Pharm Res (2012) 29:883–897
DOI 10.1007/s11095-011-0616-1

RESEARCH PAPER

Cationic Nanomicelles for Delivery of Plasmids Encoding Interleukin-4 and Interleukin-10 for Prevention of Autoimmune Diabetes in Mice

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Received: 15 September 2011 / Accepted: 24 October 2011 / Published online: 11 November 2011

ABSTRACT

Purpose To evaluate the *in vivo* transfection efficiency of N-acyl derivatives of low-molecular weight chitosan (LMWC) to deliver pVIVO2-mIL4-mIL10 plasmid encoding interleukin-4 (IL-4) and interleukin-10 (IL-10) in multiple, low-dose streptozotocin induced diabetic mouse model.

Methods N-acyl LMWC nanomicelles were characterized for size and charge. The pVIVO2-mIL4-mIL10/N-acyl LMWC polyplexes were injected intramuscularly in mice and compared for transfection efficiency with naked DNA and FuGENE® HD. Bicistronic pVIVO2-mIL4-mIL10 plasmid was compared with individual plasmids encoding IL-4 and IL-10 for efficacy. The levels of blood glucose and serum IL-4, IL-10, TNF-α and IFN-γ were monitored. The ability of plasmid administration to protect from insulitis and biocompatibility of N-acyl LMWC were studied.

Results The N-acyl LMWC led to significantly higher ($p < 0.05$) expression of IL-4 and IL-10 and reduced the levels of blood glucose, TNF-α and IFN-γ, especially in animals treated with pVIVO2-mIL4-mIL10 plasmid. The pancreas of pDNA/N-acyl LMWC polyplex treated animals exhibited protection from insulitis and the delivery systems were found to be biocompatible.

Conclusions N-acyl derivatives of LMWC are efficient and biocompatible gene delivery vectors, and the administration of bicistronic pVIVO2-mIL4-mIL10 plasmid polyplexes can protect the pancreatic islets from insulitis, possibly due to the synergistic effect of IL-4 and IL-10 encoding plasmids.

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KEY WORDS chitosan · diabetes · gene delivery · interleukin · nanomicelles

INTRODUCTION

Gene therapy represents an exciting field of interest in recent drug delivery, since it is possible to treat/prevent many genetic disorders by simply inserting the correct copy of the defective gene into target cells. It offers multiple opportunities of curing diseases rather than just treating the symptoms of diseases or disorders. Efficient delivery of gene and its subsequent stable expression *in vivo* is still a major obstacle which needs to be overcome prior to gaining clinical acceptance. Currently, the major strategies to deliver genes of interest are physical insertion and use of viral and non-viral vectors (1). The main issue associated with physical techniques, including gene gun, electroporation, and ultrasound, is the lack of sustained expression of the introduced genes in the target tissue, leading to frequent administration (2). Similarly, though use of viral vectors seems to be an effective strategy, the potential immunological problems associated with them can greatly reduce their acceptance in clinical settings. These issues along with their low safety profile have driven a need for effective non-viral gene delivery systems (3). Non-viral gene delivery using biodegradable polymeric nano-carriers, or lipid based particles, have shown to circumvent the difficulties associated with viral vectors as well as physical methods, and exhibited sustained expression of the inserted genes (4–7). It has been reported that these non-viral gene delivery vectors possess number of advantages including control over DNA release, stability, ease of modification by various functional groups to specifically target certain type of cells etc.

Type 1A diabetes, which results from the activation of T_H type 1 (T_H1) lymphocytes, is one of the candidate diseases which can potentially be treated by gene therapy. It is reported that the cell-mediated autoimmune destruction of β-cells of pancreas is the main pathologic feature of type 1A diabetes (4). Number of studies have been performed to suppress the progression of autoimmune diabetes by injecting immunosuppressive cytokines such as interferon (IFN)-γ, transforming growth factor (TGF)-β1 and interleukin (IL)-10 directly in non-obese diabetic (NOD) mice by various routes (8–12). It has been reported that biolistic-mediated interleukin-4 gene transfer, or repeated administration of IL-4 cytokine successfully prevents the onset of type 1 diabetes in NOD mice (13–15). Combined delivery of IL-4 and IL-10 cytokines was also proved to be beneficial in preventing autoimmune diabetes (16). Many studies have demonstrated the ability of direct gene transfer of naked DNA after injecting it into the skeletal muscle, liver, heart, and skin (17). However, a stable and effective gene transfer for sustained period is still questionable. The major drawback associated with the use of unformulated plasmid and viral based formulations is the short-term bioavailability of the bolus dose (18). The combined delivery of plasmid DNA encoding IL-4 and IL-10 using poly[gamma-(4-aminobutyl)-L-glycolic acid] (PAGA) polymer prevented the development of autoimmune diabetes in NOD mice (19). It has also been reported that the delivery of a chimeric plasmid encoding for both IL-4 and IL-10 is useful in delaying the autoimmune insulitis efficiently in NOD mice (20).

Increasing evidence reveals that the delivery to the skeletal muscle could be an efficient route for gene delivery (21–23). Since skeletal muscle is easily accessible, highly vascular, has larger mass (comprising about 40% of the total body mass) and has non dividing muscle fibers, makes it a suitable route for sustained gene delivery and long-lasting protein expression (24–26).

We recently synthesized a series of N-acyl substituted LMWC as an efficient gene delivery vector, and optimized the chain length and degree of unsaturation in the N-acyl grafts for gene delivery application *in vivo* (27). The synthesized polymers were able to form nanomicelles (~80–90 nm) and found to be non-toxic *in vitro* in the tested concentration range. The graft polymers, prepared by the modification of LMWC with 18-carbon chains having one or two double bonds (oleic and linoleic acids), could form stable complexes with DNA and protect it from enzymatic degradation and proved to be efficient non-viral gene delivery vector *in vitro*. These synthesized polymers also showed enhanced transfection compared to naked DNA as well as their respective LMWC control (27). In this study, we evaluated the ability of N-oleyl and N-linoleyl derivatives of LMWC to successfully deliver the therapeutic genes encoding for IL-4, IL-10, their physical mixtures (IL-4+IL-10), and a bicistronic plasmid encoding for both IL-4 and IL-10 *in vivo* in streptozotocin (STZ) induced diabetic mice model. The formulations containing pDNA/N-acyl LMWC polyplexes were injected intramuscularly at a single dose into the anterior tibialis muscle of mice, and the IL-4 and IL-10 levels in the serum of mice were measured for 6 weeks by enzyme-linked immunosorbent assay (ELISA) and compared to that of control. Since INF-γ and TNF-α are among the cytokines that lead to enhanced β cell destruction (28), their levels were also measured throughout the study period as an indication of progression of insulitis. Further, the biocompatibility of the delivery system was determined by injecting a single dose of delivery system into the muscle and then observing the presence of inflammatory cells at various time points.

MATERIALS AND METHODS

Materials

Chitosan (DD 85%, Mw 50kD) and sodium nitrite ($NaNO_2$) were procured from Sigma-Aldrich, St. Louis, MO. Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Advanced Chemtech, KY and N-hydroxysuccinimide (NHS) was obtained from Alfa Aesar, Lancashire, UK. The plasmids, pUMVC3-mIL4, encoding mouse IL-4 and pUMVC3-mIL10, encoding mouse IL-10, were generously donated by Aldevron LLC, Fargo, ND and Contour® glucometer was a gift from Bayer, Tarrytown, NY. FuGENE® HD was obtained from Roche Applied Science, Mannheim, Germany. The bicistronic pVIVO2-mIL4-mIL10 plasmid encoding both mouse IL-4 and IL-10 was purchased from Invivogen, San Diego, CA. ELISA Max™ kits for quantification of IL-4, IL-10, TNF-α and IFN-γ were procured from Biolegend, San Diego, CA. All chemicals used in the study were analytical or cell culture grade as applicable and de-ionized water was used to prepare all aqueous solutions and buffers.

Plasmids

The pUMVC3-mIL4 and pUMVC3-mIL10 plasmids encoding mouse IL-4 and IL-10 had size of 4.5 and 4.4 kb, respectively. Both plasmids had a CMV early promoter, Rabbit beta-globin polyadenylation signal and kanamycin resistance gene. The pVIVO2-mIL4-mIL10 plasmid had FerH (heavy chain) and FerL (light chain) human ferritin composite promoters with the SV40 and CMV enhancers, respectively. Also, their 5'UTRs were replaced by the 5'UTR of the mouse and chimpanzee EF1α genes and a hygromycin resistance gene was incorporated for clonal selections. The structures of the plasmids are shown on Fig. 1.

