8-3-2013

Designing Targeted F4/80 Antibody Coated TNF-α siRNA Loaded Nanoparticles: A Novel Therapeutic Approach To Treat Inflammatory Bowel Disease

Poonam Rakhya

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DESIGNING TARGETED F4/80 ANTIBODY COATED TNF-α siRNA LOADED NANOPARTICLES: A NOVEL THERAPEUTIC APPROACH TO TREAT INFLAMMATORY BOWEL DISEASE

by

POONAM RAKHYA

Under the Direction of Dr. Didier Merlin

ABSTRACT

Today, increasing evidences of systemic side effects with the treatments available for IBD limit their therapeutic outcomes. The challenge is to target anti-TNF-α agent to the inflamed tissue. Here, we have demonstrated that TNF-α siRNA can be efficiently loaded into F4-80 antibody (Ab) coated PLA-PEG-Mal nanoparticles (NPs) to target colonic macrophages (MPs). The NPs were characterized for particle size, cytotoxicity, binding characteristics, etc. These NPs are ~600 nm in diameter, non-cytotoxic, and are efficiently taken up by MPs inhibiting TNF-α secretion by the MPs in vitro. In vivo, the release of TNFα siRNA loaded inside F4/80 Ab coated NPs into colitis mice model showed higher colitis attenuation (like reduced weight loss, MPO activity and neutrophils infiltration) when compared to NPs not covered. Thus, we have formulated an efficient targeted drug delivery system in which TNF-α-siRNA NPs coated with F4/80 Ab represent an efficient therapeutic option for diseases such as IBD.

INDEX WORDS: Nanoparticles, TNF-α siRNA, Macrophages, IBD
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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the College of Arts and Sciences Georgia State University 2013
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College of Arts and Sciences
Georgia State University
August 2013
DEDICATION

This thesis is dedicated to my husband, Ashish,
for his endless love, support and encouragement.
ACKNOWLEDGEMENTS

With a deep sense of gratitude, I would like to express my sincere thanks to all who were with me all the time and encouraged me during my work.

I owe a hearty debt of profound gratitude to my honorable mentor and advisor Dr. Didier Merlin. I am indebted to him for suggesting the thesis topic to work on; his systemic and constant supervision, inspiring guidance, encouragement and suggestions propelled me into this journey and enabled me to accomplish this work. It has been my proud privilege to work under such an esteemed, eminent and masterly guide.

I sincerely thank Dr. Hamed Laroui, Assistant Professor, for helping me in all activities, for his elderly advice thinking about my betterment, and for constant encouragement and good wishes he gave during my study period. It was a great privilege for me to work with him. His kind attention, advice, towering support, incessant encouragement, constant and vigilant supervision was a great esteem for me and I most humbly express my thanks for his beneficent contributions.

I express my sincere gratitude towards my lab mates, Emilie Viennois, Moiz Charania, Mark Baker, Saravanan Ayyadurai and Bo Xiao for their kind help & timely advice that gave me the path to make it out very smoothly.
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LIST OF ABBREVIATIONS

BSA, bovine serum albumin;
COX-2, cyclooxygenase-2;
DSS, dextran sodium sulfate;
ECIS, Electrical Cell-substrate Impedence Sensing
FITC, fluorescein isothiocyanate;
IL, interleukin;
LPS, lipopolysaccharide;
MPO, myeloperoxidase;
NP, nanoparticle;
PCS, photon correlation spectroscopy;
PLA, polylactide;
PVA, polyvinyl alcohol;
TNF, tumor necrosis factor;
MP, macrophage;
PEI, poly ethylene imine;
IBD, Inflammatory Bowel Disease;
ELISA, Enzyme Linked Immuno Sorbent Assay
1. INTRODUCTION

Inflammatory Bowel Disease (IBD) is a broad term that includes both Crohn’s disease and ulcerative colitis and is characterized by inflammation of the gastrointestinal tract with chronic or recurring immune response conditions. It is important to know that Crohn’s disease and ulcerative colitis are different from each other. The symptoms of these two illnesses are quite similar, but the areas affected in the gastrointestinal tract (GI tract) are different. Crohn’s disease is a chronic inflammatory condition of the GI tract and most commonly affects the end part of the small intestine, called the ileum, and the beginning of the colon. Crohn’s disease may also affect any part of GI tract, from the mouth to anus. Ulcerative colitis is a form of colitis limited to the colon, otherwise known as the large intestine. Crohn’s disease can affect the entire thickness of the bowel wall, while ulcerative colitis only affects the innermost lining of the colon. Moreover, in Crohn’s disease, the inflammation of the intestine can skip and normal areas can be seen between the patches of diseased intestine, where as in ulcerative colitis this does not occur.

