Leukocyte-specific siRNA delivery revealing IRF8 as a potential anti-inflammatory target

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ABSTRACT

Interferon regulatory factor 8 (IRF8) protein plays a critical role in the differentiation, polarization, and activation of mononuclear phagocytic cells. In light of previous studies, we explored the therapeutic potential of IRF8 inhibition as immunomodulatory therapy for inflammatory bowel disease (IBD). To this end, we utilized siRNA-loaded lipid-based nanoparticles (siLNPs) and demonstrated a ∼90% reduction of IRF8 mRNA levels in vitro (PV < 0.0001), alongside a notable reduction in IRF8 protein. Moreover, silencing IRF8 ex vivo in splenocytes lead to a profound downregulation of IRF8 protein, followed by an immunomodulatory effect, as represented by a decrease in the secretion of TNFa, IL6 and IL12/IL23 (IL12p40) proinflammatory cytokines (PV = 0.0045, 0.0330, < 0.0001, respectively). In order to silence IRF8 in vivo, selectively in inflammatory leukocytes, we used siLNPs that were coated with anti-Ly6C antibodies via our recently published ASSET targeting approach. Through this strategy, we have demonstrated a selective binding of the targeted-LNPs (T-LNPs) to Ly6C + inflammatory leukocytes. Finally, an immunomodulatory effect was demonstrated in vivo in an IBD mouse model with a profound decrease of TNFα, IL6, IL12/IL23, and IL1β pro-inflammatory cytokines (n = 5, PV < 0.0001, < 0.0001, < 0.0001, 0.02, respectively) and an improvement of colon-morphology as assessed by colon-length measurements and colonoscopy (PV < 0.0001). Overall, using antibody-targeted siLNPs, we showed a notable reduction of IRF8 mRNA and protein and demonstrated a targeted immunomodulation therapeutic effect ex vivo and in vivo, in the DSS colitis model. We claim that a selective silencing of IRF8 in inflammatory leukocytes (such as Ly6C+) may serve as a therapeutic approach for treating inflammatory disorders.

1. Introduction

Inflammatory Bowel Disease (IBD), such as Crohn’s Disease and Ulcerative Colitis, is a group of inflammatory disorders that affect the gastrointestinal tract. The global increase in IBD incidence is closely related to environmental and lifestyle changes. However, with a multifactorial nature and a complex interaction between microbiota, genetics and environmental factors, the etiology of IBD remains unclear. [1,2] During the past few decades, progress in the development of novel immunomodulatory remedies significantly improved the patient’s quality of life. Such treatment modalities include blocking of intestinal leukocytes infiltration by anti-adhesion agents (e.g. Vedolizumab, Etrolizumab), inhibiting pro-inflammatory mediators, such as TNFα (e.g. Infliximab, Adalimumab), and IL12/IL23 (Ustekinumab). Further treatments of IBD include JAK/STAT1 inhibition (Tofacitinib), intestinal trafficking blocking (Ozanimod) and microbiome manipulation. However, several aspects are diminishing the effectiveness of clinically available treatments, including low response rate, resistance pathways, generation of antibodies against the mAb (‘ADA’ phenomena), opportunistic infections, and diverse adverse effects [3].

Healthy intestinal homeostasis is actively balanced by immune cells [4]. When this balance is disrupted, activated immune cells accumulate.
in the affected area and mediate a prominent intestinal inflammation via both humoral and cellular immune response. The numbers and subsets diversity of intestinal leukocytes is massively altered in IBD patients. For instance, CD14+ leukocytes accumulate in the intestine of IBD patients and are responsible for the secretion of pro-inflammatory mediators. In the murine dextran sodium sulfate (DSS) colitis model, which is widely used to study gastrointestinal disorders, similar phenomenon is observed by Ly6C+ population of monocytes, macrophages, and dendritic cells. Ly6C+ leukocytes are recruited to the site of inflammation and are responsible for the production of several proinflammatory cytokines, such as TNF-α and IL6. Thus, inhibiting the pro-inflammatory characteristics of Ly6C+ leukocytes have a potential as an anti-inflammatory therapeutic approach for modulating IBD and other inflammatory disorders. Consideration previous studies, we hypothesized that the inhibition of IRF8 could serve as a therapeutically relevant pathway for controlling inflammation. Here we utilized targeted LNPs to explore the role of IRF8 as a novel anti-inflammatory therapeutic approach for modulating IBD.

