Oral RNA Interference Therapy for Celiac Disease using a Polymeric Microsphere Formulation

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Research Article

Keywords: Celiac Disease, Transglutaminase-2 (TG2), Interleukin-15 (IL-15), Oral Gene Silencing, Nanoparticles-in-Microsphere Oral System (NiMOS), Poly(I:C) Mouse Model

Posted Date: March 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-311580/v1>

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Abstract

Celiac disease is a chronic inflammatory condition characterized by activation of the immune system in response to deamidation of gluten peptides brought about by tissue transglutaminase-2 (TG2). Overexpression of interleukin-15 (IL-15) in the intestinal epithelium and the lamina propria leads to the dysregulation of the immune system, leading to epithelial damage. The goal of this study was to develop an RNA interference therapeutic strategy for celiac disease using a combination of TG2 and IL-15 gene silencing in the inflamed intestine. TG2 and IL-15 silencing siRNA sequences, along with scrambled control, were encapsulated in Nanoparticle-in-Microsphere Oral System (NiMOS) and administered in poly(I:C) mouse model of celiac disease. Single TG2 and IL-15 siRNA therapy as well the combination showed effective gene silencing in vivo. Additionally, it was found that IL-15 gene silencing alone and combination in NiMOS significantly reduced other proinflammatory cytokines. The tissue histopathology data also confirmed a reduction in immune cell infiltration as well as restoration of the mucosal architecture and barrier function in the intestine upon treatment. Overall, the results of this study show evidence that celiac disease can be potentially treated with an oral microsphere formulation using a combination of TG2 and IL-15 RNA interference therapeutic strategy.

Introduction

Celiac disease is an autoimmune disorder localized in the small intestine triggered by the ingestion of dietary gluten in susceptible patient population. This chronic inflammatory disorder is characterized by enteropathy ranging from intraepithelial lymphocytosis to total villous flattening ¹. The global prevalence of this disease is 1.4% based on serological test results ². A 2012 study showed that 1 in 141 Americans had celiac disease and most participants were unaware of their diagnoses ³. The pathogenesis of this multifactorial disease involves a combination of environmental, genetic and immunological factors ⁴. Celiac disease is polygenic in that it involves both Major Histocompatibility Complex (MHC) and non-MHC genes with an estimated 6 identified MHC and 57 non-MHC genetic variants explaining 31% of celiac disease heritability ¹. In the MHC region, alleles encoding Human Leukocyte Antigen (HLA)-DQ2 and HLA-DQ8 heterodimers contribute to the celiac disease risk with 90% affected subjects expressing HLA-DQ2 molecules and the remaining expressing HLA-DQ8 ⁴.

Dietary gluten is present in cereals such as wheat, rye, barley, contain glutenin polymers and gliadin monomers which activates celiac disease ^{5,6}. These peptides contain large amounts of prolines and glutamines, in particular a 33-mer α -gliadin which has been shown to be resistant to enzymatic hydrolysis ⁵. Because of their inability to be digested ^{7,8}, these prolines and glutamines tend to accumulate and activate the innate and adaptive immune system in genetically predisposed population ⁹. As gliadin passes through the epithelial barrier, tissue transglutaminase-2 (TG2) enzyme deamidates it creating long peptides and a negative charge which enables it's strong binding to HLA-DQ molecules, eliciting a T-cell response that cascades and causes mucosal inflammation ¹⁰⁻¹². In response to dietary gluten, celiac disease patients develop auto-antibodies specific for TG2 enzyme ¹². The detection of anti-TG2

antibodies is considered a characteristic feature of the disease. Current approach to mitigate celiac disease focuses entirely on limiting gluten from the diet.