1.1 Epidemiology of IBD

It is estimated that as many as 1.4 million persons in the United States suffer from these diseases. IBD is more prevalent between the ages of 15 and 30, although it may occur at any age. About 10% of cases occur in individuals younger than 18 years. These chronic diseases tend to run in families and affect males and females equally. The case of ulcerative colitis in males is slightly more common than women, whereas there is a slight female predominance in Crohn’s disease [1]. The etiology of IBD is a complex interaction of genetic, immunologic, and environmental factors that seem to play role as evidenced by the following: The greatest relative risk of IBD disease is found among first-degree relatives, suggesting a strong genetic component. Studies have shown that 5% to 20% of affected individuals have a first – degree relative (parents, child,
or sibling) with one of the diseases [2]. Both illnesses, Crohn’s disease and ulcerative colitis, do have one strong feature in common that they are marked by an abnormal response by the body’s immune system. Normally, the cells and proteins that make up the immune system protect the body from infections. In people with IBD, however, the food, bacteria, benign or beneficial cells, and other materials in the intestine are mistaken for foreign/invading substances and the immune system mounts a response. The body sends white blood cells into the lining of the intestines, where they produce chronic inflammation and ulceration, thickening of the intestinal wall, and eventually causing patient symptoms. This destructive inflammatory response directed toward a self-antigen such as mucin, goblet cells, colonocytes, or other cells has been proposed as one of the factors underlying the basis of IBD [3-6]. The environment in which we live also appears to play a role in pathogenesis of IBD. IBD is more common in developed countries rather than undeveloped countries, more frequent in urban communities rather than rural areas. Moreover, there is a noted variation in northern communities compared with those living in southern climates. These observations are the result of changes in diet, smoking, and differences in exposure to sunlight, pollution, and industrial chemicals [7]. Smoking is one of the more notable environmental factors. Studies have shown that ulcerative colitis is more prevalent among ex-smokers and nonsmokers, whereas Crohn’s disease is more prevalent among smokers [8, 9]. Other factors such as diet, oral contraceptives, perinatal and childhood infections, measles infection or vaccination, or atypical mycobacterial infections have been suggested but not proven to play a role in expression of IBD [1].

1.2 Signs and symptoms of IBD

It is often difficult to diagnose which form of IBD a patient is suffering from because the illnesses, Crohn’s Disease and ulcerative colitis, show some common symptoms. Symptoms
related to GI tract inflammation include persistent diarrhea; rectal bleeding; urgent need to move bowels; abdominal cramps and pain; sensation of incomplete evacuation; and constipation that can lead to bowel obstruction. Although the disease is not always limited to the GI tract, it can also affect the joints, eyes, skin, and liver. Fever, loss of appetite, weight loss, fatigue, night sweats, loss of normal menstrual cycle are some of the non GI tract related symptoms. Until recently, it was thought that mainly ulcerative colitis patients have an increased risk of cancer, but it is now known that increased risk of colon cancer exists for Crohn’s patients as well.

