD3⁺CD69⁺ cells, and CD3⁺CD4⁺ T cells did not show significant changes. The treatment resulted in the recovery of CD11b⁺CD11c⁺ dendritic cell counts, while the numbers of CD11b⁺CD11c⁺ dendritic cells remained largely unchanged (Fig. 3C). A specific decline was observed in B220⁺CD21^{High}CD23^{Low} marginal zone B cells but not in B220⁺CD21^{Int}CD23^{High} follicular B cells following treatment, as shown in Fig. 3D. Additionally, plasmablast and plasma cells exhibited reduced numbers in the treatment group (Fig. 3E).

Abnormal serum cytokine levels in the transgenic mice were ameliorated after treatment

The broad changes in the cell subsets of splenocytes observed above inspired us to investigate the functional aspects of the immune cells. Briefly, the serum collected from mice was applied to the protein microarray slide for target binding, followed by the detection using detection antibody and fluorescent labeling reagents. The outcomes are presented in Fig. 4. The panel on the left represents the normalized comparison between TG mice and WT controls for each molecule analyzed. The panel on the right displays the normalized comparison between transgenic mice treated with LNP-ASO or placebo. Relative to their littermates, the B6.*MeCP2*^{Tg1} mice exhibited marked increases in the levels of IL2, IFN- γ , TNF- α and TGF- β 1 levels. While increases in IL5, IL-22, IL-23, and CCL20 levels were observed, they did not reach statistical significance. Following treatment with LNP-ASO, a notable reduction in serum cytokine levels, including TNF- α , IL5, IL-23 and CCL20, was observed in the transgenic mice compared to those receiving placebo treatment. A downward trend was also noted for IFN- γ and IL-22 levels. These findings indicate that B6.*MeCP2*^{Tg1} mice exhibit abnormal serum cytokine profiles, and targeted knockdown of MeCP2 in the spleen has the potential to mitigate these abnormalities to a certain extent.

Autoantibody levels can be mitigated after treatment

We employed an autoantigen array featuring 93 antigens to perform an extensive screening for autoantibodies across three distinct groups: WT, TG, and TG(ASO). The findings were depicted in a detailed cluster heatmap, as illustrated in Fig. 5A. This analysis allowed for a precise comparison of autoantibody levels across the groups. Figure 5B shows a significant increase in autoantibody levels in the TG group compared to the WT group. In contrast, the TG(ASO) group exhibited a reduction in autoantibody levels compared with TG, suggesting a mitigation of the autoimmune phenotype.

We compared the autoantibody profiles between the TG and TG(ASO) groups to assess the treatment's efficacy. Figure 5C, D summarize the 14 autoantibodies that demonstrated a significant reduction in the TG(ASO) group relative to the disease group. A fold change comparison was conducted for these specific molecules, highlighting the potential of LNP-ASO treatment in alleviating autoimmune responses in this model.

LNP-ASO treatment decreased proteinuria levels and reduced IgG deposition

To examine if spleen-targeted treatment mitigates kidney damage, we assessed kidney function recovery post-treatment by measuring proteinuria levels. Initially, TG mice displayed a marked increase in proteinuria by nine weeks of age before receiving any treatment (Figure S2A). Subsequent observations were made at ages 14 and 18 weeks. While no significant change was observed in proteinuria levels between ASO-treated and placebo-treated mice at 14 weeks, a significant reduction in proteinuria levels was noted in ASO-treated mice by 18 weeks, and the levels are comparable to WT groups, without significant difference, indicating an improvement in kidney function (Figure S2B).