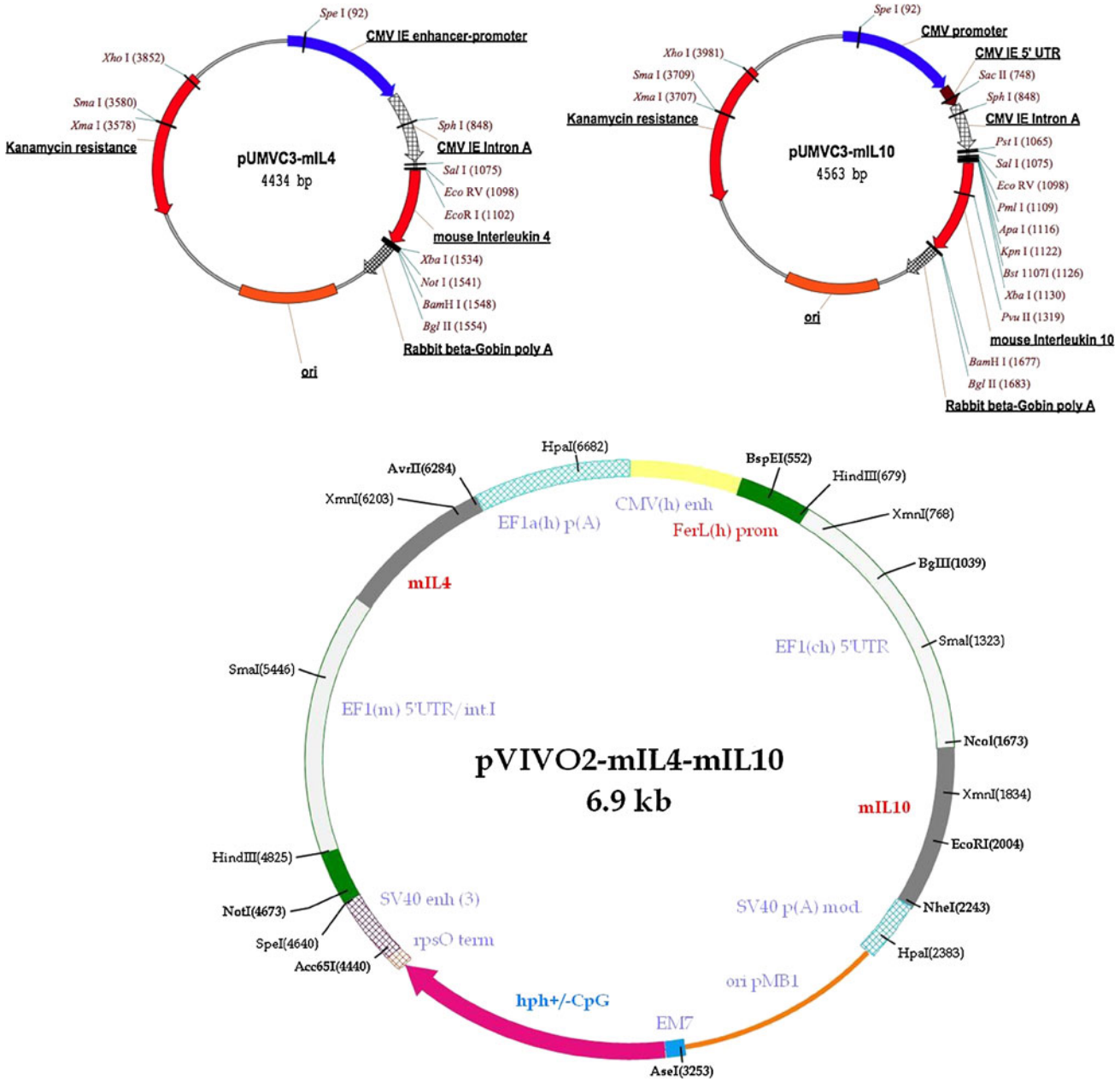

Fig. 1 Structures of pUMVC3-mIL4, pUMVC3-mIL10 and pVIVO2-mIL4-mIL10 plasmids.

Synthesis and Characterization of N-acyl LMWC and Preparation of Polyplexes

LMWC and N-oleyl and N-linoleyl derivatives of LMWC were synthesized and characterized as per the procedures described in our earlier publication; however, some essential parameters were determined again for reproducibility (27). The structure and degree of substitution of the derivatized polymers were confirmed by Infrared (IR) spectroscopy, while critical micellar concentration (CMC) was determined by pyrene probe method. The essential characteristics such as hydrodynamic size and zeta potential were also re-determined and are presented in Table I.

The aqueous solutions of N-acyl LMWC were prepared by adding N-acyl LMWC in deionized water, stirring at 50 RPM for 10 min, sonicating the solutions at 75 W and 20 kHz using a probe sonicator (Model 150 V/T, Biologics, Manassas, VA) to re-disperse the nanomicelles and filtering the solutions through a 0.8 μ syringe filter. The pH of the solutions was adjusted to 7.4 using 0.1 N HCl and 0.1 N NaOH as required. The polyplexes between pDNA and N-acyl LMWCs were prepared by self-assembly at a previ-

Table I Properties of N-acyl LMWC

Parameters	N-oleyl LMWC	N-linoleyl LMWC
Degree of substitution (%)	~35	~36
CMC (mg/ml)	<0.0156	<0.0313
Hydrodynamic size: nanomicelles (nm) ± SD	95.1 ± 7.89	89.7 ± 6.43
Hydrodynamic size: pDNA/nanomicelles polyplexes (nm) ± SD	103.2 ± 5.77	97.7 ± 4.89
Zeta potential: nanomicelles (mV) ± SD	30.1 ± 0.48	34.1 ± 0.63
Zeta potential: pDNA/nanomicelles polyplexes (mV) ± SD	19.7 ± 0.55	15.3 ± 0.80

ously optimized N:P ratio of 20:1. In short, the pDNA was incubated with the aqueous solution of N-acyl LMWC and allowed to incubate for 30 min at room temperature. The complex was formed due to the ionic interactions between negatively charged DNA and positively charged polymer. The polyplex formation was confirmed by agarose gel retardation assay and the polyplexes were characterized for their hydrodynamic size and zeta potential using dynamic light scattering as shown in Table I.

In Vivo Studies

Male, 5–6 week old, BALB/c mice were obtained from Harlan, Indianapolis, IN and housed in a pathogen free environment and maintained at 12 h dark and 12 h light cycle. The animals were acclimatized for a week before starting the study and food and water were provided *ad libitum* for the duration of study. The animal studies were performed in compliance with the "Principles of Laboratory Animal Care" (NIH publication #85–23, revised in 1985) with the approval from Institutional Animal Care and Use Committee (IACUC) at North Dakota State University (protocol A10052). The mice were divided into 18 groups of six animals each. The animals in the control group were treated with saline, while streptozotocin treated animals were used as a negative control. Passive control groups consisted of animals treated with either naked plasmids (IL-4 or IL-10) or a physical mixture of equal volumes of plasmids (IL-4 + IL-10) or bicistronic plasmid encoding for both IL-4 and IL-10, directly administered into the anterior tibialis muscle without any polymeric delivery system. Animals in the positive control groups were treated with either IL-4, or IL-10 or a mixture of IL-4 and IL-10, or a bicistronic plasmid encoding for both IL-4 and IL-10 using FuGENE® HD as a delivery vector. The treatment groups were divided into 8 different groups and the details about the grouping structure is depicted in Table II.

The formulations were injected as a single dose, corresponding to 50 μg of pDNA per animal, into the anterior tibialis muscle of mice. Except for the animals in the saline control group, all other groups received intraperitoneal injections of streptozotocin (40 mg/kg of body weight) for 5 consecutive days. The blood glucose levels of the animals were monitored on a daily basis, and the animals were considered diabetic if the random blood glucose level for the animal is 200 mg/dl or more for two consecutive days. After first week, blood sampling was performed after on a weekly basis using tail vein puncture. Blood glucose levels were measured using Bayer Contour® glucometer. The blood samples, collected at specific time points, were centrifuged at 4°C in a cooling centrifuge, and the serum was separated. The serum was stored at −20°C until further analysis. ELISA was used to determine the levels of IL-4, IL-10, TNF-α and Interferon-γ (IFN-γ) in the serum samples. The expression of the proteins IL-4 and IL-10 was compared throughout the study. At the end of the 6 week period, the animals were euthanized by injecting pentobarbital (150 mg/kg body weight) intravenously through tail vein. The pancreas and anterior tibialis muscle were dissected and studied for histopathological changes to assess the ability of formulation to protect pancreatic beta cells and the biocompatibility of the formulations, respectively.

Table II The Grouping Structure for *In Vivo* Studies

1.	Saline (Control)
2.	Streptozotocin Control (Negative control)
3.	Naked IL-10 Plasmid (Passive Control)
4.	Naked IL-4 Plasmid (Passive Control)
5.	Mixture of naked IL-4 and IL-10 Plasmids (Passive Control)
6.	Bicistronic IL-4 and IL-10 Plasmid (Passive Control)
7.	FuGENE® HD+ IL-4 Plasmid (Positive Control)
8.	FuGENE® HD+ IL-10 Plasmid (Positive Control)
9.	FuGENE® HD+ Mixture of IL-4 and IL-10 Plasmid (Positive Control)
10.	FuGENE® HD+ Bicistronic IL-4/ IL-10 Plasmid (Positive Control)
11.	N-oleyl LMWC+ IL-4 Plasmid
12.	N-oleyl LMWC+ IL-10 Plasmid
13.	N-oleyl LMWC+ Mixture of IL-4 and IL-10 Plasmids
14.	N-oleyl LMWC+ Bicistronic IL-4/IL-10 Plasmid
15.	N-linoleyl LMWC+ IL-4 Plasmid
16.	N-linoleyl LMWC+ IL-10 Plasmid
17.	N-linoleyl LMWC+ Mixture of IL-4 and IL-10 Plasmids
18.	N-linoleyl LMWC+ Bicistronic IL-4/IL-10 Plasmid

Blood Glucose Level Determination

A glucometer (Bayer Contour® Glucometer, Mishawaka, IN, USA) was used to measure the blood glucose levels of mice.