Interleukin-15 (IL-15) is a cytokine present on the surface of all cell types, and is overexpressed under stress or inflammatory conditions¹². In celiac disease, it is overexpressed both in the intestinal epithelium and the lamina propria (LP) affecting various immune mechanisms, contributing to the pathogenesis of the disease^{12,13}. In the LP, the loss of oral tolerance to gluten is characterized by IL-15 influencing the polarization of dendritic cells (DCs) where IL-15 leads to the expansion of CD4 + T-cells in the presence of retinoic acid via the induction of IL-12-p70, producing IFN- γ ^{13,14} instead of a regulatory T-cell response. In the intestinal epithelium, IL-15 influences the gluten-independent T-cell receptor (TCR) $\alpha\beta$ intestinal epithelial lymphocytes (IELs) to kill intestinal epithelial cells (IECs) that express stress signals^{12,13,15}. IL-15 also counteracts Smad3 rendering IELs resistant to TGF β , an anti-inflammatory cytokine^{13,16}. Taken together, it is well established that IL-15 overexpression brings about the perpetuation of epithelial cell damage and T-cell proliferation in celiac disease^{12,17,18} making it a lucrative target in alleviation of the disease.

Based on emerging scientific evidence suggesting involvement of TG2 and IL-15 in the pathogenesis of celiac disease; we have hypothesized that reducing expression of either or both these proteins will potentially result in effectively reducing disease symptoms and inflammatory damage in the small intestine associated with this disease. Here, we have tested this hypothesis by evaluating impact of siRNA mediated oral silencing of TG2 and IL-15 proteins on the inflammatory and histological markers of the disease in a mouse model of celiac disease.

Our group has pioneered the development of multicompartamental formulation strategies for delivery of nucleic acid constructs including solid-in-solid polymeric Nanoparticles-in-Microsphere Oral System (NiMOS)^{19–23}. Previous studies have shown that NiMOS can be effectively used for delivery and transfection of plasmid DNA as well as siRNA in the specific regions of the gastrointestinal tract (GIT)^{19–23}. NiMOS formulation offers one or more compartment(s) that protects the payload from the harsh environment of the GIT whilst enabling delivery to targeted sites based on environmentally responsive release. This versatile platform can have different properties such as hydrophilicity to incorporate different types of drugs in the respective compartments. In these systems, type B gelatin nanoparticles (GNP) containing a nucleic acid payload were encapsulated within poly(ϵ -caprolactone) (PCL)-based biodegradable microspheres to be efficient in promoting efficient intracellular delivery of encapsulated payload at target sites in the GIT^{19–23}.

In the past, our research has shown that *TG2* and *IL-15* silencing siRNA encapsulated in GNP, is associated with RISC in the Caco-2 cells for a long period of time showing sustained effects even after administration²⁴. In an *in vitro* celiac disease model, the group showed the best suppression of TNF- α and IFN- γ when *TG2* and *IL-15* silencing siRNA were encapsulated in combination. Biodistribution and PK studies using orally administered TG2-NiMOS showed local delivery of the payload to small intestines

with minimal systemic exposure²⁵. After promising results from *in vitro* and biodistribution studies, the NiMOS formulation needed to be tested for efficacy in a diseased animal model.

In this study, we have utilized the polyinosinic/polycytidylic acid [poly(I:C)] based mouse model of celiac disease for evaluating efficacy of single and combination *TG2* and *IL-15* silencing using NiMOS for the treatment of celiac disease. We show successful oral silencing of *TG2* and *IL-15* genes, reduction in the pro-inflammatory cytokine expression, reduction in neutrophil infiltration and tissue myeloperoxidase (MPO) activity, and improved tissue healing and barrier function upon siRNA delivery.