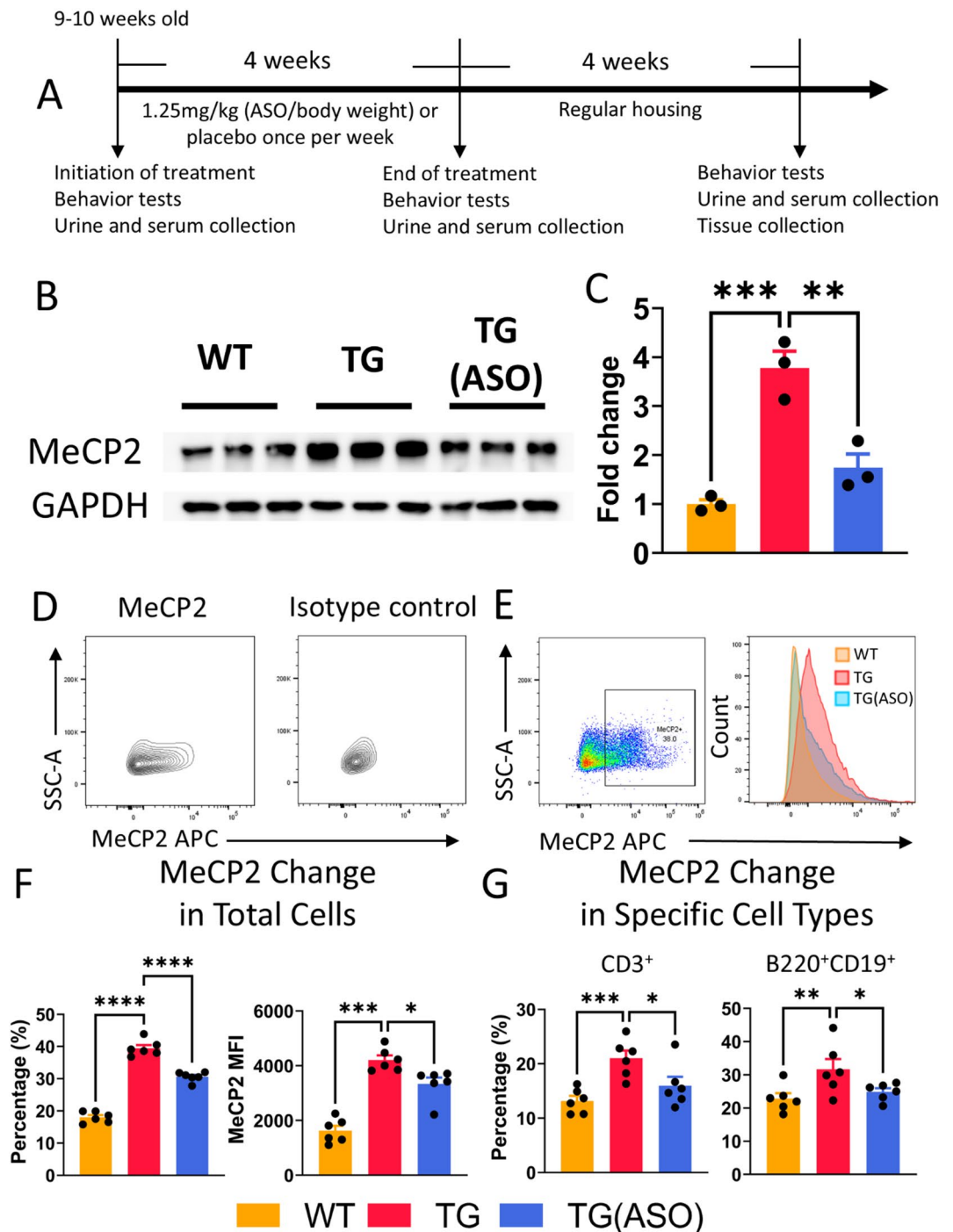


Fig. 2. Evaluation of the efficiency of spleen-specific MeCP2 knockdown in B6.*Mecp2*^{Tg1}. **A** Animal study design flow chart. **B** Western blot revealing the expression of MeCP2 in the spleen collected from different groups, including WT, TG and TG(ASO). **C** Fold change comparison of MeCP2 expression in the spleen. The signals were normalized to the internal control (GAPDH) expression levels. $n = 3$ for each group. **D** Flow cytometry validation of MeCP2 intracellular staining in a representative TG sample compared to the isotype control staining. **E** Flow cytometry gating strategy with a comparative analysis among WT, TG and TG(ASO) groups. **F** Quantification of MeCP2 expression level changes in total splenocytes across different groups, including MeCP2⁺ total cell numbers in the spleen (left) and mean fluorescence intensity (MFI) (right). $n = 6$ per group. **G** Quantifying MeCP2 expression level changes in specific splenocyte cell types across different groups, including CD3⁺ splenocytes and CD19⁺B220⁺ splenocytes. $n = 6$ per group. *Means changes between mouse groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

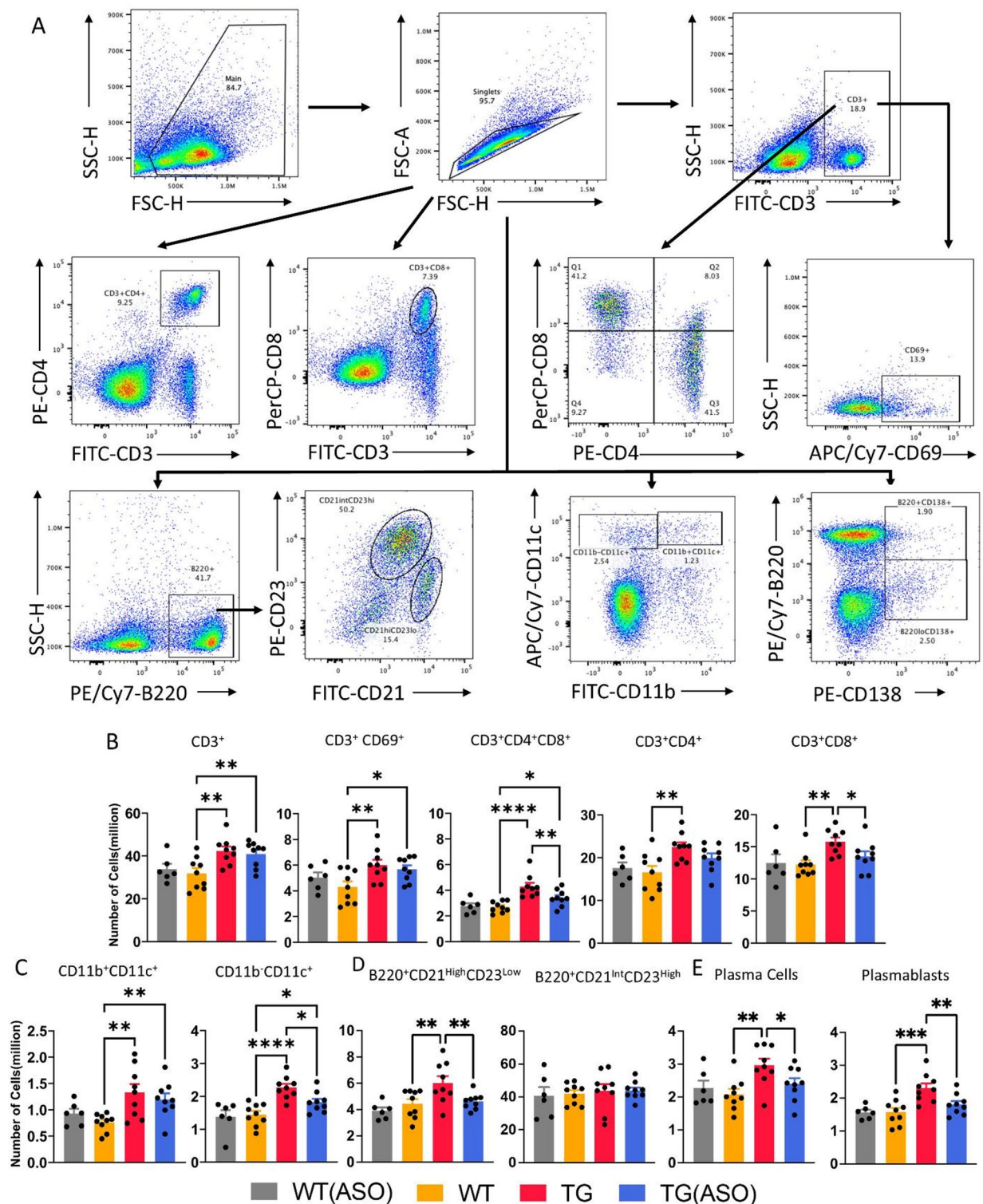


Fig. 3. Analysis of immune cell subsets in splenocytes using flow cytometry four weeks post-treatment. **A** Gating strategy for T cell subsets. **B** Changes in CD3⁺ T cells, CD3⁺CD69⁺ cells, double-positive T cells (CD3⁺CD4⁺CD8⁺ cells), CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells. **C** Changes in CD11b⁺CD11c⁺ dendritic cells and CD11b⁻CD11c⁺ dendritic cells. **D** Changes in B220⁺CD21^{High}CD23^{Low} marginal zone B cells and B220⁺CD21^{Int}CD23^{High} follicular B cells. **E** Changes in plasma cells and plasmablasts. $n = 6$ for WT(ASO) group and $n = 9$ for other groups. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