ELISA for Determination of IL-4, IL-10, IFN-γ and TNF-a in Serum

BioLegend's ELISA Max™ Deluxe Set sandwich Enzyme Linked Immunosorbent Assays were used to determine the concentration of IL-4, IL-10, IFN-γ and TNF-α cytokines in the blood samples of mice (29). The procedures were followed as per manufacturer's protocol. In short, prior running the ELISA, mouse specific capture antibodies for IL-4, IL-10, IFN-γ and TNF-α were coated on the plates by incubating at for 16 h at 4°C. The plates were washed thoroughly with wash buffer (Phosphate-Buffered Saline + 0.05% Tween-20, pH 7.4) and residual buffer was removed by tapping the plate. The plates were then incubated with assay diluent at room temperature (RT) for 1 h to block the non-specific binding. Plates were washed again, and suitable dilutions of standard stock solution of IL-4, IL-10, IFN-γ and TNF-α as well as the samples were added to the plates, followed by incubation at RT for 2 h. After incubation, plates were washed followed by addition of biotinylated anti-mouse detection antibodies for IL-4, IL-10, IFN-γ and TNF-α, respectively, to produce an 'antibody-antigen-antibody sandwich'. After 4 h of incubation, an enzyme, avidin-horseradish peroxidase (avidin-HRP) was added to the plates, and the plates were incubated a RT under shaking conditions followed by addition of TMB substrate. The plates were incubated in dark for 20 min until the blue color was developed, followed by addition of stop solution (150 µl of 2 N H_2SO_4). The reaction color changed from blue to yellow after addition of the stop solution. The intensity of yellow color developed was measured at 450 nm with a microplate reader. The standard curves plotted using the dilutions of standard stock solutions prepared in the ranges of 2–125 pg/ml, 31.3–2,000 pg/ml, 15.6–1,000 pg/ml and 7.8–500 pg/ml were used to estimate the concentration of IL-4, IL-10, IFN-γ and TNF-α, respectively, in mice serum.

In Vivo Biocompatibility of the Delivery System

At 1, 4, and 6 week after the administration of formulations, the animals were euthanized by injecting pentobarbital (150 mg/kg body weight) intravenously through tail vein. The injection site of the formulations (anterior tibialis muscle) was dissected, fixed in 10% neutrally buffered formalin solution, sectioned, stained with hematoxylin-eosin stain and studied for histopathological changes to assess the biocompatibility of the formulations. The presence of inflammatory cells, mononuclear infiltration, fibrosis, and necrosis if any, was noted.

Histopathological Changes in Pancreas

At 1 week, 4 week and 6 week time after the administration of formulations, the mice were euthanized, the pancreas were removed and fixed in 10% neutrally buffered formalin solution, sectioned and stained with hematoxylin-eosin stain. The the progression of insulitis in the pancreatic cells of treated animals was asseseed and copared with control (untreated) pancreas to determine the therapeutic efficacy of expressed IL-4 and IL-10 to supress the autoimmune insulitis.

Statistical Analysis

For all *in vivo* studies, 6 replicates were used and single factor ANOVA was performed using SAS 9.2 (SAS Institute, NC). A probability value of less than 0.05 was considered to be significant.

RESULTS

Preparation of pDNA/ N-acyl LMWC Polyplexes

N-oleyl and N-linoleyl derivatives of LMWC were synthesized successfully by the procedure described in our earlier publication (27). The major chitosan fraction with average molecular weight of approx. 6,500 Da was selected and N-oleyl and N-linoleyl LMWC were synthesized. We have previously described the structural characterization of the N-acyl LMWCs using IR spectroscopy and the results were validated using elemental analysis (27). The IR spectra of N-acyl LMWC confirmed the structure of the polymer synthesized, and the degree of substitution of the derivatised polymers was found to be 35–36%, while critical micellar concentration (CMC) was <0.0312 mg/ml for both the polymers as determined by pyrene probe method. The key features of the N-oleyl LMWC and N-linoleyl LMWC are summarized in Table I.

Blood Glucose Level Determination

The blood glucose levels in mice treated with the polymeric delivery systems as well as the control groups are presented in Fig. 2 (A-D). It was observed that the blood glucose levels of mice in passive control groups (Fig. 2-A) (naked plasmid DNA encoding for IL-4, IL-10, mixture of IL-4 and IL-10, and bicistronic plasmid encoding for both IL-4 and IL-10, injected intramuscularly) were significantly higher compared to saline control and were comparable with STZ

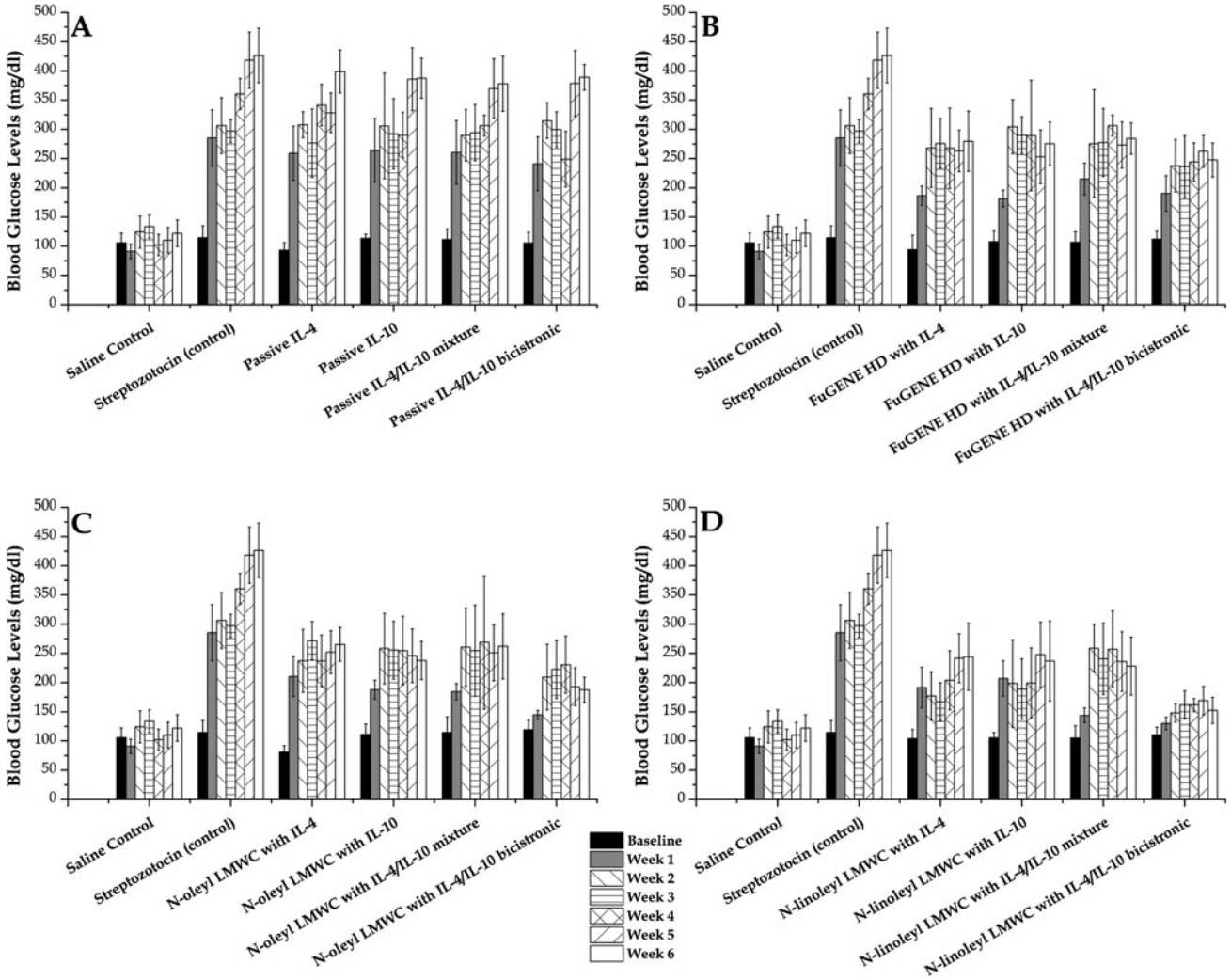

Fig. 2 Mean blood glucose levels (mg/dl). Vertical bars indicate standard deviation ($N = 6$).

treatment group from week 1 to 6. This indicates that there is no appreciable advantage of direct injection of naked plasmids *in vivo*. The animals in the positive control groups where FuGENE® HD was used as a delivery vector showed higher blood glucose levels compared to saline control, but were significantly lower than STZ treatment group, indicating the need of a delivery system to deliver the plasmid DNA *in vivo* (Fig. 2-B). Among these four positive control groups the blood glucose levels in the animals treated with bicistronic plasmid were lower compared to that of IL-4, IL-10 or mixture of IL-4 and IL-10.