2. Materials and methods

2.1. Monoclonal antibodies used in these studies

αLy6c (clone Montes1, BioXcell) Rat IgG2a isotype control (clone 2A3, BioXcell)
αLy6c (clone HK1.4, Biolegend)
αIRF8 (clone V3GYWCH, eBioscience)
Mouse IgG1 kappa isotype control (clone P3.6.2.8.1, eBioscience)
aCD45 (clone 30-F11, Biolegend)
aCD19 (clone 6D5, Biolegend)
aCD11b (clone M1/70, Biolegend)
aCD4 (clone GK1.5, Biolegend)
aCD8 (clone 53.6.7, Biolegend)
aCD3 (clone 145-2C11, Biolegend)
aIRF8 polyclonal (ThermoFisher)
aRabbit conjugated to HRP (Jackson ImmunoResearch)
aRat conjugated to HRP (Jackson ImmunoResearch)

2.2. Cell lines

RAW 264.7 cells (ATCC, TIB-71). All cells were routinely checked every two months for Mycoplasma contamination using EZ-PCR Mycoplasma Test Kit (Biological Industries) according to the manufacturer’s protocol.

2.3. siRNAs

Chemically modified Dicer-substrate siRNAs against IRF8, negative control siRNA NCS and NCS-Cy5 (siCy5) were synthesized at IDT (Coralville, Iowa, USA) using standard phosphoramide chemistry and the following sequences.

IRF8-3 siRNA : 5‘ GUCUGUGAGCUAAGAGAAUUCGGAa 3‘, 5‘ UUUCUGAGAUCUGAUUGCCAC3‘ IRF8-9 siRNA : 5‘ GCCGCAAACUUGAUAAAGAUUC 3‘, 5‘ GAAGACUUUUCACAGGGUUCGCCGC 3‘, where uppercase bases are RNA, uppercase underlined are 2‘-O-methyl RNA, and lowercase are DNA.

2.4. Lipids

DSPC, Cholesterol, DMG-PEG 2000 and DSPE-PEG 2000 were purchased from Avanti Polar Lipids. Dlin-MC3-DMA (MC3) was synthesized according to a previously described method.

2.5. Primers

2.6. Preparation of LNPs with entrapped siRNAs

LNPs were prepared according to previously described method (10). Briefly, one volume of lipid mixture (MC3, DSPC, Cholesterol, DMG-PEG, and DSPE-PEG-Ome at 50:10:5:38:140.1 mol ratio) in ethanol and three volumes of siRNA (1:16 w/w siRNA to lipid, 1:1 mol ratio of siIRF8-3 and siIRF8-9) in an acetate buffer were mixed in a microfluidic mixing device Nanoassemblr (Precision Nanosystems) at a combined flow rate of 2ml/min (0.5ml/min for ethanol and 1.5ml/min for aqueous buffer). The resultant mixture was dialyzed against phosphate buffered saline (PBS) (pH 7.4) for 16 h to remove ethanol. For Cy5-labelled particles, 15% Cy5-labelled NCS siRNA were used for flow cytometry analysis and confocal microscopy.

2.7. Size distribution

LNPs were measured by dynamic light scattering using a Malvern nano-ZS Zetasizer (Malvern Instruments Ltd).

2.8. Transmission electron microscopy

A drop of aqueous solution containing siRNA loaded LNPs was placed on a carbon-coated copper grid and dried and analyzed using a JEOL 1200 EX transmission electron microscope.