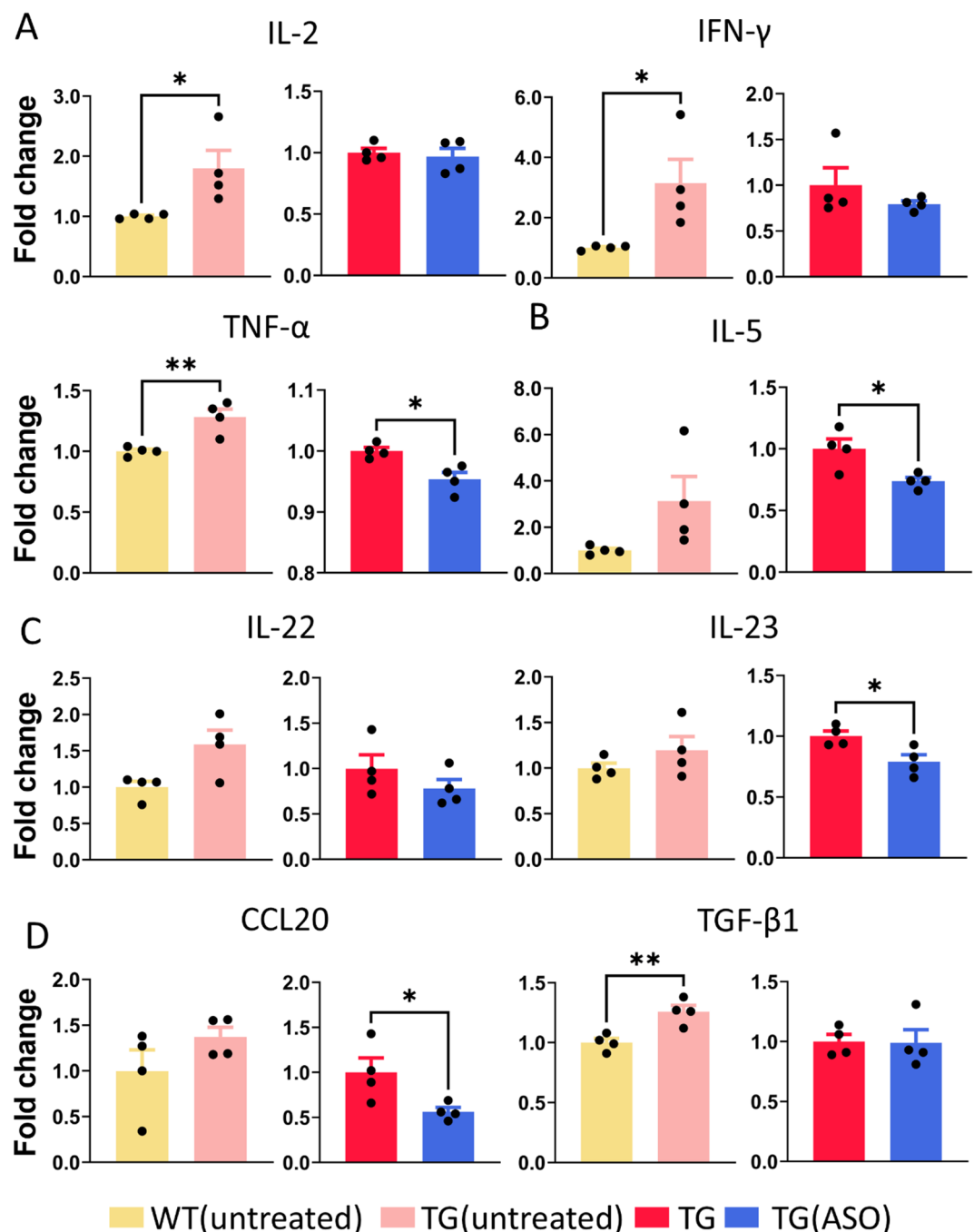


Fig. 4. Assessment of serum levels of cytokine and chemokine using a protein microarray. Comparative microarray results highlight cytokine level differences in different cytokine types, including **A** TH1-associated cytokines; **B** TH2-associated cytokines; **C** TH17-associated cytokines and **D** cytokines associated with multiple T helper cells. The left panel illustrates normalized cytokine levels between untreated transgenic mice and their littermates; the right panel compares cytokine levels between LNP-ASO-treated transgenic mice and placebo. $n = 4$ per group. * means changes between groups. * $P < 0.05$, ** $P < 0.01$.

Immunofluorescence was employed to evaluate IgG deposition levels and analyze kidney phenotypes further. Figure S2C presents representative images that highlight IgG deposition in the glomeruli and its colocalization with nuclei (stained with DAPI). These images reveal that IgG deposition levels in the kidney glomeruli were reduced following ASO treatment, suggesting a mitigating effect on nephritis.

Spleen-targeted therapeutic effects on neuropsychiatric phenotypes

Given the severe neuropsychiatric symptoms linked to MeCP2 dysfunction, we explored whether treatments targeting the spleen could mitigate these effects. Immunofluorescence imaging, as presented in Fig. 6A,

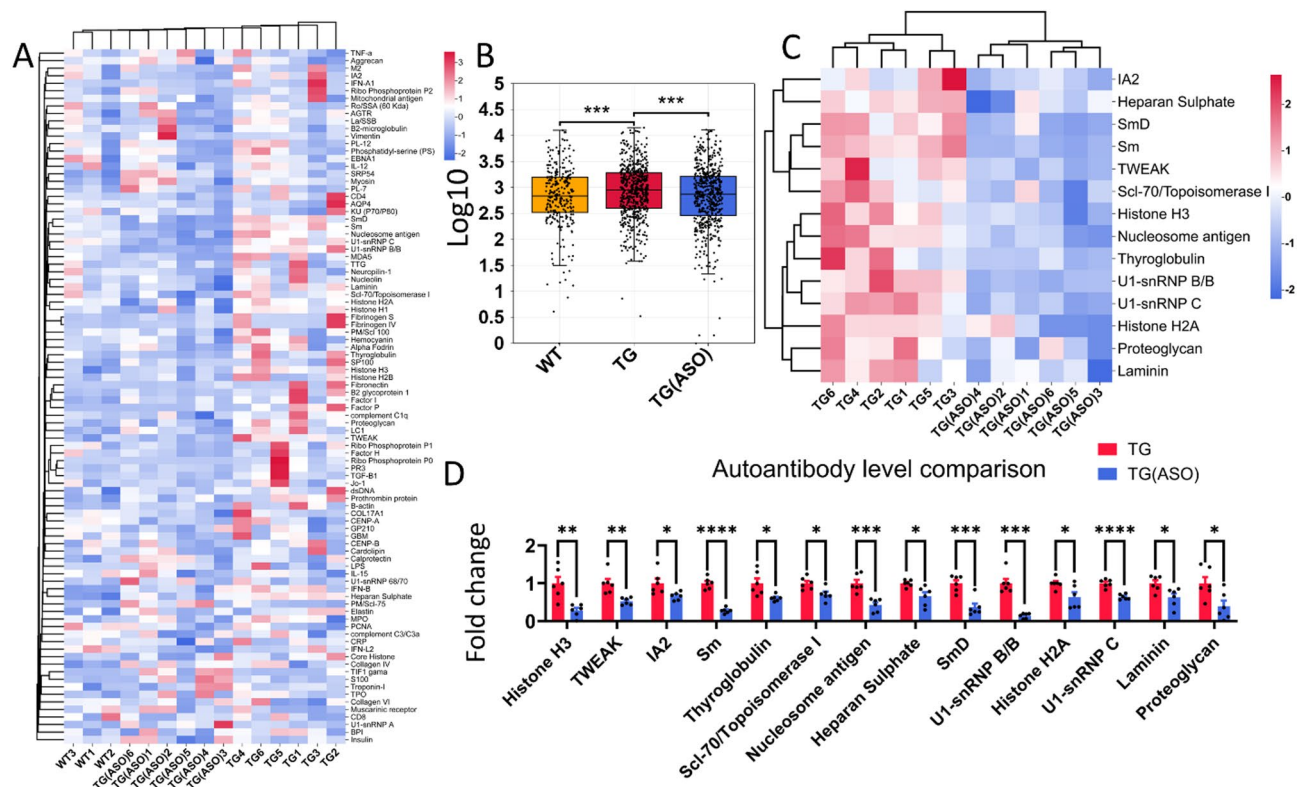


Fig. 5. Profile of changes in systemic autoimmune-associated autoantibodies. **A** Heatmap of autoantibody profiles; the expression level was calculated using z-score. **B** Overall comparison of all IgG autoantibody levels. **C,D** Representative autoantibodies showing significant downregulation post-treatment. Z-score was used for normalization in **C**, and the fold change comparison of the autoantibody levels between TG and TG(ASO) is shown in **D**. $n = 3$ for the WT group and $n = 6$ for both TG and TG(ASO) groups. Heatmaps and box plots were created by chipshot.com. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