The blood glucose levels of the mice in the treatment groups 11–14, where N-oleyl LMWC was used as a delivery vector is presented in Fig. 2-C. It was observed that the blood glucose levels were significantly lower ($p<0.05$) than the streptozotocin control. Even though the mice treated with bicistronic plasmid (group 14) performed better compared to the rest treatment groups, the blood glucose levels were higher than the saline control at all time points.

The animals treated with plasmid DNA in a N-linoleyl LMWC micellar system, showed significantly ($p<0.05$) low blood glucose levels as compared to STZ control. When the treatment groups 15–18 were compared among themselves, it was noted again that the group containing N- linoleyl LMWC delivery system containing bicistronic plasmid performed considerably better. The blood glucose levels were comparable to saline control and were significantly lower ($p<0.05$) than the groups treated with single gene expression plasmids or physical mixture of IL-4 and IL-10 plasmids (Fig. 2-D).

Comparison of IL-4 Expression

The results of IL-4 expression determined by ELISA are presented in Fig. 3 (A-D). Figure 3-A depicts the IL-4 level of animals treated with naked plasmids (passive controls, groups 3, 5 and 6). It was observed that the expression of IL-4 was significantly higher in the passive control groups

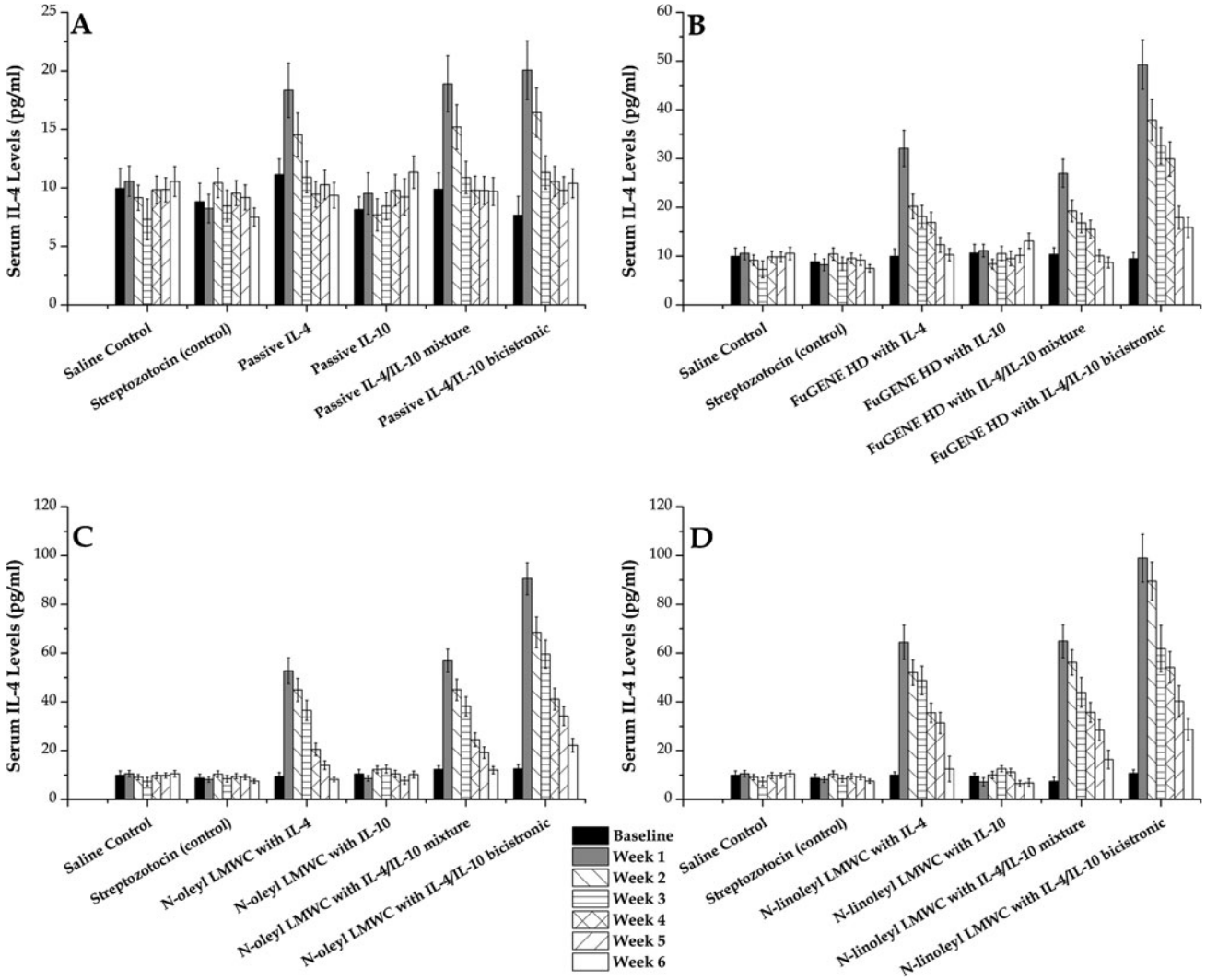

Fig. 3 Mean serum IL-4 levels (pg/dl). Vertical bars indicate standard deviation ($N = 6$).

treated with naked IL-4 plasmid (group 3), mixture of IL-4 and IL-10 (group 5), and bicistronic plasmid (group 6) initially at weeks 1 and 2. But, the levels of IL-4 dropped significantly after week 3 and were comparable to that of saline control as well as STZ control at the end of 6 week period. This indicated the transient expression resulted due to the injection of naked plasmids.

The expression levels of IL-4 in the FuGENE® HD treated groups is shown in Fig. 3-B. The delivery system containing FuGENE® HD performed significantly better at all time points compared to saline and STZ control. IL-4 expression was significantly higher ($p < 0.05$) in the group treated with bicistronic plasmid delivered with FuGENE® HD, as compared to other positive controls, saline treated as well as STZ treated groups.

The expression results for IL-4 using N-oleyl LMWC and N-linoleyl LMWC are presented in Fig. 3-C and 3-D, respectively. When N-oleyl LMWC was used as a gene carrier, it was observed that the IL-4 cytokine expression increased significantly from baseline (11.4 ± 1.6 pg/ml) to 52.7 ± 5.3 pg/ml for IL-4 plasmid, 56.9 ± 4.7 pg/ml for physical mixture (IL-4+IL-10), and 90.5 ± 6.6 pg/ml for bicistronic plasmid at week 1. Similar trend was observed in case of treatment groups containing N-linoleyl LMWC polymeric delivery system. The expression of IL-4 was 9.9 ± 1.7 pg/ml at baseline, and was highest at 1 week. The IL-4 levels were found to be 64.4 ± 7.1, 64.89 ± 6.8, and 98.9 ± 9.9 pg/ml for treatment groups 18, 17 and 18, respectively. The IL-4 levels then continuously decreased over the period of 6 weeks, for all treatment groups but were still significantly higher than saline and STZ control groups. In all cases, the IL-4 expression was significantly higher in the group treated with bicistronic plasmid compared to that of single expression plasmids or physical mixture group.

Comparison of IL-10 Expression

IL-10 cytokine expression results are shown in Fig. 4 (A-D). The expression pattern for IL-10 was similar to IL-4. Even though the expression of IL-10 was significantly higher ($p < 0.05$) initially for 2 weeks in the passive control groups treated with naked IL-10 plasmid (group 3), mixture of IL-4 and IL-10 (group 5), and bicistronic plasmid (group 6), the levels fall significantly after 3 weeks period. The IL-10 levels were comparable to that of saline and STZ control at week 6, indicating a temporary expression due to the injection of naked plasmids.

Mice treated with IL-10 plasmid in FuGENE® HD delivery system, showed significantly higher ($p < 0.05$) IL-10 levels till 6 weeks compared to that of saline and STZ controls (Fig. 4-B). Significantly ($p < 0.05$) higher IL-10 expression was observed in the group treated with bicistronic plasmid delivered with FuGENE® HD, as compared to other positive controls, saline treated as well as STZ treated groups.

IL-10 expression results for the polymeric micellar delivery system containing N-oleyl LMWC and N-linoleyl LMWC are presented in Fig. 4-C and 4-D, respectively. The baseline IL-10 expression observed was 41.2±6.3 pg/ml. IL-10 expression increased significantly in the N-oleyl LMWC treated group and observed to be 329.0±34.1 pg/ml for pUMVC3-mIL-10, 358.9±29.0 pg/ml for physical mixture (IL-4+IL-10), and 628±43.4 pg/ml for bicistronic plasmids at week 1.