2.9. ASSET LNP incorporation and TsiLNP assembly

ASSET was incorporated into the LNPs as previously described method (10), by an incubation of ASSET micelles with LNPs for 48 h at 4 °C (1:36, ASSET:siRNA weight ratio).

2.10. siRNA encapsulation efficiency

The efficiency of siRNA encapsulation was determined by Quant-iT RiboGreen RNA assay (Life Technology) as described previously (10). Briefly, 2 µl of LNPs or dilutions of siRNA at known concentrations were diluted in a final volume of 100 µl of TE buffer (10 mM Tris–HCl, 20 mM EDTA) and 0.5% Triton X-100 (Sigma-Aldrich) in a 96-well fluorescence plate (Costar, Corning). The plate was incubated for 10 min at 40 °C to allow particles to become permeabilized before adding 99 µl of TE buffer and 1 µl of RiboGreen reagent to each well. Plates were shaken at room temperature for 30 min and fluorescence (excitation wavelength 485 nm, emission wavelength 528 nm) was measured using a plate reader (Biotek).

2.11. In vitro knockdown

RAW 264.7 cells (ATCC, TIB-71) (60% confluence) were treated with 0.2 µg/ml of siIRF8 or siNC5 entrapped in LNPs. After 24 h the cells were washed and activated with 2.5 ng/ml IFN-γ (Peprotech). 48 h after the transfection, cells were harvested and mRNA was isolated using EZ-RNA (Biological Industries), and cDNA was prepared using a cDNA synthesis kit (Quanta Biosciences). IRF8 mRNA levels were analyzed via RT-qPCR, normalized to mouse GAPDH as endogenous control. 72 h after transfection the medium was collected for cytokine analysis and the cells were taken to a dot blot assay.

2.12. Dot blot analysis

In vivo, ex vivo and in vitro samples were prepared using a solution of 150 mM NaCl, 50 mM Tris – HCl, 1 mM EDTA, 1% Triton X-100, 1% Na deoxycholic acid, protease inhibitor. Protein samples from 10 [5] cells were blotted onto a nitrocellulose membrane. After blocking in 5% low-fat milk in PBS buffer, the membrane was incubated with rabbit anti-mouse IRF8 antibodies (ThermoFisher) followed by HRP-conjugated goat anti-rabbit (Jackson ImmunoResearch), anti-mouse CD45 antibodies labeled with Alexa Flour 488 (Biolegend) and anti-Rat HRP (Jackson ImmunoResearch) as a control. ECL (Thermo Scientific Pierce) was used as a substrate solution. Signals were analyzed using Amer sham Imager 600 (GE Healthcare Life Sciences) and PXi (Syngene) instruments.

2.13. Intracellular flow cytometry

72 h after transfection with LNPs (1 µg/ml siRNA), IRF8 protein levels were assessed via intracellular Flow cytometry. RAW 264.7 cells or primary mouse splenocytes were fixed and permeabilized using FoxP3/transcription factors fixation/permeabilization assay (ebioscience), stained with membrane antibodies and directly conjugated anti-IRF8 antibodies or isotype control. Fluorescence levels were analyzed using CytoFLEX instrument (Beckman Coulter).

2.14. IRF8 associated genetic pathways

In order to gain some insights on the anti-inflammatory mechanisms of IRF8 silencing, RAW 264.7 cells (ATCC, TIB-71) (60% confluence) were treated with 0.2 µg/ml of siIRF8 or siNC5 entrapped in LNPs. After 24 h the cells were activated with 2.5 ng/ml IFN-γ (Peprotech). 72 h after the transfection, cells were harvested and mRNA was isolated using EZ-RNA (Biological Industries), and cDNA was prepared using a cDNA synthesis kit (Quanta Biosciences). mRNA levels of Cyb, Spil, CCL5, Slc11a1 and STAT1 were analyzed by RT-qPCR, normalized to mouse HPRT as endogenous control.