immunofluorescence examines the integrity of the BBB in B6.*Mecp2^{Tg1}* mouse models. In these images, CD31 serves as an endothelial marker to highlight blood vessels, and albumin, a 69 kDa protein typically found in blood, indicates the presence of BBB leakage as it is not supposed to infiltrate healthy individuals' brains and elevated albumin levels in the brain of B6.*Mecp2^{Tg1}* mice imply the occurrence of BBB damage, augmented brain inflammation, and subsequent neuropsychiatric disorders. We looked deeper into the patterns of IgG deposition, depicted in Fig. 6B, which illustrates a comparative study of IgG levels between the TG(ASO) mice and the TG mice. The imaging data reveals a decline in IgG accumulation within the TG(ASO) group, suggesting that the targeted intervention may effectively reduce the IgG deposition in the brain, which may be partly due to the leakage across the blood-brain-barrier.

We also conducted a brain and neuronal-associated protein microarray analysis to examine whether autoantibodies against these brain proteins change after spleen-targeted treatment in the B6.*Mecp2^{Tg1}* mouse model. This antigen array was tailored to assess alterations pertinent to central nervous system (CNS) related diseases. Autoantibodies that exhibited significant changes are displayed in Figure S3A. Figure S3B depicted the fold changes of those autoantibodies that were notably downregulated in LNP-ASO-treated mice. Notably, the levels of four autoantibodies against YWHA, OLIG2, GAD2, and CX43 were significantly altered after treatment.

Next, behavioral assessments, including anxiety-like behavior and locomotor dysfunction, were performed. As shown in Fig. 7A, the representative plots from the elevated plus maze (EPM) test illustrate that TG(ASO) mice exhibit a marked increase in exploration of the open-arm zones and demonstrate greater overall mobility, as evidenced by their longer distances covered within the test arena, in comparison to the TG control group. Figure 7B, C reveal no improvement was noted at 13 weeks old (4 weeks post-treatment); however, by 17 weeks old, there was significant mitigation. Specifically, the time spent in the closed arm by mice in the TG(ASO) group was significantly reduced, indicating a reduction in anxiety-like behavior. Moreover, these mice covered longer distances in the closed arm within a shorter duration, suggesting improved locomotor function compared to the TG group. Figure 7D displays results from the light and dark behavior tests, showing alleviated anxiety-like behavior in the TG(ASO) group by 17 weeks, consistent with Fig. 7B. Interestingly, signs of improvement were observed as early as 13 weeks old, marking an early onset of behavioral rescue. Although not all phenotypes were fully rescued, TG(ASO) reached levels comparable to WT in some phenotypes at 17 weeks old, with no significant differences observed between the two groups (Fig. 7C, D and 17-week).

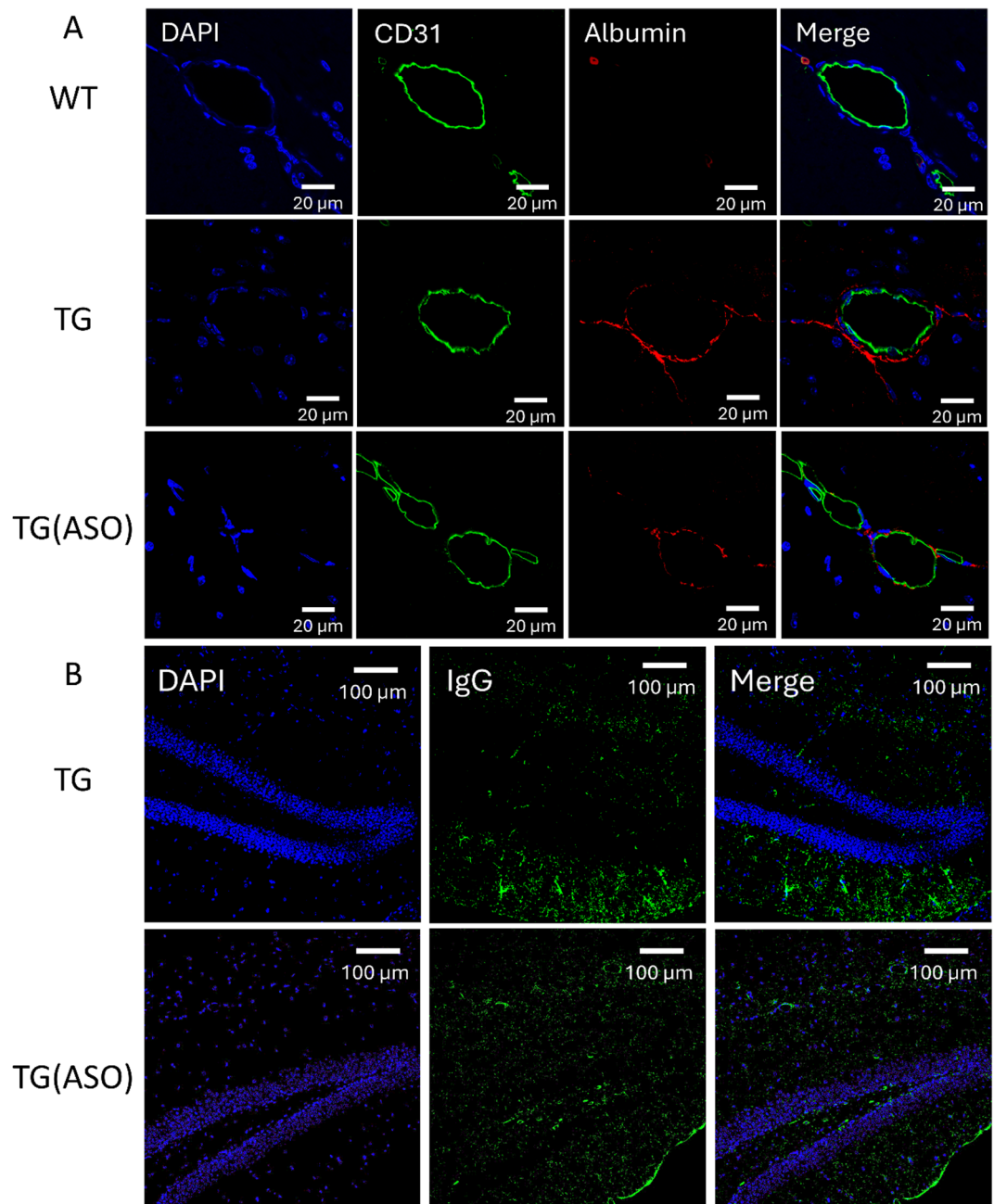


Fig. 6. Spleen-targeted treatment mitigates IgG deposition in the brain of B6.*Mecp2*^{Tg1} mice. Immunofluorescence images collected from rodent brain. **A** Visualization of blood-brain barrier leakage. Blue: DAPI staining for nuclei; green: CD31; red: albumin. **B** Visualization of IgG deposition of the hippocampus in brain slices. Blue: DAPI staining for nuclei; green: IgG.