Materials And Methods

1.1. siRNA encapsulated NiMOS formulation development and characterization

The formulation of gelatin nanoparticles and NiMOS encapsulating siRNA has been described previously^{24,25}. Briefly, gelatin nanoparticles were formulated with use of an ethanol-water solvent displacement method; 25 mg of type B gelatin of bloom strength 225 (Sigma-Aldrich, St Louis, MO) was dissolved in 20 ml of deionized water at 37°C; the pH of the solution was adjusted to 7.0 (with 0.2M NaOH solution). siRNA at 1 % w/w concentration of gelatin was added to this solution, followed by precipitation of gelatin nanoparticles by addition of absolute ethanol to final concentration of 65% v/v. The resulting gelatin nanoparticles were cross-linked by drop-wise addition of 500µL 4% glyoxal solution, under constant stirring. Excess cross-linking reagent was quenched by addition of 100µl of 1mM glycine solution. Tangential flow filtration (TFF) was performed by using a TFF filter cartridge with a molecular weight cut-off of 20,000 KDa, to remove ethanol. The aqueous gelatin nanoparticle suspension thus obtained was then concentrated to a final gelatin concentration of 5 mg/mL and used for preparation of NiMOS. 500 µL of 2.5 mg/mL suspension of gelatin nanoparticles encapsulating desired siRNA was homogenized with 5mL of 0.5% (w/v) PCL (14 KDa Sigma Aldrich (St. Louis, MO)) in dichloromethane at 9000 rpm, using a Silverson lab mixture (Model L4RT-A, Silverson Lab Machines, Bucks, England) for ten minutes. The above system was further homogenized with 10 mL of 0.1% (w/v) poly(vinyl alcohol) (PVA) in deionized water at 9000 rpm for another 5 minutes. The resulting system was magnetically stirred overnight for removal of dichloromethane, by evaporation and hardening of microspheres. On the following day, the dispersion was centrifuged at 5,000 rpm for 20 minutes and washed thrice with deionized water for removal of excess PVA, followed by freeze drying and storage at 4°C, until used.

1.2. Development of poly(I:C) mouse model of celiac disease

The animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Alnylam Corporation (Cambridge, MA).

Male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Poly(I:C) was purchased from Sigma-Aldrich (St. Louis, MO). Mice were divided into two groups, with three animals in each group. To determine if we can reproduce the poly(I:C) mouse model described in the literature²⁶, poly(I:C) was administered to mice in Group 1 by intraperitoneal route at a dose of 30 mg/kg. Mice in Group 2 served as controls, and were administered with PBS by intraperitoneal route. At four hours post dose, animals were euthanized and upper portion of jejunum was collected for evaluating expression levels of IL-15, TG2, TNF- α and IFN- γ by qPCR²⁷.

A pilot study with six mice was conducted to investigate poly(I:C) based induction of proinflammatory response in the small intestine. Mice were administered with either poly(I:C) or PBS by intraperitoneal route and euthanized at 4h post dose. A 1.6-fold increase in the expression levels of TG2 mRNA, 3.8-fold increase in expression levels of IL-15 mRNA, 3.0-fold increase in expression levels of tumor necrosis factor-alpha (TNF- α) mRNA and a 4.5-fold increase in expression levels of interferon-gamma (INF- γ) was observed after a single intraperitoneal administration of poly(I:C). These results indicated that poly(I:C) can induce an acute inflammatory response in the small intestine consistent with data reported in the literature^{28,29}.

1.3. Oral administration of siRNA encapsulated NiMOS in a poly(I:C) based mouse model

A poly(I:C) based mouse model of celiac disease was developed using C57BL/6 mice, as described previously²⁸. Male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Poly(I:C) was purchased from Sigma-Aldrich (St. Louis, MO). The animals were dosed with PBS or poly(I:C) by intraperitoneal route, and dosed orally with the control or test article as per study design shown in Fig. 1 and Table 1. The animals were divided into six groups. Animals in Group 1 received QDx6 oral doses of PBS (injection volume 200 μ L). Animals in Group 2 received QDx6 daily doses of 30 mg/kg poly(I:C) via the intraperitoneal route and QDx6 oral doses of PBS. All animals in Group 3 through 6 received QDx6 doses of poly(I:C), administered via the intraperitoneal route (lower left quadrant). Four hours post each poly(I:C) injection, animals in Group 3 received an oral dose of scramble NiMOS (NiMOS containing scramble siRNA sequence), animals in Group 4 received an oral dose of IL-15-NiMOS (NiMOS containing siRNA targeting murine IL-15 gene), animals in Group 5 received an oral dose of TG2-NiMOS (NiMOS containing siRNA targeting murine TG2 gene), animals in Group 6 received an oral dose of IL-15 + TG2-NiMOS (NiMOS containing siRNA sequences targeting murine IL-15 and TG2 genes). Dose volume for all intraperitoneal as well as oral administrations were less than or equal to 200 μ L. The experimental animals were fasted for up to a maximum of 14 hours (animals in groups 2 through 6) prior to administration of each oral dose of test article. Animals were observed for signs of distress during the first 4 hours of each Poly(I:C) injection. Animal body weights were monitored once daily during the course of the study. Animals were euthanized on Day 6 at 4, 24 and 72h post last test article dose.