2.15. Animal experiments

All animal protocols were approved by Tel Aviv University Institutional Animal Care and Usage Committee and in accordance with current regulations and standards of the Israel Ministry of Health. All allocation and administered treatments. Mice were randomly divided in a blinded fashion in the beginning of each experiment.

2.16. Ex vivo experiments

siRNA loaded LNPs were incubated with fresh mouse serum for 30 min in 37 °C. Splenocytes from C57BL/6 mice, 2 × 10⁶ cells/ml in RPMI supplemented with P/S/N, L-Glu, 1% sodium pyruvate, 1% NEAA, 0.1% β-mercaptoethanol (life-technologies), were incubated for 1 h with siIRF8 or siNC5 loaded LNPs (1 µg/ml). 1 h after transfection 10% PBS was added to the medium and 24 h later the cells were activated with 10 ng/ml LPS (Peprotech). IRF8 levels were analyzed 72 h after transfection by intracellular flow cytometry and dot blot western. Cytokines levels in the medium were analyzed 72 h after transfection using IL-6, TNFα, IL12p40 and IL1β ELISA kits (R&D Systems).

2.17. Confocal microscopy

Splenocytes from C57BL/6 mice were incubated for 30 min at 4 °C with LNPs encapsulating 15% NC5-cy5 siRNA, self-assembled with ASSET and αLyz6c (bioXccll) or isotype control antibody (bioXcell). Cells were further stained with Hoechst 33342 (Sigma Aldrich) and Alexa Fluor 488 αLyz6c (Biolegend). Cells were washed, and images were analyzed using a Leica SP8 confocal microscope (Leica microsystems).

2.18. In vivo TsiLNPs binding

Cy5 labeled T-LNPs and I-LNPs were injected intravenously to C57BL/6 mice. 1 h after injection, splenocytes were isolated and stained with αLyz6c (Biolegend) mAbs. Cy5 fluorescence levels in Ly6C⁺ cells were analyzed using CytoFLEX instrument (Beckman Coulter).
2.19. In vivo IRF8 silencing

T-LNPs or I-LNPs, encapsulating siIRF8 or siNC5, were injected intravenously to 10-weeks-old C57BL/6 mice. 1 h after LNP injection the mice were euthanized and leukocytes were harvested from the spleens. Splenic leukocytes were then sorted to Ly6C+ and Ly6C− cells. The cells were cultured, 2 × 10^6 cells/ml in RPMI supplemented with 10% FBS, P/S/N, L-Glu,1% sodium pyruvate, 1% NEAA, 0.1% β-mercaptoethanol (life-technologies) and 10 ng/ml LPS (Peprotech), for 72 h and then lysed to evaluate IRF8 protein levels via western dot blot analysis.

2.20. IBD model

Colitis was induced in 10-weeks-old female C57BL/6 mice (Harlan laboratories) by dextran sodium sulphate (DSS). Mice were given 2% (wt/vol) DSS in the drinking water for 8 days. Suspensions (200 μ l in PBS) of TsilNPs loaded with siRNAs against IRF8 or negative control siRNA NC5, and self-assembled with cLyd6C or isotype control primary antibodies (BioXcell), were injected intravenously on days 3, 5 and 7 from the start on DSS treatment, at 1.5 mg/kg. Body weight was monitored every other day. On day 8 colitis severity was assessed by colonscopy, using the Murine Endoscopic Index of Colitis (MEICS). All MEICS scoring was determined based on three impartial assessments. The length of the entire colon from cecum to anus was measured. Colon sections were homogenized using a lysis solution (150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 1% Triton x-100, 1% Na deoxycholic acid and protease inhibitor), then assessed cytokines by IL-6, TNFα, IL12p40 and IL1β ELISA kits (R&D Systems). Splenocytes were isolated and stained with antibodies against CD45, CD11B, Ly6C, CD3, CD4 and CD19 (Biolegend) for 30 min at 4 °C. Splenic leukocytes subsets were analyzed using flow cytometry (CytoFLEX instrument, Beckman Coulter).