MeCP2-mediated signaling pathway related to neuropsychiatric disorder and autoimmunity

To explore the role of MeCP2, we compared protein expression levels across three groups, as depicted in Fig. 8. Based on our previous work, we investigated two molecules: cyclic AMP response element (CRE) -binding protein (CREB) and mammalian target of rapamycin (mTOR). Figure 8A demonstrates that LNP-ASO treatment led to the downregulation of both total CREB and its phosphorylated form (ser133). Additionally, Fig. 8B reveals that in the TG(ASO) group, the levels of total mTOR and its phosphorylated form at ser2448 were reduced more remarkably than the decreasing in the phosphorylation at ser2481, compared to the TG group. Figure 8C presents further quantification of the changes in both total and phosphorylated protein levels of CREB, and mTOR. In summary, MeCP2 is a positive regulator for both CREB and mTOR, including their phosphorylated forms, suggesting a potential pathway through which MeCP2 influences the neuropsychiatric disorder and autoimmunity.

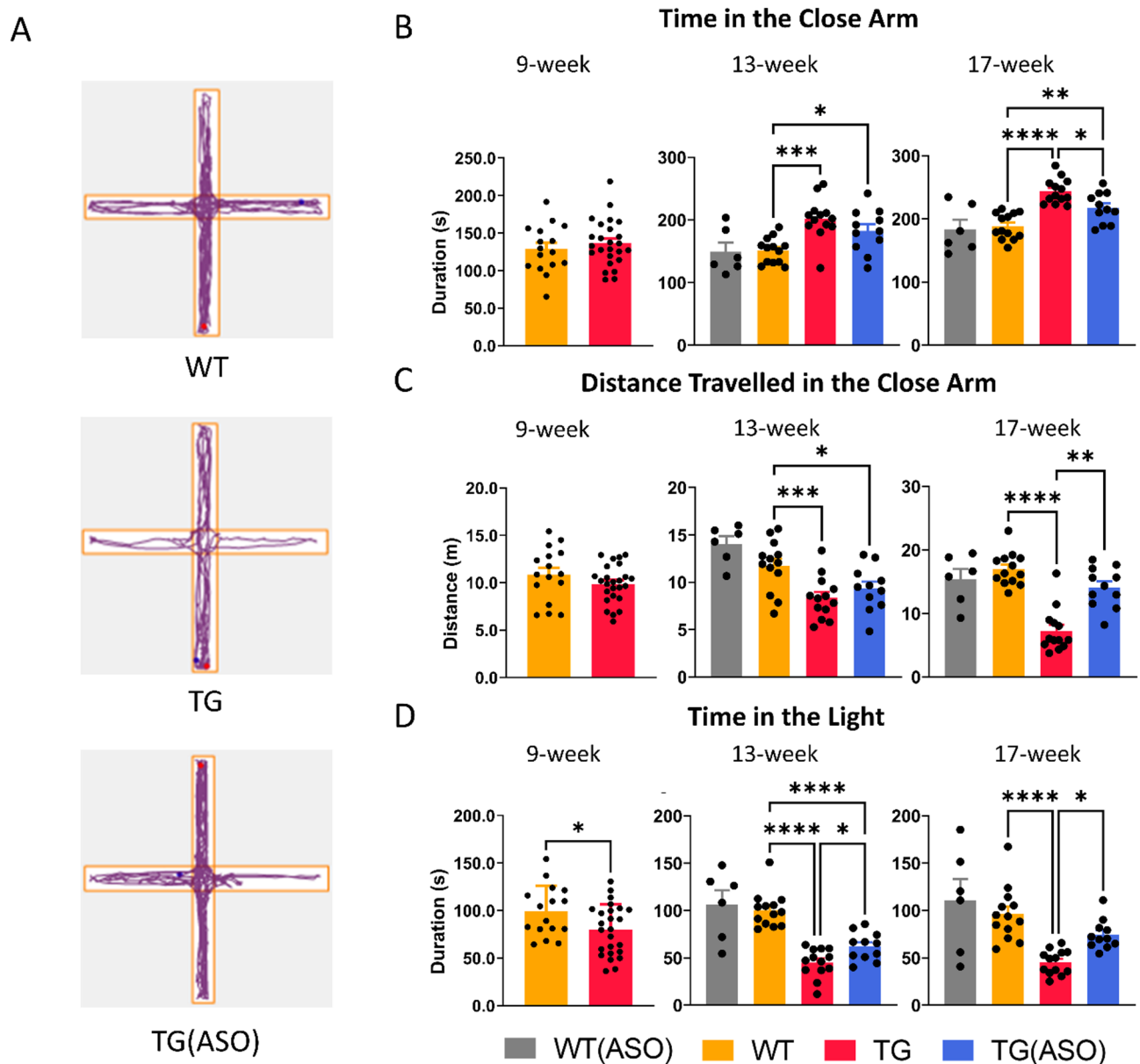


Fig. 7. The effects of targeted MeCP2 knockdown in the spleen on behavior impairment at different time points. **A** Representative track plots of rodents in Elevated plus maze (EPM) test. **B,C** At different time points, the time mice spent (**B**) and distance travelled (**C**) in the closed-arm zone. **D** Data collected from light and dark test. Figures represent time spent in the light chamber. Each data point represents one mouse in an individual experiment. For 9-week experiments, $n=16$ to 25 in each group. At other time points, $n=6$ for WT(ASO) group and $n=11$ to 13 in other groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Discussion

This study aimed to evaluate the efficacy of delivering ASOs targeting MeCP2 specifically to the spleen using LNPs and to assess the therapeutic impacts. Our results demonstrate successful spleen-targeted delivery of ASOs, which led to a specific knockdown of MeCP2 in this organ. This targeted intervention not only significantly ameliorated autoimmune features, including modulation of immune cell populations, normalization of serum cytokine levels and reduction in autoantibody production, but also shows improvement in extra splenic phenotypes, including behavioral deficits, and amelioration of kidney phenotypes. These findings align with previous reports highlighting MeCP2's role in immune regulation and expand on the therapeutic potential of spleen-targeted RNA interference.

LNPs represent a cutting-edge platform for the delivery of nucleic acid-based therapeutics, such as RNA interference (RNAi) molecules. The traditional four-component formulation of LNPs typically includes an ionizable lipid, phospholipids, cholesterol and PEG-lipids. However, because of the natural affinity of lipid nanoparticles for the liver, side effects in the liver could be severe. Adding a fifth lipid to the conventional