Table 1

Study design for oral administration of *TG2* and *IL-15* gene silencing siRNA in NiMOS in a poly(I:C) mouse model of celiac disease

Group ^a	siRNA Dose (mg/kg)	Test Article	Number of C57/BL6 Mice	Poly(I:C) (Y/N)	Regimen	Time Points
1	0	PBS	9	N	QDx6	4, 24 and 72 h post last test article dose
2	0	PBS	9	Y	QDx6	
3	0	Scramble NiMOS	9	Y	QDx6	
4	5	IL-15-NiMOS	9	Y	QDx6	
5	5	TG2-NiMOS	9	Y	QDx6	
6	5 + 5	IL-15 + TG2-NiMOS	9	Y	QDx6	
^a Animals in Groups 2 through 6 were administered with poly(I:C) at 30 mg/kg by intraperitoneal route on Days 1 through 5. On each dosing day, test article was administered by oral route at 4-hour post poly(I:C) dose. The experimental animals in Group 1 were dosed orally with 1x PBS only.						

At each time point small intestine and blood was collected for the determination of relative IL-15 and TG2 mRNA expressions, along with profiling for proinflammatory cytokines. Tissue samples were flash frozen in liquid nitrogen and stored in a freezer set to maintain – 70°C. Blood samples were collected in K2EDTA tubes and processed for plasma separation prior to storage in a freezer set to maintain – 70°C. A small portion of the upper jejunum from the small intestinal tissue was collected in labeled 15 mL jars containing neutral buffered formalin solution for tissue histology (Heamatoxylin and Eosin (H&E) staining). A portion of upper jejunum will be flash frozen in liquid nitrogen and stored at -80°C until analysis.

1.4. qPCR assay for TG2, IL-15 and pro-inflammatory cytokine expression profiling

Total RNA was extracted from jejunal tissues using High Pure RNA Isolation Kit (Roche), as per protocol from the kit manufacturer. Relative gene expression analysis was performed for IL-15, TG2 as well as proinflammatory cytokines including interleukins (IL)-1 α , IL- β , IL-2, IL-6, IL-17, IL-18, TNF- α , IFN- γ , and Monocyte Chemotactic Protein (MCP)-1, using custom built Taqman 384-well qPCR plates (Life technologies, NY) with quantification based on the delta-delta Ct method³⁰.

1.5. H&E tissue stating and microscopic evaluations

H&E tissue sample staining was performed on histological sections prepared from tissue samples (upper jejunum) obtained from different groups. After staining, the sections were imaged using bright-field microscopy. Images were evaluated for signs of inflammatory damage such as change in mucosal architecture, cellular infiltration, villous atrophy, crypt hyperplasia and goblet cell depletion.

1.6. Tissue myeloperoxidase (MPO) activity assay

MPO is a peroxidase enzyme most abundantly expressed in the neutrophil granules and is a reliable measure of neutrophil infiltration. Upper jejunal tissue from each animal was pulverized using a Geno/Grinder (Spex, NJ). MPO Activity was determined by using FluoroMPO assay obtained from Cell Technology (Mountain View, CA). Pulverized samples (50mg) were minced with hexadecyltrimethylammonium bromide buffer (0.5% in 50mM phosphate buffer) on ice and homogenized. The homogenate was sonicated and centrifuged at 10,000 r.p.m. for 3 minutes. The detection mix containing hydrogen peroxide, a non-fluorescent detection reagent, 1x assay buffer and a known amount of tissue lysate supernatant were combined. A fluorescent analog was produced by oxidation of hydrogen peroxide in presence of MPO, was measured at an excitation wavelength of 530 nm and emission of 590 nm. Reported values were normalized to total protein content for individual samples.