Similarly, in case of treatment group containing N-linoleyl LMWC, the expression of IL-10 was 427.3±47.7, 379.3±39.6 and 665.8±66.3 pg/ml at week 1 for groups treated with pUMVC3-mIL-10, mixture of IL-4 and IL-10, and bicistronic plasmid, respectively. The expression reduced gradually till the end of 6 week period, but was significantly higher in all treatment groups compared to saline and STZ control. The expression of IL-10 was significantly higher in animals treated

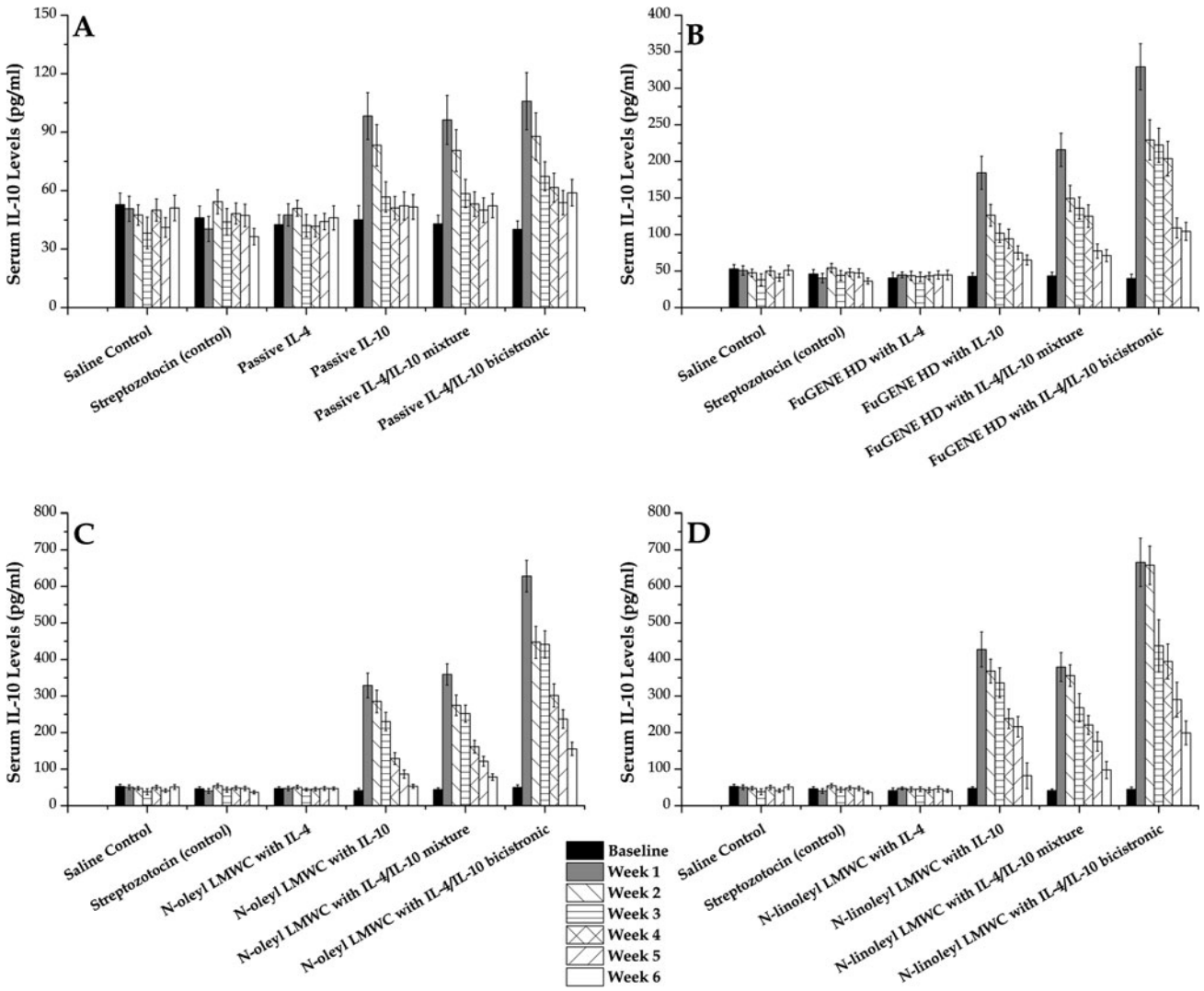

Fig. 4 Mean serum IL-10 levels (pg/dl). Vertical bars indicate standard deviation ($N = 6$).

with bicistronic plasmid compared to all other groups in the experiment.

Determination of INF-γ and TNF-a in Mouse Serum

The serum levels of type 1 cytokines; INF-γ and TNF-α were also evaluated to determine the progression of autoimmune diabetes induced by multiple low doses of streptozotocin (STZ) (Figs. 5 and 6). Considerable elevation in the plasma level of INF-γ and TNF-α were observed in passive control groups compared to saline control. The serum levels of both the cytokines remained elevated till the end of 6 week period in streptozotocin control group and well as all passive control groups.

INF-γ and TNF-α levels were significantly lower in FuGENE® HD treated mice at all time points compared to STZ control. But, it was observed that, though the levels of both cytokines reduced gradually, they were significantly higher ($p<0.05$) in all groups (7–10) compared to saline control.

The INF-γ level reduced considerably in case of mixture (IL-4+IL-10, group 13), and bicistronic (group 14) plasmid treated mice. Similar trend was observed in case of the mice treated with the delivery system containing N-linoleyl LMWC. Except for initial 1 week period, this polymeric system performed significantly better when used for bicistronic plasmid delivery.

At the end of 6 weeks, the serum INF-γ levels in the mice treated with bicistronic plasmids were comparable to saline control, and were significantly lower ($p<0.05$) as compared to the animals in STZ, IL-10, IL-4, and IL-10+IL-4 mixture treatment groups.

In case of animals treated with naked unformulated plasmids (groups 3–6), the levels of TNF-α were significantly higher ($p<0.05$) compared to baseline as well as saline control and remained higher till the end of 6 weeks.

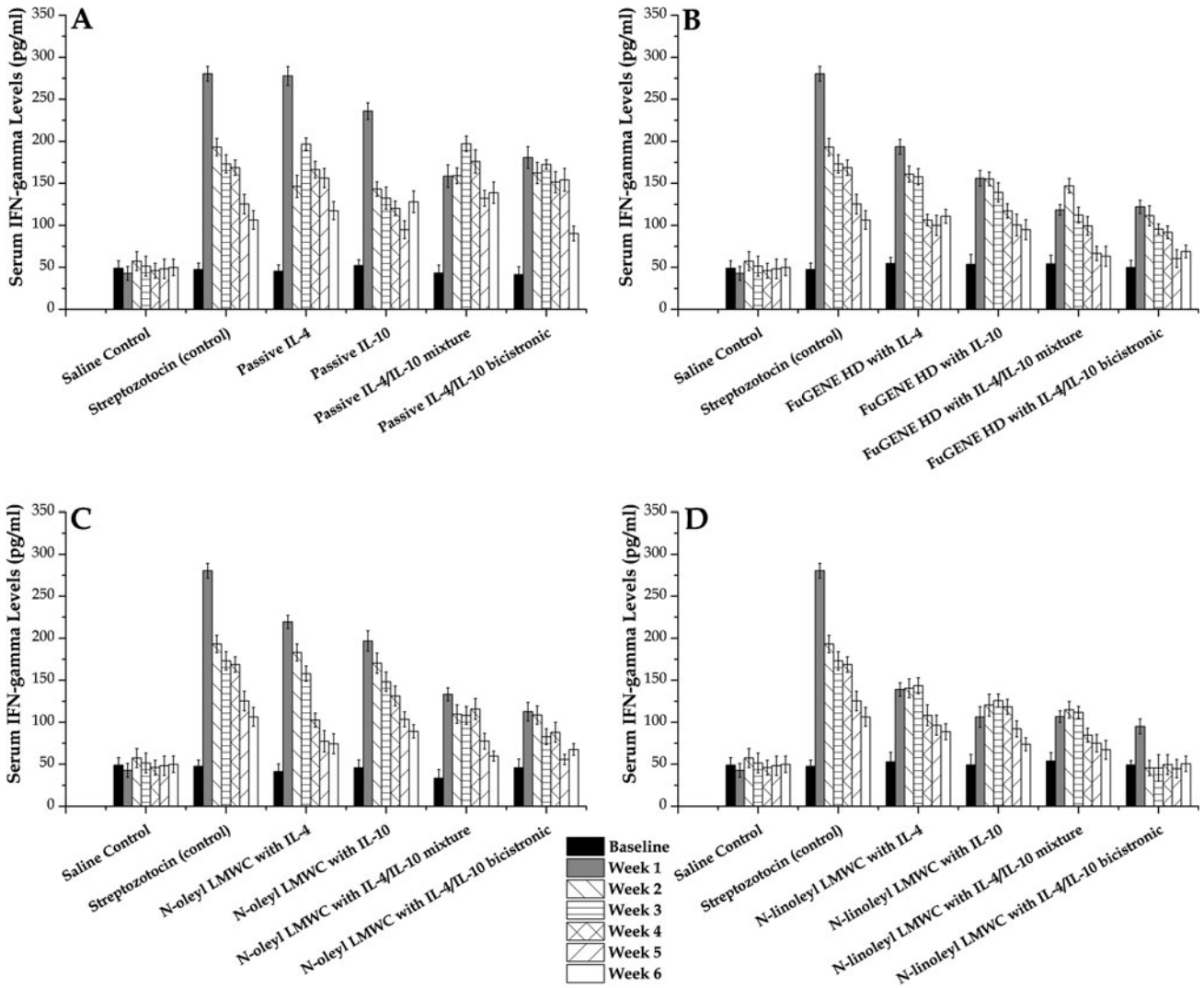

Fig. 5 Mean serum IFN-γ levels (pg/dl). Vertical bars indicate standard deviation ($N=6$).