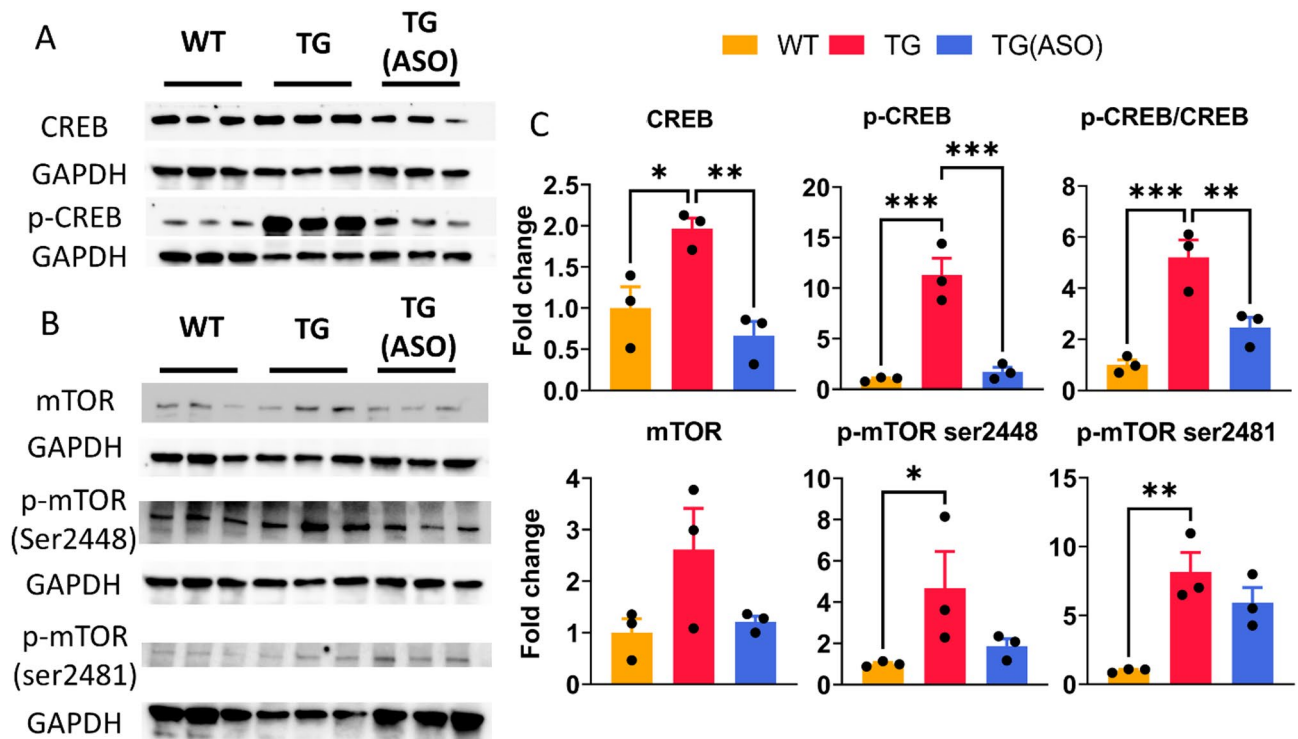


Fig. 8. The effect of spleen-specific *Mecp2* knockdown on downstream signaling pathways. **A** CREB and phosphorylated CREB expression post-treatment. **B** mTOR and phosphorylated mTOR expression post-treatment. **C** the quantification of **A,B**. The expression level is normalized to WT group. $n = 3$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

LNP components enables specific targeting of the nanoparticles to the spleen, offering a promising strategy for autoimmune diseases given the spleen's crucial role in both innate and adaptive immunity⁴⁸. Our data demonstrates that the targeted delivery of LNPs to the spleen facilitates gene therapy across various cell types and modulates immune cell functions, thus attenuating autoimmune responses. Also, in the WT and WT(ASO) groups, we observed no significant alterations in splenic cell types, suggesting the high safety profile of the LNP intervention (Fig. 3). This focused approach not only boosts the therapeutic impact by ensuring a higher concentration of the drug directly at the spleen but also minimizes the exposure of the rest of the body to the therapeutic agent. As a result, the potential for side effects could be significantly reduced, offering a more targeted and safer treatment option.

The observed decrease in splenic immune cell populations, particularly CD220⁺CD19⁺ cells and CD3⁺ cells, after treatment with LNP-ASO, highlights the direct impact of MeCP2 modulation on immune regulation. This reduction is notable in plasma B cells, often implicated in autoimmune pathogenesis due to their role in autoantibody production⁵⁶. Correspondingly, the diminished levels of autoantibodies against typical nuclear antigens post-treatment indicate a successful amelioration of the autoimmune responses. The reduction in different types of proinflammatory cytokines further elucidates the anti-inflammatory effects of spleen-targeted LNP-ASO therapy. These cytokines are crucial mediators of autoimmune pathology, and the decrease of pro-inflammatory cytokines suggests a suppression of inflammatory signaling pathways⁵⁷. These findings support the hypothesis that targeted delivery of therapeutic agents to the spleen can modulate immune cell function and autoantibody production, offering a promising strategy for treating autoimmunity-related disorders.

Our findings suggest that MeCP2 contributes to the progression of autoimmune diseases, potentially through modulation of the CREB and mTOR signaling pathways. Specifically, MeCP2 is identified as a regulator of CREB⁵⁸, a stimulus-induced transcription factor essential for immune responses via Ser133 phosphorylation⁵⁹. This regulation is supported by our data showing altered CREB activity consistent with previous reports on its role in immune responses⁶⁰ including cytokine regulation^{61–64} and lymphocyte function^{65,66}. Further, MeCP2's regulation of the mTOR signaling pathway emphasizes its potential relevance in the molecular dysfunction. mTOR has two complexes, mTORC1 and mTORC2, marked by ser2448 and ser2481 respectively⁶⁷. MeCP2 is involved in the regulation of the mTOR signaling pathway^{68–70}, whose dysregulation has been linked to various disorders, including the development of autoimmune conditions^{71,72} and neuropsychiatric symptoms^{73,74}. The mTOR pathway involves various cellular processes in autoimmune diseases, including the proliferation and differentiation of immune cells^{75,76}, and the secretion of inflammatory cytokines⁷⁷. By modulating this pathway, it may be possible to design treatments that specifically correct the dysregulated immune responses characteristic of these conditions^{78–80}. Our findings on cytokine alterations, changes in immune cell subsets, and autoantibody

secretion provide insights into the mechanisms facilitated by the interaction of MeCP2 with CREB and mTOR pathways.

In this study, spleen-specific targeted therapy improved the spleen itself, kidney function and behavioral phenotypes. The spleen modulates the ‘cytokine storm’, a hallmark of systemic inflammation⁸¹. It is increasingly recognized as an organ with a significant role in immune function and systemic inflammation, which can affect kidney functions and the central nervous system⁴⁸. In autoimmune diseases, autoantibody production and deposition in the kidney glomeruli are critical pathological steps^{82,83}. By targeting the spleen, we observed a reduction in plasma cell numbers and a downregulation of autoantibody levels, which likely explain the improvements in kidney phenotypes. As for the brain-spleen axis, our research found surprising results in ameliorating behavior defects. The disruption of BBB in B6.*Mecp2*^{Tg1} mouse models underscores a critical pathophysiological mechanism wherein the compromised barrier allows for the infiltration of IgG and proinflammatory cytokines from the bloodstream into the brain. This observation aligns with previous research highlighting the role of BBB in the neurological manifestations of autoimmune and neuroinflammatory conditions⁸⁴. Notably, we found that LNP-ASO treatment led to a decreased accumulation of IgG in the brain. Meanwhile, identifying reduced levels of autoantibodies against brain antigens such as YWHAЕ, OLIG2, GAD2, and CX43 after treatment points to a complex interplay between the immune system and neuropsychiatric disorders. In the behavioral assessments upon targeted therapy, reductions in anxiety-like behaviors and locomotor dysfunction were observed among treated mice. This behavioral improvement underscores the potential for targeted therapies to facilitate neuropsychiatric conditions’ molecular and immunological underpinnings and their manifest clinical symptoms. However, this approach requires a comprehensive understanding of the immune system’s contribution to neurodevelopment. It also requires detailed understanding of the complex interplay between peripheral immune occurrences and their consequent effects on the central nervous system. Such an understanding is crucial to unravel the bidirectional communication pathways that link immunological processes with neurological health and disease.