1.7. Data analysis and statistics

R packages: tidyverse³¹ and ggpubr³² were used for statistical data analysis and visualizations. For comparing more than two groups, one-way ANOVA (analysis of variance) with a post hoc (Tukey) test for multiple comparisons was performed. $p < 0.05$ was regarded as statistically significant.

Results

2.1 bPoly(I:C)-induced mouse model of celiac disease

A 1.6-fold increase in the expression levels of TG2 mRNA, 3.8-fold increase in expression levels of IL-15 mRNA, 3.0-fold increase in expression levels of tumor necrosis factor-alpha (TNF- α) mRNA and a 4.5-fold increase in expression levels of interferon-gamma (INF- γ) was observed after a single intraperitoneal administration of poly(I:C) (Figure 2). These results indicated that poly(I:C) can induce an acute inflammatory response in the small intestine consistent with data reported in the literature^{28,29}.

2.2 TG2 and IL-15 gene silencing in the small intestine with NiMOS

Blank and siRNA encapsulated NiMOS formulations were developed. The particle size of the final formulation was around 2.60 μm and the siRNA encapsulation efficiency was found to be around 55% (Supplementary Figure 1).

TG2 and IL-15 mRNA expression levels in the upper jejunum of the small intestines using scrambled NiMOS and siRNA-NiMOS is shown in Figure 3. Results obtained from this analysis showed that NiMOS

mediated delivery of siRNA by oral route resulted in knockdown of target genes in the small intestine. Approximately 60% reduction from baseline of target TG2 mRNA and IL-15 mRNA was observed 4h after the last dose with around 40% reduction observed after 72h of the last dose. This shows that NiMOS were able to elicit a notable and sustained knockdown of target genes in the small intestine after oral delivery.

2.3 Proinflammatory cytokine profiling by qPCR

Profiling for proinflammatory cytokines was performed to further investigate efficacy of TG2 and IL-15 knockdown in reversing poly(I:C) mediated inflammatory damage. Expression levels of TNF- α , IFN- γ , IL-1 α , IL-1 β , IL-2, IL-6, IL-17, IL-18 and MCP-1 in different poly(I:C) treated groups relative to naïve control are shown in Figure 4. Results obtained from cytokine profiling showed statistically significant decrease in levels of proinflammatory cytokines TNF- α , IFN γ , IL-6, IL-18, MCP-1 and IL-1 β , when poly(I:C) treated mice are dosed with scramble-NiMOS is compared to IL-15- and IL-15-TG2-NiMOS. IL-1 α only shows statically significant decrease between scramble-NiMOS and IL-15-NiMOS groups. The decrease in expression levels of these cytokines was more prominent at early time points (4h post last poly(I:C) dose) when compared to those at later time points (Figure 4 and Supplementary Figure 2).

2.4 Changes in animal body weight

Percent change in animal body weights from baseline (Day 0) are shown in Figure 5. Mice in Group 1 (naïve mice) showed slight increase in body weights for the duration of the study, with a maximum increase from baseline of ~2.4%, observed on Day 6. Mice in Group 2, receiving poly(I:C) showed a gradual decrease in body weights with a maximum decrease from baseline of ~7.6%, observed on Day 6. Mice in Group 3 and 4, receiving poly(I:C)+Scramble NiMOS and poly(I:C)+TG2-NiMOS, respectively, showed a maximum decrease in body weights from baseline of ~6 to 7%, on Day 6. Mice in Group 4 and 5, receiving poly(I:C)+IL-15-NiMOS and poly(I:C)+[TG2+IL-15-NiMOS], respectively, did not show any apparent change in body weights from the baseline value. These results indicate that inhibition of TG2 expression in the small intestine does not help with normalizing poly(I:C) mediated loss in body weights. Inhibition of IL-15 expression in the small intestine had a statistically significant ($p < 0.0001$) effect in minimizing poly(I:C) mediated weight loss. Inhibition of TG2 in combination with IL-15 did not appear to have a synergistic effect in normalizing poly(I:C) mediated reduction in body weights.