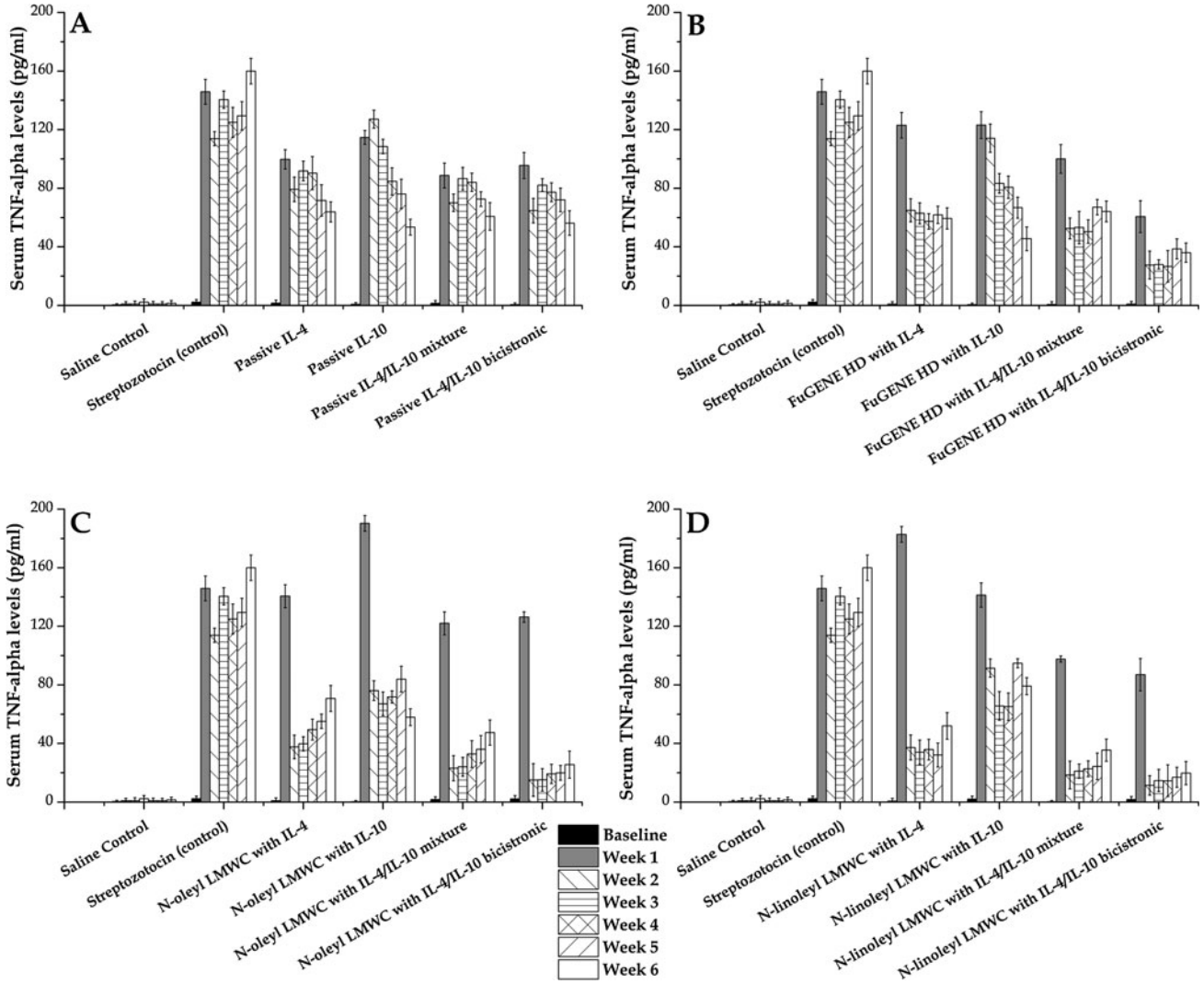

Fig. 6 Mean serum TNF-α levels (pg/dl). Vertical bars indicate standard deviation ,$N = 6$).

Similarly, in case of FuGENE® HD delivery vector, it was noted that there is a significant rise in the TNF-α serum concentration even in case of bicistronic plasmid delivery group. The TNF-α concentration was higher in both N-acyl LMWC treatment groups at week 1, and then reduced sufficiently. Though the TNF-α levels were significantly lower ($p < 0.05$) in bicistronic plasmid/N-oleyl LMWC and bicistronic plasmid/N-linoleyl LMWC treatment groups as compared to all other treatment groups, they remained significantly higher as compared to saline control throughout the study period.

Histopathological Changes in Pancreas

The level of destruction of pancreatic β cells due to immune infiltration and progression of insulitis if any was observed and compared here, and is presented in Fig. 7. Figure 7-A represents histology of normal untreated pancreas. STZ injection, showed severe destruction of pancreatic islets, with high degree of infiltration of lymphocytes and macrophages. The islets were surrounded by the mononuclear cells as shown in Fig. 7-B. The histology of pancreas treated with polymer treatment groups are represented in Fig. 7 C-D. Pancreatic histology of mice treated with unformulated plasmids showed some beta cell infiltration but was lower as compared to STZ treatment group. The histology of pancreas of the mice in the treatment group showed the presence of very few mononuclear cells, indicating the protecting effect exerted by the expressed IL-4 and IL-10.

Biocompatibility of the Delivery System

The histological changes in the anterior tibialis muscle were studied to evaluate the *in vivo* biocompatibility of the polymeric nanomicelles containing pDNA and presented in Fig. 8. It was observed that 1 week after injection of the delivery system into

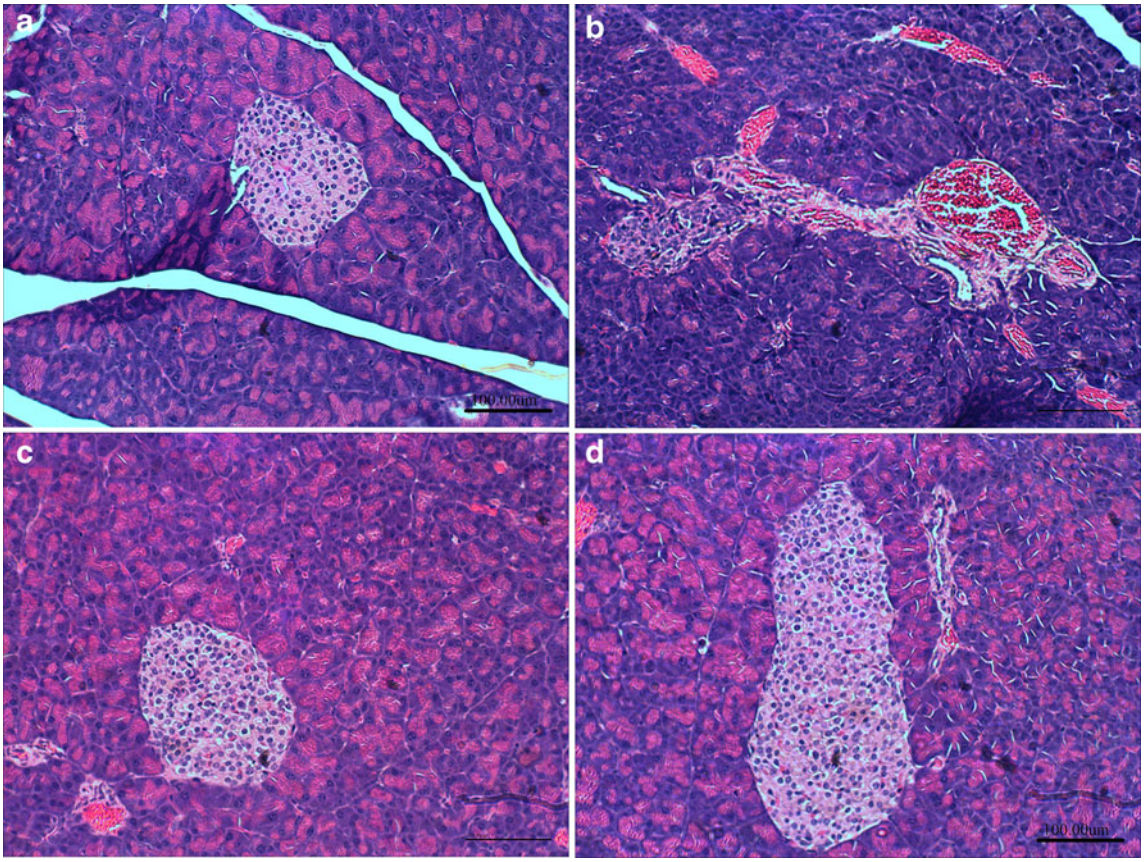

Fig. 7 Pancreatic islets of animals from (**a**) control group, (**b**) STZ treated group, (**c**) N-oleyl LMWC treated group and (**d**) N-linoleyl LMWC treated group at 6 weeks post administration. The scale bar represents 100 μm.

the muscle, large number of macrophages and lymphocytes were observed at the site of injection. But, after 2–3 weeks of treatment, the inflammation subsided considerably indicated by the presence of less number of inflammatory cells. The muscle histology was comparable to that of control at the end of 6 weeks showing the biocompatible nature of the delivery system. However, even after 6 weeks post-administration, the injection site muscles of the animals treated with FuGENE® HD exhibited signs of inflammation (Fig. 8-B).