In conclusion, our study presents an advancement in the treatment of autoimmune symptoms through the targeted delivery of antisense oligonucleotides encapsulated in spleen-specific lipid nanoparticles. The knockdown of MeCP2 in the spleen led to a notable improvement in clinical symptoms, including reduced cytokine levels, diminished autoantibody production, modulation of immune cell subsets, decreased proteinuria levels and rescued behavior phenotypes. These findings emphasize the pivotal role of MeCP2 in the pathogenesis of autoimmunity and underscore the potential of spleen-targeted therapies. Using spleen-specific targeting lipid nanoparticles as a delivery system for ASO represents a novel and promising approach, offering a more precise and potentially less toxic treatment option for autoimmune diseases.

There are several limitations in this study. First, our findings are derived from the B6.*Mecp2*^{Tg1} model, a specific framework for autoimmune disease. Further investigation is warranted to study transcriptional signaling pathways to better understand the underlying mechanisms. This can help us understand whether spleen-targeted therapies and MeCP2 targeted therapies would exhibit comparable efficacy and relevance across a broader range of autoimmune disorders. Second, the age dependence of the proposed treatment was not evaluated. Future studies will explore both preventive interventions at earlier disease stages and therapeutic applications during later progression. Third, although spleen-targeted immunomodulation is a promising strategy for reducing neuroinflammation and enhancing mental health, its application remains experimental. Further research is necessary to refine its effectiveness, understand individual variability, and ensure safety before it can be a mainstream therapeutic approach.

Overall, spleen-targeted immunomodulation offers a novel and innovative approach to addressing neuroinflammation-related psychiatric and neurological disorders. Future research should focus on optimizing therapeutic strategies, identifying biomarkers for patient selection, and integrating spleen-targeted approaches with existing treatments to maximize translational potential and clinical benefits.

Materials and methods

Materials

DLin-MC3-DMA was purchased from Medkoo Biosciences. DSPC, DMG-PEG2000 and 18PA were purchased from Avanti Polar Lipids. Cholesterol was purchased from Sigma-Aldrich. DIR was purchased from Biotium. Firefly luciferase mRNA was purchased from Trilink Biotechnologies. D-luciferin was purchased from Goldbio. ASO was synthesized by IDT with 20 chemically modified nucleotides (MOE gapmer). The sequence is 5′-GG TTTTCTCCTTTATTATC-3′ with phosphorothioate modification on the backbone and the central gap of 10 deoxynucleotides is flanked on its 5′ and 3′ sides by five 2′-O-(2-methoxyethyl) (MOE)-modified nucleotides. All antibodies used in this study have been listed in Supplementary Table 1.

Preparation of LNP

ASO-loaded LNP formulations were formed using the ethanol dilution method. The original 4-component LNP (0%PA LNP) preparation was carried out by dissolving all lipids in ethanol and ASO in 10 mM citrate buffer (pH 3.0). The two solutions were mixed rapidly with a volume ratio of 3/1 (aq./ethanol) and weight ratio of 1/40 (ASO/lipids). The obtained solution was incubated at room temperature for 10 min before further operation. To prepare spleen-specific delivery lipid nanoparticles, PA was dissolved in THF and then mixed with ethanol formulations to obtain a specific molar ratio of the 5 components. The molar ratio of the five components DLin-MC3-DMA/DSPC/cholesterol/DMG-PEG2000/PA is 50/10/38.5/1.5/X (X equals to 0 for 0%PA LNP and X equals 25 for 20%PA LNP). After LNP formation, the fresh LNP formulations were diluted with PBS to make final ethanol concentration lower than 5% for size and zeta potential detection using a Malvern Zetasizer machine (Nano-ZS, Malvern Instruments, Malvern, UK). For DIR-stained lipid nanoparticles, DIR of 10% the weight of total weight of lipids was dissolved in ethanol with lipids and then follow the same protocol to

formulate the lipid nanoparticles. For in vivo and in vitro studies, the prepared LNP was dialysed against PBS with Pur-A-Lyzer Dialysis Kits, MWCO 3.5 kDa overnight at 4 centigrade and concentrated with Amicon ultra centrifugal filter, MWCO 30 kDa.

Biodistribution visualization

C57BL/6 mice of 8 to 10 weeks were i.v. injected with lipid nanoparticles via tail vein, formulated with or without 20% PA. The lipid nanoparticles were encapsulated with Luc mRNA, otherwise encapsulated with ASO and stained with DIR. Six hours after the iv injection, a group of Luc mRNA injected mice were i.p. injected with D-luciferin following instructions from the manufacturer. After 10 min, IVIS was done on the mice to visualize the bioluminescence. The other mice were sacrificed, and the main organs were collected for ex vivo imaging.

Measurement of LNP encapsulation efficiency

The LNP encapsulation efficiency measurement is performed based on the manufacturer's protocols for the Qubit ssDNA assay kit (Fisher Scientific, Catalog #Q10212). After preparing the ASO-encapsulated LNPs, the nanoparticles were washed with PBS and filtered through an Amicon ultra centrifugal filter, MWCO 30 kDa, three times. The nanoparticles were incubated with 1% Triton X-100 to release the ASO. Considering the Qubit ssDNA assay kit has a limited tolerance capacity for Triton X-100, the standards and broken nanoparticles were dialyzed against PBS for two hours with Pur-A-Lyzer Dialysis Kits, MWCO 3.5 kDa. Then, the broken LNP and standards were loaded, and the signals were read using a fluorescent plate reader (SpectraMax M3, Molecular devices). The encapsulation efficiency (EE) was calculated by:

$$\%EE = \frac{\text{encapsulated amount of ASO}}{\text{initial amount of ASO}} \times 100$$

In vitro LNP cytotoxicity test

Jurkat cell line purchased from ATCC was used for cytotoxicity test. This cell line was cultured in RPMI-1640 (ATCC, Catalog#30–2001) supplemented with 10% of fetal bovine serum (Fisher Scientific, Catalog#16-000–044). Lipofectamine 3000 Transfection Reagent (Fisher Scientific, Catalog#L3000001) was a benchmark control. The LNP in vitro cytotoxicity test followed the manufacturer's instructions for cell counting kit 8 (CCK8, Abcam, catalog#ab228554). Briefly, cells were seeded in a 96-well plate at a density of 10,000 cells per well, incubated with tested substances for 24 h, followed by incubation with CCK8 solutions for 3 h. The absorbance increase was measured at 460 nm. The viability ratio was calculated by normalizing the signals to the blank control (cells without adding transfection reagent).