2.21. Statistical analysis

All data are expressed as median ± min to max or mean ± SD. Statistical analysis for comparing two experimental groups was performed using two-sided Student's t-test. In experiments with multiple groups we used one-way ANOVA with Dunnett’s multiple comparison post hoc test. Analyses were performed with Prism 5 (Graph pad Software). A value of p < 0.05 was considered statistically significant. Differences are labeled * for p < 0.05, ** for p ≤ 0.01, *** for p ≤ 0.001 and **** for p ≤ 0.0001. Sample size of each experiment was determined to be the minimal necessary for statistical significance by the common practice in the field. Similarity between variances of each statistically compared groups were verified by F test. Pre-established criteria for removal of animals from experiment were based on animal health, behavior and well-being as required by ethical guidelines; no animals were excluded from the experiments.

3. Results

3.1. Down regulating IRF8 in vitro via siRNA loaded LNPs

siRNA loaded LNPs were assembled using a microfluidic mixing of siRNA molecules and a lipid mixture (MC3, DSPC, Cholesterol, DMG-Peg, and DSPE-Peg at 50:10.5:38:1.4:0.1 mol ratio) under acidic conditions, using NanoAssemblr® system. The siRNA loaded LNPs were further characterized through DLS (57.63 ± 3.2 nm in diameter) with a ζ potential of 0.7 ± 0.35 mV, and visualized via TEM (Fig. 1, SI appendix, table S1). siRNA encapsulation efficiency was evaluated by Ribogreen assay (99 ± 3.1; SI appendix, table S1). The level of silencing IRF8 using siRNA loaded LNPs was assessed in vitro in Raw 264.7 cells. Raw 264.7 cells were incubated with 0.2 μg / ml siRNA encapsulated in LNPs and were activated to 2.5 ng / ml IFNγ; siIRF8-LNPs reduced IRF8 mRNA compared to negative control 5 siRNA (NC5si, IDT), by ~90% (PV < 0.0001, n = 5, Fig. 1b). A notable reduction in IRF8 protein levels by IRF8 siRNA, compared to the NC5 siRNA control, was demonstrated using flow cytometry by an intracellular staining of IRF8 protein (Fig. 1c, SI appendix, Fig. S1a–b). IRF8 downregulation was accompanied with a noticeable decrease in TNFα pro-inflammatory cytokine as assayed by ELISA (n.s., SI appendix, Fig. S1c). To gain insights into the anti-inflammatory mechanism of IRF8 silencing in Raw 264.7 cells we analyzed the transcription levels of genes that were previously found to be regulated by IRF8 protein [23–27]. The mRNA levels of genes that were previously found to be associated with IRF8 protein were examined 72 h after LNP transfection, by RT-PCR. These genes were chosen based on a comprehensive literature analysis and with the use of STRING database [28]. While the NC5 siRNA-loaded LNPs increased the transcription of Cybb, Slic11a1, and Spi1 genes, IRF8 downregulation inhibited the transcription of these genes by 2, 2 and 1.6 folds, respectively (PV = 0.005, = 0.003, < 0.0001, respectively, n = 10, SI appendix, Fig. S2a-c). CCL5 mRNA levels were significantly reduced by 1.5 folds with siNC5-LNPs treatment and 6-fold following IRF8 silencing (PV < 0.0001, n = 10, SI appendix, Fig. S2d). Furthermore, although upregulated by the NCS-siRNA loaded LNPs, a trend of STAT1 downregulation by IRF8 silencing was seen (PV = 0.018, n = 10, SI appendix, Fig. S2e). 3.2. Anti-inflammatory effect by down regulating IRF8 ex vivo