DISCUSSION

A large number of cationic polymers which effectively condense the DNA and mask their negative charge have been investigated for gene therapy (30–34). Though the viral vectors were initially used as gene carriers, the benefits of non viral polymeric delivery systems are now being fully appreciated. The obvious advantages of non viral vectors, including superior safety profile, ease of modification, amenability to attachment of targeting ligands and absence of the possibility of insertional mutagenesis add to their attractiveness. Chitosan, a cationic polymer, has been explored as a safe and versatile gene delivery carrier. However; due to its low and pH dependent solubility, and low transfection efficiency makes it poor candidate for more widespread use. In our previous studies, the problems associated with chitosan were circumvented by a dual approach. We prepared soluble, low-molecular weight chitosan and derivatized it further with a series of fatty acids. The alkyl chain length, degree of unsaturation, and ratio of pDNA/polymer were varied and an optimized cationic nanomicellar delivery system consisting of N-acyl LMWC was prepared. The delivery system was investigated for its transfection efficiency *in vitro* and compared with a marketed transfection reagent, FuGENE® HD. *In vitro* studies performed to determine/quantify the expression of therapeutic cytokines IL-4 and IL-10, using N-oleyl and N-linoleyl LMWC showed that there is approximately a 8 and 35 fold increase in expression as compared to LMWC and naked DNA, respectively (27). The delivery systems were as efficient as FuGENE® HD ($p \geq 0.05$) *in vitro*. The reason for this high transfection might be due to the buffering ability, small size or due to fast endosomal escape of the nanomicelles. After conversion of ~35% of free primary amine groups into amide moieties, the polymers retain sufficient buffering ability (unpublished data) and this might lead to their eventual endosomal escape due to the 'proton sponge'

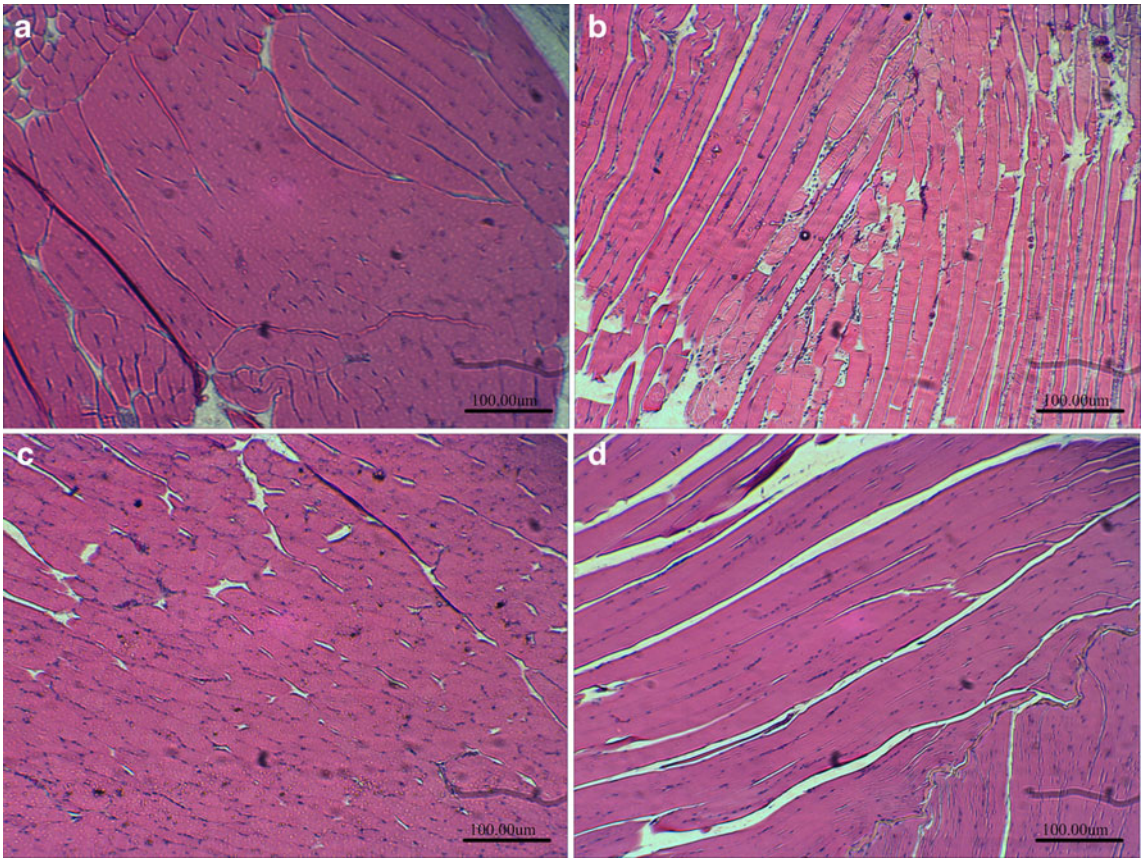

Fig. 8 Anterior tibialis muscle at the injection site of animals from (**a**) control group, (**b**) FuGENE® HD treated group, (**c**) N-oleyl LMWC treated group and (**d**) N-linoleyl LMWC treated group at 6 weeks post administration. The scale bar represents 100 μm.

effect as described by Behr (35), resulting in superior transfection. In this study, the ability of these hydrophobically modified low molecular weight chitosans was evaluated for therapeutic plasmids delivery *in vivo*.

Type 1A diabetes is generally explained by T_H1/T_H2 balance model which concludes that autoimmunity is caused due to dominance of T_H1 cytokines. T_H1 cells with their cytokine effectors such as IFN-γ, IL-2 and TNF-α elicit cell-mediated responses; whereas T_H2 cells through their cytokines such as IL-4 and IL-10 elicit humoral responses. Restoration of balance between T_H1 and T_H2 cytokines by upregulation of TH2 cytokine expression may lead to prevention of autoimmune diabetes. Delivery of cytokines, however, is not feasible due to high cost and very short plasma half-life. Delivery of genes encoding T_H2 cytokines has the potential to eliminate these shortcomings by facilitating *in situ* expression of cytokines. It may also circumvent the problems associated with immune reaction against foreign cytokines (30,36,37).

It has been reported that the use of multiple, low-dose STZ approach partially damage pancreatic islets, and trigger an inflammatory response which leads to further destruction of β-cells and results in insulin deficiency leading to hyperglycemia. This phenomenon usually closely resembles the pathogenesis and morphologic changes that occur due to type 1 diabetes mellitus (T1DM) (38). Though multiple, low-dose STZ induced diabetic mouse model has been less studied as compared to other models of type 1A diabetes such as NOD mouse and bio-breeding rats, it offers multiple advantages including a quicker and more reliable onset of diabetes and ability to produce diabetes irrespective of genetic background of the mice (39). Lee *et al.* (20) have demonstrated the advantages of intravenous administration of a chimeric plasmid to prevent insulitis in NOD mice, but no study has evaluated the effects of administration of a bicistronic plasmid in multiple, low-dose STZ induced diabetic mouse model via the intramuscular route. It has already been demonstrated that the use of skeletal muscle for gene therapy results in sustained and systemic expression of therapeutic proteins.(26) A single intramuscular (IM) injection of β-galactosidase (AAV-lacZ) gene or human erythropoietin (AAV-Epo) using viral vector into adult BALB/c mice resulted in long-term protein expression.