2.5 H&E staining and tissue histological assessment of efficacy

Evaluation of changes in tissue histology serves as an important aspect to investigate feasibility and efficacy of anti-inflammatory treatment. Bright-field images of H&E stained intestinal sections (upper jejunum) captured (Day 7, 24h post last oral dose) using a light microscopy are shown in Figure 6. Tissue section from healthy control mice served as a baseline control for purpose of comparison. H&E staining for healthy control mice did not show any signs of inflammatory infiltration or abnormal tissue damage. In comparison to this, tissue sections from poly(I:C) treated mice showed clear signs of inflammation with profound villous atrophy, crypt hyperplasia, depletion of goblet cells and infiltration of

inflammatory cells. In contrast, tissue sections from IL-15-NiMOS and IL-15+TG2-NiMOS treated group showed a relatively healthy cellular architecture, with substantial reduction in inflammation, along with apparent signs of tissue regeneration and morphology similar to the healthy baseline group.

2.6 Neutrophil infiltration and tissue myeloperoxidase (MPO) activity measurement

Another marker for inflammatory infiltration, was assessed by determining neutrophil infiltration and MPO activity, as it is a direct measure for presence of neutrophil granulocytes. Results for MPO activity are shown in Figure 7. In healthy controls (naïve mice), MPO activity was estimated to be approximately 1.1mU/mL per total protein. MPO activity was similar in poly(I:C), poly(I:C)+Scramble NiMOS and poly(I:C)+TG2-NiMOS treated mice, with elevated levels observed at initial time points of 4h and 24h post last oral dose, and it dropped closer to the baseline at 72h post last oral dose. In contrast, MPO activity levels were markedly reduced for the IL-15-NiMOS and TG2+IL-15-NiMOS treated dose groups with the value close to that observed in naïve mice after 24h post last oral dose.

Discussion

Celiac disease is an autoimmune disorder affecting the small intestine in genetically predisposed population in response to dietary gluten. Currently, the only possible treatment option for the patients affected by this disease is to follow a strict gluten-free diet. In this study we have evaluated the impact of local orally delivered siRNA mediated silencing of intestinal TG2 and IL-15 genes on the therapeutic effect and reduction in inflammatory markers associated with the pathogenesis of celiac disease.

Several types of celiac disease models have been developed by researchers, which recapitulate different aspects of celiac disease^{33,34}. Poly(I:C) based mouse model is one such system that has been used by several researchers for inducing signs and symptoms of celiac disease³⁴. Poly(I:C) is a toll-like receptor-3 (TLR3) agonist that has shown to induce intestinal enteropathy similar to that observed in celiac disease³⁵. In the first study which showed the effects of poly(I:C) on intestinal histopathology by Garside et al.³⁶, (CBAXBALB/c) F1 mice were injected intraperitoneally with poly (I:C), which resulted in a significant villous atrophy and crypt hyperplasia within one day post injection and recovery by 3 days post injection. In a later study, it was found that intestinal epithelial cells express high levels of IL-15 upon treatment with poly(I:C), causing an expansion of cytotoxic intraepithelial lymphocytes (CD8 α +) ³⁷. As per other reports, intraperitoneal injection of poly(I:C), has resulted in transient small intestinal injury along with activation of tissue TG2 ^{28,29}. Collectively, these results indicate that poly(I:C) based small intestinal tissue damage recapitulates majority of signs and symptoms of celiac disease. In our studies, we have shown that poly(I:C) causes upregulation of proinflammatory cytokines (Fig. 4) in the small intestine along with increased neutrophil infiltration and disruption of healthy intestinal architecture (Fig. 6) that are similar to those observed in patients with celiac disease³⁸.