To test whether IRF8 silencing has a therapeutic anti-inflammatory potential, we analyzed the effect of IRF8 downregulation on primary leukocytes ex vivo. Primary splenocytes, isolated from C57BL/6 mice were incubated with 1 μg / ml siRNA loaded in LNPs, and were further activated by 10 ng / ml LPS. LPS was chosen as it demonstrates a wider spectrum of pro-inflammatory activation, which recapitulate better leukocytes activation in DSS treated mice. 72 h after transfection with LNPs the medium was collected for cytokine analysis and the cells were lysed for IRF8 protein levels analysis. siIRF8 but not siNC5 loaded LNPs mediated a notable reduction in IRF8 protein levels as demonstrated by dot blot assay, using splenocytes lysate (Fig. 1d). To assess the anti-inflammatory effect of IRF8 inhibition, we quantified pro-inflammatory cytokines in splenocytes conditioned media by ELISA (n = 4). Secreted TNFα levels were reduced by 23.5% with IRF8 silencing (PV = 0.0045), alongside a significant reduction in IL-6 medium concentration (PV = 0.0330, Fig. 1e–f). In addition, the concentration of the common subunit of IL-12 and IL-23, IL12p40, was decreased by 42.2% compared to NC5 control (Fig. 1g, PV < 0.0001).

3.3. Selective binding of antibody-targeted siRNA-loaded LNPs to Ly6C+ leukocytes

Ly6C+ leukocytes population was previously demonstrated to play an important pro-inflammatory role in IBD pathology [10,11]. Therefore, we aimed to selectively repogram Ly6C+ cells. cLy6C targeting antibody or isotype control were introduced to the LNPs using the ASSET platform to form targeted- (T-LNPs) and isotype control- (I-LNPs) (schematic illustration Fig. 2a, SI appendix, table S1). ASSET-LNPs were further characterized by TEM (Fig. 2b) and DLS and found to be 64.95 ± 2.4 nm in diameter with a ζ potential of 0.7 ± 0.35 mV, and visualized via TEM (Fig. 1, SI appendix, table S1). siRNA encapsulation efficiency was evaluated by Ribogreen assay as 98 ± 1.7% (SI appendix, table S1). The specificity of the T-LNPs to bind selectively Ly6C+ cells was demonstrated previously [10,11] and further assessed ex vivo by confocal microscopy and in vivo by flow cytometry using Cy5 labeled LNPs (Fig. 2c-d, SI appendix, Fig. S3a-g). Cy5 T-LNPs, but not I-LNPs, bound selectively to Ly6C+ cells. Furthermore, non-specific binding was not observed in Ly6C− cells (Fig. 2c-d, SI appendix, Fig. S3a-g). The flow cytometry analysis of TsilNPs binding in vivo demonstrates a significant increase in Cy5 fluorescence signal of Ly6C+ cells and can be appreciated by the movement of the Ly6C+ population (Fig. S3e, g).

3.4. Selective reprogramming of Ly6C+ cells

Following the anti-inflammatory effect observed ex vivo, and the establishment of a selective transfection of Ly6C+ leukocytes, we chose to test the feasibility to reprogram Ly6C+ cells through IRF8 inhibition. T-LNPs or I-LNPs, encapsulating siIRF8 or siNC5 as a control, were injected intravenously into C57BL/6 mice. Splenocytes were isolated 1 h after LNPs injection and sorted to Ly6C+ and Ly6C− cells (SI appendix, Figs S4a–c, gating strategy). The cells were cultured for 72 h and then lysed to evaluate IRF8 protein levels via western dot blot analysis which revealed a significant reduction in IRF8 levels, by T-LNPs encapsulating siIRF8, only in the Ly6C+ cells (Fig. 3a–b), compared to CD45 protein levels. A minor reduction in IRF8 protein levels was noticed when using siNC5 T-LNPs and siIRF8 I-LNPs.