Animal studies

All mouse experiments described in this study were approved by the University of Houston Institutional Animal Care and Use Committee (IACUC) conducted in accordance with relevant guidelines and regulations, including the ARRIVE guidelines for reporting animal research. B6.*Mecp2^{Tg1}* mouse breeders are generous gifts from Dr. Huda Zoghbi. Littermates bred without the target gene served as wildtype controls. All the mice were housed in a climate-controlled room with a 12-h light, 12-h dark cycle and free access to food and water. For experiments involving anesthesia, mice were anesthetized with 2–3% isoflurane (MWI Animal Health, Boise, ID, USA) in oxygen, delivered via a precision vaporizer. Mice were euthanized using CO₂ inhalation followed by cervical dislocation as a secondary method to ensure death.

LNP-ASO treatment

After genotyping, the animals were randomly assigned to experimental groups receiving either placebo or LNP-ASO injections. The treatment of mice starts at 9 to 10 weeks old. The treatment group was intravenously injected via the tail vein with LNP encapsulated with ASO at the dosage of 1.25 mg/kg (ASO/body weight) once a week for four weeks. The LNP solutions were diluted based on the weight of the mice to make the final volume injected at 200 µl. Control groups were intravenously injected with an equal volume of PBS. After the final injection, the mice were housed continually for another four weeks before sacrifice for organ collection.

Proteinuria

Mice were housed in metabolic cages individually, with free access to water but restricted food access. Urine collection continues for 24 h, and collected samples were stored appropriately to prevent degradation. At the end of the collection period, urine volumes were measured, and protein levels in the samples were quantified using Pierce Bradford Plus Protein Assay Reagent (Catalog #23238; Thermo Scientific) following the manufacturer's instructions. Each sample was assayed in duplicate.

Western blotting

Harvested tissues were lysed on ice for 30 min with vortexing and then sonicated with Q125 Sonicator from Qsonica (Newtown, CT, USA). Lysates were centrifuged for 15 min at four centigrade, and the protein concentration was measured using a BCA kit (Catalog #23225; Thermo Scientific). The lysates were diluted to equal concentrations to run on SDS-PAGE. Following electrophoresis, proteins were transferred onto PVDF membranes and incubated with primary antibodies at suggested concentrations from the manufacturer overnight at four centigrade. After washing, the membrane was incubated in a diluted secondary antibody for one hour at room temperature. ECL kit was used before imaging, and the software ImageLab (version 6.1, Bio-Rad Laboratories, Hercules, CA, USA; <https://www.bio-rad.com/>) was used for quantification. Each band

represent one biological sample, and the experiment was repeated three times. Figure S4 shows the full-length, uncropped blots corresponding to those presented in the manuscript.

Detection of autoantibodies

The autoantigen slides were purchased from Genecopoeia (Catalog #PA001 and #PA002). Each autoantigen printed on the slide was spotted in duplicate. The experiment was operated following the manufacturer's instructions. Briefly, the serum sample was treated with DNase I, diluted 1:100, and incubated with an autoantigen array. The array was then incubated with fluorescent dye-labeled secondary antibody and was scanned with GenePix 4400 A microarray scanner (Molecular Devices, San Jose, CA, USA). The averaged net fluorescent intensity (NFI) of each autoantigen was normalized to internal controls.

Cytokine microarray

The Quantibody Mouse TH17 Array from Raybiotech (Catalog #QAM-TH17-1-1) was utilized per the supplied protocol. Each cytokine printed on the slide was spotted in quadruplicate. Briefly, serum samples, diluted in a 1:2 ratio, and provided standards, were incubated on the array, followed by incubation with a fluorescently labeled secondary antibody. The GenePix 4400 A microarray scanner (Molecular Devices, San Jose, CA, USA) was used for scanning, and fluorescent intensities were quantified against the provided standards.

Flow cytometry

The spleens were collected freshly from mice and processed into single-cell suspensions. The cells were quantified and adjusted to a concentration of 10 million per 1 milliliter and seeded into a 96-well plate with 100 μ l per well. The cells were blocked with anti-mouse CD16/32 (Biolegend, catalog#101320) and then incubated with desired surface staining antibodies. Afterward, the cells were fixed with fixation buffer (Biolegend, catalog#420801), permeabilized using Permeabilization Wash Buffer (Biolegend, catalog#421002), and incubated with MeCP2 monoclonal antibodies followed by APC conjugated secondary antibodies. The cells were then ready for flow cytometry. For each sample, 50,000 to 70,000 cells were analyzed. The collected data was analyzed using FlowJo software (version 10.8.1; BD Life Sciences, Ashland, OR, USA; <https://www.flowjo.com/>). Information about the used antibodies can be found in Supplementary Table 1.

Immunofluorescence

Mice were sacrificed with perfusion. The brains and kidneys were collected in 10% neutral buffered formalin, incubated at four centigrade for 24 h, followed by incubation in 20% sucrose for 24 h and then 30% sucrose for 24 h. Afterward, tissue samples were sectioned and collected in PBS. The sections were incubated with 0.25% triton-X and then blocked with 10% goat or mouse (consistent with the host of secondary antibody) serum (Abcam, Catalog #ab7481 or Abcam, Catalog #ab7486) in PBS, followed by incubation with specific antibodies. For BBB immunofluorescence, the slices were incubated with CD31 antibody and albumin antibody simultaneously overnight at four centigrade, followed by incubation with secondary antibodies at room temperature for two hours. For IgG deposition immunofluorescence, slices were directly incubated with Alexa flour 488 conjugated goat anti-mouse secondary antibodies. Following additional PBS washes, sections were mounted on slides using ProLong Gold Antifade Mountant with DAPI (Thermo Scientific, Catalog #P36935) for nuclear counterstaining. After curing at room temperature for 24 h, the slides were imaged using a confocal microscope (Olympus FV3000, Nikon, Japan).

Elevated plus maze test

The apparatus consists of two open arms and two closed arms extending from a central platform elevated above the floor. At the start of the test, the animal was placed at the center of the maze. The animal's behavior was then tracked for 5 min, during which the time spent in the open and closed arms was noted, and the track the animal traveled was also recorded using ANY-maze (version 6.14, Stoelting Co., Wood Dale, IL, USA; <https://www.any-maze.com/>).

Light and dark test

This apparatus consists of two compartments: one transparent and brightly lit and the other dark and covered, connected by an opening that allows the animal to move freely between the two sections. At the beginning of the test, the animal was placed in the light zone, and its movements were tracked for 5 min using ANY-maze (version 6.14, Stoelting Co., Wood Dale, IL, USA; <https://www.any-maze.com/>). This software is also used for data analysis.

Statistics

Statistical comparisons were performed with Prism 6.0 (GraphPad, San Diego, CA, USA; <https://www.graphpad.com/>) using the unpaired Student's t-test for two groups if they passed the normality test; otherwise, comparisons were performed using the Mann-Whitney test. For comparison that occurred within 3 or more groups, a one-way ANOVA test was conducted. Data are presented by mean $\pm$ sem.

Data availability

Data supporting the findings of this study are available from the corresponding author on reasonable request.

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Author contributions

TW and SZ conceived this work. SZ performed the experiments with the help of YL, BY and AT. SZ wrote the manuscript, which TW, CM, SS, SM, and AT edited and revised. SM helped measure the size, zeta potential and encapsulation efficiency of the lipid nanoparticles. FA and SS helped with the animal behavior tests.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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