While there are many advantages of using siRNA in causing controlled silencing of specific genes, the delivery and the subsequent endosomal release of these labile molecules, especially after oral

administration in the harsh GIT environment, is challenging, Since the activation and progression of celiac disease is restricted to the small intestines, oral siRNA delivery and local transfection would be ideal as a treatment option.

The multi-compartmental NiMOS used in this study uses type B gelatin nanoparticles that physically entraps the siRNA and is encapsulated further in acid- and protease-resistant PCL microsphere that protects the payload from the stomach and releases specifically in the small intestine in response to local lipases with the digestive fluid ³⁹. The physical entrapment of nucleic acids by type b-gelatin aids in cellular uptake and cytosolic release of encapsulated siRNA ²⁴, shown to be essential for promoting targeted mRNA silencing efficacy ²⁴. PCL on the other hand would protect the gelatin against pH and protease degradation in the stomach, thereby promoting delivery of encapsulated siRNA(s) to the intestinal sites ^{25,39} - making this an ideal platform for oral local intestinal delivery of nucleic acids.

The data shown in this study indicate that NiMOS mediated oral siRNA delivery results in efficient target gene silencing in the small intestine. While the silencing of TG2 expression through TG2-NiMOS did not appear to correct the poly(I:C) mediated upregulation of proinflammatory cytokines, silencing of IL-15 expression through IL-15-NiMOS resulted in a significant reduction in expression of TNF- α , IFN γ , IL-1 α , IL-1 β , IL-6, IL-18 and MCP-1 at 4 and 24h time points post dose. IL-15 knockdown did not appear to influence elevated levels of IL-2 and IL-17. Results obtained with adjunct knockdown of TG2 and IL-15 through TG2 + IL-15-NiMOS were similar to those obtained with specific IL-15 silencing (through IL-15-NiMOS), indicating that TG2 silencing did not influence anti-inflammatory effects of IL-15 inhibition. The percent change in animal body weight as compared to Day 0 was maintained for IL-15-NiMOS and TG2 + IL-15-NiMOS. H&E stained upper jejunum histopathology post 24h of last oral dose, shows significant recovery for IL-15-NiMOS and TG2 + IL-15-NiMOS formulation treated groups, compared to other controls. There was also a notable reduction in MO activity at the 24h post last dose time point in IL-15-NiMOS and TG2 + IL-15-NiMOS formulation treated groups. Collectively, the present study along with work of others ⁴⁰ suggest that silencing of overexpressed IL-15 in celiac disease has the potential to be an efficacious treatment option for patients with celiac disease, and warrants further non-clinical and clinical research.

Inhibition of TG2 expression via TG2-NiMOS did not show a noticeable impact on alleviating proinflammatory effects of poly(I:C) in the small intestine. These results were not unexpected, as the TG2 (overexpressed in the poly(I:C) mouse model ²⁹) inhibition in this mouse model could only impact the ongoing inflammatory cascade. It is important to note that while the poly(I:C) animal model recapitulates the inflammatory features of celiac disease, it is not sensitive to gluten challenge – meaning irrespective of gluten challenge, poly(I:C) would lead to inflammatory damage in the small intestine. In patients with celiac disease, TG2 inhibition may potentially be beneficial as TG2 play a role prior to activation of the inflammatory pathway, where it mediates deamidation of the α -gliadin peptides, forming immunogenic peptides that subsequently trigger the pathway. Having shown here that TG2-NiMOS and IL-15 + TG2-NiMOS can reduce TG2 expression in the small intestine (Fig. 3), our strategy involving co-administration

of siRNAs against TG2 and IL-15 may prove to be effective in patients with celiac disease and warrants further non-clinical development.

Inhibition of IL-15 by either IL-15-NiMOS or IL-15 + TG2-NiMOS had a significant impact on neutralizing proinflammatory effects of poly(I:C) in the small. These results indicate that overexpression of IL-15 cytokine is a major contributor for inflammatory damage caused by poly(I:C) in mice. Further, this also indicates that inhibition of IL-15 may prove to be an efficacious strategy for treating inflammatory diseases such as celiac, where IL-15 is overexpressed.