3.5. IRF8 is a potential anti-inflammatory target in DSS induced colitis mice

T-LNPs or I-LNPs, encapsulating siIRF8 or siNC5 as a control, were injected intravenously to Dextran Sodium Sulfate (DSS) induced mice (the timeline of the experiment is detailed in Fig. 3c). To assess the disease severity, the mice were weighed on a daily basis, and colon morphology was evaluated by colonoscopy on day 8 from the start of the DSS induction. After euthanization, colon-length was measured as a marker for inflammation and proteins were extracted from the colon using a lysis solution for further analysis. Although no difference in weight loss was observed (Fig. 3d), a significantly healthier colon morphology was observed in mice treated with T-LNPs encapsulated siIRF8, compared to the controls. Blinded assessment was performed via mouse colonoscopy evaluation and murine endoscopic index of colitis.
severity (MEICS) scoring. Mice treated with T-LNPs silencing IRF8 demonstrated ~20% improvement in colon length compared to the controls (PV < 0.0001, n = 5), and colonoscopy, with ~35% decrease in MEICS scoring, measuring five morphological parameters: the amount of colonic fibrin, colonic transparency, colonic blood vessels, colonic granularity, and feces’ integrity (PV < 0.0001, n = 5, Fig. 3e-f). A profound improvement in colonic transparency as well as reduced amounts of colonic blood vessels and bleeding was demonstrated following a treatment with T-LNPs encapsulating siIRF8, compared to the controls (Fig. 3g).

Colonic pro-inflammatory cytokines were quantified as an indication for the severity of the intestinal inflammation (n = 5). Colonic TNFα levels were drastically reduced to baseline (PV < 0.0001, Fig. 4a). In addition, IRF8 silencing decreased the concentration of colonic IL6 cytokine and IL12p40 protein by ~60% and ~40%, respectively (PV < 0.0001, compared to T-NC5 control, Fig. 4b-c). A partial inhibition of colonic IL1β cytokine was demonstrated in all treatments, with a significant reduction by T-IRF8 treatment (PV = 0.02, Fig. 4d). Finally, the levels of Ly6C+ inflammatory monocytes in the spleen were examined as an aspect of leukocytes recruitment from the bone marrow following a peripheral inflammation. Splenic population of CD11b+ Ly6C+ inflammatory monocytes was significantly lowered following the treatments with T-IRF8 LNPs (n = 4, PV < 0.05, Fig. 4e-f, SI appendix, Fig. S5a-c).