Comparison among the Polymer Groups

In our studies, we have compared the expression of plasmids encoding for IL-4, IL-10 and a bicistronic plasmid

encoding for both IL-4 and IL-10 using hydrophobically modified LMWC. We have also determined the effect of successful IL-4 and IL-10 gene delivery on multiple, low-dose STZ induced insulitis. The blood glucose levels as well as serum cytokine levels of animals treated with IL-4, IL-10 and bicistronic plasmid polyplexes were compared with those of untreated and naked plasmid treated animals. There is gradual increase in blood glucose levels in animals treated with IL-4 plasmid with either of the delivery systems. Though low blood glucose levels were observed in animals treated with the delivery system containing N-linoleyl LMWC compared to passive, STZ, FuGENE® HD and N-oleyl LMWC groups, N-linoleyl LMWC was superior to rest of the treatments. When the serum IL-4 levels were analyzed, it was noted that both the N-oleyl LMWC and N-linoleyl LMWC showed marked ($p<0.05$) increase in IL-4 expression compared to passive or FuGENE® HD controls. Such improved transfection by N-acyl LMWCs can be explained on the basis of their low cytotoxicity and stable nature of their polyplexes with pDNA. Serum IL-4 levels reduced eventually at the end of 6 weeks, but remained elevated in N-linoleyl LMWC group. Although there is a drop in IL-4 expression observed over the period of time, the levels remained significantly higher ($p<0.05$) than physiological levels (compared to saline control). Similar findings have been reported by Basarkar and Singh (30), explaining that the reduction in expression may be a result of deactivation of promoter or due to the turnover of muscle fibres.

Similar results were observed in case of mice treated with IL-10 plasmid. Animals treated with hydrophobically modified chitosan showed considerably low blood glucose levels compared to STZ and passive treatment groups. Higher IL-10 concentration was observed as a result of higher expression of IL-10 plasmid formulated in N-oleyl LMWC and N-linoleyl LMWC delivery systems. The expression reduced over the period of 6 weeks, but was high enough to reduce the destruction of pancreatic islets and resultant progression of insulitis. This high expression resulted in marked decrease in IFN-γ and TNF-α levels, which eventually lengthened the progression of disease. In all cases, N-linoleyl LMWC was found to be superior compared to naked plasmids, FuGENE® HD as well as N-oleyl LMWC treatments.

Since it has been reported that the combined administration of IL-4 and IL-10 plasmid is effective in preventing the autoimmune insulitis, a physical mixture of IL-4 and IL-10 was prepared at a weight ratio of 1:1 and incorporated into the micellar delivery system. The effect of co-administration of both the plasmids was evaluated and compared. Though the blood glucose levels were significantly ($p<0.05$) low in case of N-oleyl LMWC, N-linoleyl LMWC and FuGENE® HD treated groups compared to STZ, it is difficult to obtain the exact correlation between the effect and the plasmid expression. At the same time it could be noted that the N-linoleyl LMWC treatment group showed lower glucose levels.

IL-4 and IL-10 cytokines were highly expressed in animals treated with IL-4+IL-10 mix in N-oleyl LMWC and N-linoleyl LMWC, indicated the polymeric delivery systems (N-oleyl and N-linoleyl LMWC) worked with better efficacy as compared to passive and FuGENE® HD treatments. The marked reduction in the IFN-γ and TNF-α serum levels also supports the fact that the co-administration of both plasmids in N-oleyl LMWC and N-linoleyl LMWC worked efficiently as compared unformulated or FuGENE® HD controls. It was noted that, though the levels of INF-γ were higher in the animals treated with plasmids in N-oleyl LMWC delivery system initially and reduced gradually at the end of 6 week period. An initial transient increase in IFN-γ has been previously observed (30) and is mainly attributed to the acute inflammation caused due to needle injury or due to the presence of the delivery system.

Maximum expression of IL-4, and IL-10 was observed in case of mice treated with bicistronic plasmid formulated in N-linoleyl LMWC delivery system. The expression gradually reduced but the concentrations were sufficient enough to prevent the autoimmune diabetes progression. The amount of IL-4 and IL-10 copies in the bicistronic plasmids are higher than the single expression plasmids in the mixture groups but lower than single expression plasmids in the individual plasmid groups. Lee *et al.* (20) have reported that such difference in the number of plasmid copies as well as the plasmid size do not have any significant effect on the transfection and subsequent expression of therapeutic proteins. From these results it could be said that the delivery vector (N-linoleyl LMWC) enhanced the transfection efficiency of the plasmid incorporated irrespective of the size and number of plasmid copies administered. This can be explained on the basis of the excellent binding and effective condensation of pDNA by hydrophobically modified LMWCs. Such polymer/pDNA interaction led to formation of nanomicelles-based polyplexes which are easily taken up by the cells and resulted in sustained transgene expression after a single IM injection.

Though FuGENE® HD exhibited high transfection efficiency *in vitro*, its *in vivo* biocompatibility is questionable. It is reported to be moderately cytotoxic in a dose dependant manner *in vivo* (40). We found that the acute inflammatory responses were more pronounced at the injection site of FuGENE® HD. Such toxicity might have led to destruction of the injected skeletal myocytes resulting in reduced expression of the delivered genetic cargo as compared to nontoxic N-acyl LMWCs.

Comparison among the Plasmid Groups

When the treatment groups were compared on the basis of plasmid efficiency, mice treated with bicistronic plasmid, showed significantly low plasma glucose levels. Moreover, the animals treated with bicistronic plasmid/ N-linoleyl LMWC group exhibited by low blood glucose levels which were comparable to saline control at all time points. Mice injected with bicistronic plasmid in the N-oleyl LMWC and FuGENE® HD showed marked reduction in blood glucose, but was significantly higher than saline control as well as N-linoleyl LMWC treatment groups. Unformulated plasmid treated animals did not show any change in the glucose level and the levels remained elevated till the end of 6 weeks.

Even though the expression levels of individual expression plasmids, or physical mixture of IL-4 and IL-10, are good with N-linoleyl LMWC as a delivery vector, bicistronic plasmid treated animals showed the maximum expression of the cytokines. Animals treated with naked unformulated plasmids showed transient expression of IL-10 and IL-4 cytokines. The serum levels of both the cytokines dropped rapidly after 2 weeks indicated that frequent administration is required to maintain the therapeutic protein levels. Similar results have been reported by Basarkar and Singh (30).

Proinflammatory cytokines such as TNF-α, and IFN-γ, produced primarily by T_H1 cells play an important role in the pathogenesis of type 1A diabetes by causing activation and migration of more inflammatory cells into the pancreas (41). The levels of inflammatory markers IFN-γ and TNF-α were observed to determine the inhibitory effect of IL-4 and IL-10 cytokines expressed. The levels of IFN-γ and TNF-α were significantly high initially (week 1) but lowered over 6 week period, indicating inhibitory effect of IL-4 or IL-10 on IFN-γ production and reduction in the progression of insulitis in case of N-oleyl LMWC, N-linoleyl LMWC and FuGENE® HD treated animals. The inhibitory effect was sustained for animals treated with N-oleyl LMWC and N-linoleyl LMWC throughout the study duration. The initial acute immune response might be due to needle injury or due to the presence of polymeric formulation. Animals in passive control group (naked IL-4 plasmid injection) showed reduction in IFN-γ levels at week 2 but later on increased in IFN-γ concentration showed that there was a transient expression of IL-4 resulted in temporary inhibitory effect on IFN-γ. The levels remain higher and were comparable to that of STZ control till 6 weeks.

Histopathology of pancreas showed severe infiltration by the inflammatory cells in STZ group. Even though the pancreas of animals treated with unformulated plasmids showed reduction in infiltration initially at week 1, it was not sustained, and severe inflammation was observed after 4 weeks of treatment. Thus, the expression of IL-4 and IL-10 cytokines was temporary and did not persist long and sufficient enough to protect the pancreas from progression of insulitis. On the other hand, the pancreas preserved their morphology, and no inflammation was seen in case of animals treated with N-oleyl and N-linoleyl LMWC polyplexes, probably due to the higher and sustained expression of IL-4 and IL-10.

Since cationic polymers are known to cause immune response, the muscle histology of anterior tibialis muscle was studied after injection of the formulations. Except for transient inflammation observed at week 1, there was no infiltration/inflammatory response seen in case of either of the formulations. In contrast, the injection site muscles of the animals treated with FuGENE® HD exhibited marked signs of inflammation at 4 and 6 weeks post-administration. At the end of 6 weeks, the histology of muscles of polymer treated animals was comparable to untreated control indicates that the delivery systems were biocompatible and safe to delivery using IM route.

CONCLUSIONS

The potential of oleic acid and linoleic acid grafted LMWC to deliver therapeutic plasmids *in vivo* was investigated. The comparable gene delivery efficiencies of oleic acid and linoleic acid grafted LMWC showed that they can be used as safe and efficient alternatives to currently marketed non-viral vectors. The efficient expression of IL-4, and IL-10 delivered intramuscularly using the hydrophobically modified LMWC efficiently protected the pancreatic beta cells from inflammation and insulitis and was found to be superior to FuGENE® HD. No signs of chronic inflammation of the injection site muscle were observed in histological studies indicating the biocompatible nature of the delivery systems. The nanomicelles-based polyplexes lowered the induction of inflammatory cytokines as compared to naked pDNA as well as FuGENE® HD, indicating the safety of N-acyl LMWC based gene delivery systems. The bicistronic plasmid expressed IL-4 and IL-10 more effectively, and demonstrated the feasibility of bicistronic plasmid/N-acyl LMWC delivery system for the prevention of autoimmune diabetes.

ACKNOWLEDGMENTS & DISCLOSURES

This work was supported by the grant from the Fraternal Order of the Eagles and Presidential Fellowship by North Dakota State University.

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