Conclusion

Previously, we have shown that NiMOS holds immense promise to deliver nucleic acid payload locally to the different regions of the GIT. In this study, we further explored the platform to deliver TG2 and IL-15 siRNA to alleviate the inflammation caused in celiac disease using a poly(I:C) murine model. The formulations explored comprised of individual as well as combination of TG2 and IL-15 siRNA. IL-15- and IL-15 + TG2-NiMOS showed alleviation in inflammation as seen by gene knockdown, statistically significant reduction in proinflammatory cytokines, no apparent body weight changes, relatively healthy cellular architecture and reduced immune cell proliferation when compared with scramble-NiMOS. While TG2-NiMOS didn't reduce proinflammatory cytokines in the poly(I:C) murine model, it does show silencing of TG2 mRNA in the small intestines of the murine model. Taken together, these results show that TG2 and IL-15 siRNA encapsulated NiMOS has the potential to be utilized in the treatment of celiac disease.

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Table

Table 1. Study design for oral administration of *TG2* and *IL-15* gene silencing siRNA in NiMOS in a poly(I:C) mouse model of celiac disease

Group ^a	siRNA Dose (mg/kg)	Test Article	Number of C57/BL6 Mice	Poly(I:C) (Y/N)	Regimen	Time Points
1	0	PBS	9	N	QDx6	4, 24 and 72 h post last test article dose
2	0	PBS	9	Y	QDx6	
3	0	Scramble NiMOS	9	Y	QDx6	
4	5	IL-15-NiMOS	9	Y	QDx6	
5	5	TG2-NiMOS	9	Y	QDx6	
6	5+5	IL-15+TG2-NiMOS	9	Y	QDx6	

^a Animals in Groups 2 through 6 were administered with poly(I:C) at 30 mg/kg by intraperitoneal route on Days 1 through 5. On each dosing day, test article was administered by oral route at 4-hour post poly(I:C) dose. The experimental animals in Group 1 were dosed orally with 1x PBS only.

Figures

Figure 1

Schematic representation of dosing schedule with control and test formulations.

Figure 2

Poly(I:C)-induced mouse model of celiac disease. Induction of tissue transglutamine-2, interleukin-15, tumor necrosis factor- α , and interferon- γ by poly(I:C) treatment relative to untreated control.

Figure 3

Intestinal transglutaminase-2 and interleukin-15 gene silencing with NiMOS. Relative expression profile of (A) transglutaminase-2 (TG2) mRNA and (B) interleukin-15 (IL-15) mRNA in the small intestine after multiple oral doses of control (scrambled sequence) and target siRNA containing NiMOS formulations in poly(I:C) treated mice; **** $p < 0.0001$, *** $p < 0.001$; $n = 3$.

Figure 4

Relative expression of pro-inflammatory cytokines in the small intestine. Relative expression of proinflammatory cytokines the small intestines at 4-hours post last dose in control and treatment groups; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; $n = 3$.

Figure 5

Relative change in body weight. Percent change in animal body weights normalized to Day 0, over the duration of the study after intraperitoneal injection of poly(I:C) followed by oral siRNA delivery with NiMOS formulations; **** $p < 0.0001$; $n = 9$.

(A) Naïve Control

(B) Poly(I:C)+Scramble NiMOS

(C) Poly(I:C)+TG2 NiMOS

(D) Poly(I:C) + IL-15 NiMOS

(E) Poly(I:C)+[TG2+IL-15 NiMOS]

Figure 6

Histological assessment of intestinal tissue. Microscopic evaluation of intestinal tissue (upper jejunum) histopathology (H&E staining) for tissues harvested from control and test groups, 24h post last oral dosing.

Figure 7

Neutrophil infiltration and tissue myeloperoxidase activity. Tissue myeloperoxidase (MPO) activity in the small intestine normalized to the total protein content in each sample; * $p < 0.05$, ** $p < 0.01$; $n = 3$.

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