4. Discussion

IRF8 transcription factor was previously demonstrated to have a central role in directing hematopoiesis towards the mononuclear phagocytic pathway as well as the differentiation of monocytes and several dendritic cells populations. Moreover, several studies implicated IRF8 in macrophages activation, supporting pro-inflammatory cytokines secretion and promoting Th1 and Th17 polarization in mice. Together with valuable experimental data, human genetic analysis revealed a correlation between IRF8 genetic variations and an increase susceptibility to various inflammatory disorders, such as IBD. [15] Although several studies indicated IRF8 as a pro-inflammatory mediator in various diseases [17,18,20], a profound immunomodulation therapy through IRF8 inhibition was not demonstrated. Here we show, via targeted lipid nanoparticles approach, a therapeutic anti-inflammatory effect by IRF8 silencing in Ly6C+ monocytes which were previously shown to play a critical role in IBD. IRF8 silencing ex vivo in leukocytes demonstrated promising anti-inflammatory properties, indicated by the decreased levels of inflammatory cytokines, which were previously shown to play a critical role in IBD. We have demonstrated a selective binding of TsiLNPs to Ly6C+ cells ex vivo and in vivo. Due to reasonable technical reasons, the ex vivo binding experiment showed high binding capabilities that includes also a clustering of the receptors and the shielding of αLy6C labeled-mAbs binding, while the in vivo binding experiment showed a milder, and yet selective, TsILNPs binding. This can be explained by the relative concentration of the LNPs, physiological conditions (such as temperature) and whole animal aspects such as LNPs’ clearance. We established a selective delivery of siIRF8 loaded LNPs to Ly6C+ cells and demonstrated how these LNPs mediated IRF8 down-regulation in Ly6C+ cells and an anti-inflammatory effect in vivo, in a murine colitis model. A slight decrease in IRF8 levels was detected in Ly6C+ cells when using siIRF8 loaded 1-LNPs and siNC5 loaded T-LNPs. This phenomenon can be explained by a minor unspecific uptake of I-LNPs by the phagocytic Ly6C+ cells, and through the anti-inflammatory effect of αLy6C primary Abs conjugated to the LNPs, respectively. Yet, we have previously reported [10] that ASSET platform is shielding the Fc region of the Rat IgG2a mAbs from Fc receptors recognition. IRF8 silencing led to a significant decrease in the secretion of inflammatory cytokines, such as TNFα, IL6, IL12p40 and IL1β, both ex vivo and in vivo. While colonic IL1β was reduced in all treated groups to some extent, perhaps due to anti Ly6C Abs, which possess an anti-
inflammatory capability or the negligible unspecific uptake of the LNPs by phagocytic cells, a significant reduction was demonstrated with the siIRF8-loaded T-LNPs treatment. Moreover, the inhibition of IRF8 resulted in a decreased amount of circulating CD11B⁺ Ly6C⁺ cells. Although no difference in weight loss was observed in the DSS colitis model, a significant improvement of colon’s morphology was demonstrated. This phenomenon can be explained by the acute nature of the DSS model or perhaps by other pathways by which Ly6C⁺ affect
metabolism and adipose tissues. Indications for siIRF8 anti-inflammatory mechanism were demonstrated in Raw 264.7 cells with the downregulation of the transcription of several known IRF8-related pathways, Cybb, Spi1, CCL5, Slc11a1 and STAT1 [23–27]. While several of these inflamatory genes were upregulated due to the treatment with the LNPs, as can be expected in such artificial technique with the use of siRNA and ionizable cationic lipids [29–31], a clear downregulation of these pathways was demonstrated with the use of siIRF8. Moreover, it is likely that IRF8 downregulation induce changes in multiple inflammatory genes. This should be explored in a follow-up study.

While several studies identified IRF8 as a tumor suppressor gene, controlled downregulation of IRF8 in inflammatory leukocytes has a therapeutic potential for balancing the immune response and thus chronic inflammation [32,33]. For instance, IRF8 depletion in murine colonic epithelial cells was demonstrated to result in increased incidence of colon cancer [32]. However, our selective and transient T-LNPs approach for reprograming Ly6C+ inflammatory cells, via IRF8 silencing, has a potential to overcome such side effects and to mediate a profound anti-inflammatory effect.

In this study we have established a substantial transfection ex vivo of the notoriously hard to transfec leukocytes. This technique can serve as a valuable research tool for a wide range of applications. We further demonstrated how our versatile gene manipulation platform, ASSET-based T-LNPs, can be utilized to identify new therapeutic targets, such as IRF8. Altogether, we propose that siIRF8 loaded T-LNPs, targeting inflammatory Ly6C+ cells, can serve as a new immunomodulatory modality for treating IBD and other inflammatory disorders. Furthermore, we have demonstrated the research capabilities of our targeting platform, serving not only as a powerful therapeutic technology but also as a research tool, capable of characterizing new targets for potential therapy.

Authors contribution

N.V. and D.P. conceived the study. N.V, M.G, and S.R performed the research, N.V., Y.D., E.E., M.B., I.B. and D.P. analyzed the data. N.V., Y.D, D.R and D.P. wrote the manuscript.
Data and material availability

All relevant data are available from the authors upon reasonable request.

Declaration of competing interest

D.P. declare financial interest in Quiet Therapeutics and ART Biosciences. The rest of the authors declare no other conflict of interests.

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Appendix A. Supplementary data

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