**1.3 Treatments available for IBD**

Patients with IBD show defects in intestinal epithelial cell barrier function, thus allowing bacteria to colonize colonic epithelia [10, 11]. Inappropriate access of bacterial antigens through dysfunctional barrier function to the mucosal immune system represents an important element in the pathogenesis of IBD, because, as the bacterial antigens are presented to the mucosal immune system, the dendritic cells and macrophages secrete pro-inflammatory cytokines to the lamina propria, triggering recruitment of circulating immune cells via expression of adhesion molecules on endothelial and immune cells [12]. These pathogenic processes are the targets of modern IBD therapeutic approaches that can be divided into three categories, specifically, development of inhibitors of inflammatory cytokines (e.g., anti-TNF-α) that induce T-lymphocyte apoptosis; identification of anti-inflammatory cytokines that downregulate T-lymphocyte proliferation; and synthesis of selective adhesion molecule (SAM) inhibitors suppressing T-lymphocyte trafficking into the gut epithelium[13-23]. Pharmaceutical treatment of IBD includes 5 major classes of drugs, namely amino salicylates (5-ASA), steroids, immune modifiers (azathioprine, 6-MP, and methotrexate), antibiotics (metronidazole, ampicillin, ciprofloxacin, others), and biologic therapy (infliximab). Traditionally, these drugs are usually administered in high doses and/or
systemically, and thus complications arise due to their non-specific effects on the immune system, leading to significant systemic side effects. Because of their limited therapeutic efficacy, these drugs are effective in only few IBD patients. Two-thirds to three-quarters of patients with Crohn's disease will require surgery at some point during their lives. In one-quarter to one-third of patients with ulcerative colitis, medical therapy is not completely successful and complications arise. Under these circumstances, surgery becomes necessary when medications can no longer control the symptoms. This operation involves the removal of the colon (colectomy). Unlike Crohn's disease, which can recur after surgery, ulcerative colitis is cured once colectomy is done [24-26].

1.4 TNF-α and IBD

Advances in our knowledge of the pathophysiology of IBD have highlighted the importance of proinflammatory cytokines in the inflammatory process. Tumor necrosis factor alpha (TNF-α) is one of the most well recognized proinflammatory mediators involved in the pathogenesis of IBD and plays an important role by contributing to the recruitment of immuno-competent cells that amplify T cells, macrophages, and mucosal cells inflammatory response [27-31]. Indeed, studies have shown that TNF-α levels are increased in both serum and mucosa of IBD patients [32-35]. Moreover, significantly increased TNF-α levels in animal models of IBD (2, 4, 6-trinitrobenzenesulphonic acid and dextran sulphate sodium (DSS)-induced colitis) have also been shown [36, 37]. TNF-α also has deleterious effects on tight junctions, impairing intestinal epithelial cells barrier function, and thus enhancing immune challenge by presenting luminal antigens [38, 39]. Thus, TNF-α is likely an important component in the pathophysiology of IBD, and therefore agents targeting TNF-α in IBD have been studied. Recent multiple clinical trials have confirmed that biological therapies are effective in the treatment of IBD. An example of
this kind of treatment is the use of anti TNF-α agents, such as monoclonal antibodies infliximab, adalimumab, natalizumab, etanercept or onercept. Among these antibodies, infliximab is the only one approved for use in IBD [40]. Although anti-TNF-α antibodies are highly effective in general, few patients showed at least one of the adverse effects such as pneumonia, cancer, or acute inflammation, largely due to systemic TNF-α suppression [41]. Several studies have demonstrated that Infliximab is useful for the treatment of Crohn’s disease; however, it is not useful in ulcerative colitis. Infliximab treatment has some drawbacks, such as the development of anti-infliximab antibodies, which makes the treatment less effective and leads to hypersensitive reactions. Another drawback is the development of autoimmune reactions, such as that related with antinuclear antibodies. The reactivation of infections such as tuberculosis has also been reported among one of the adverse effects of Infliximab and in fact its screening is required before administration for tuberculosis [40]. Thus, a major drawback in the development of therapeutic strategies for diseases such as IBD is the inability to target drugs in sufficient quantities to the site of inflammation, such that the local drug concentration is maximized while systemic side-effects are minimized. Also, the requirement of frequent intake of drug at high doses to exert measurable clinical activity is one of the challenges in treating IBD.

1.5 RNA interference and IBD

Dysregulated TNF-α secretion by macrophages (MPs) and dendritic cells (the two main colonic cell lines that secrete TNF-α) in the colon has been shown in IBD. Thus, to prevent intestinal inflammation and reduce systemic side-effects, it seems reasonable to hypothesize that direct and local inhibition of TNF-α secretion from MPs/dendritic cells is needed. Small-interfering RNA (siRNA)-mediated knockdown of pro-inflammatory cytokines at the messenger RNA level (termed RNA interference) has turned out to be one of the attractive therapeutic strategies to
overcome inflammatory conditions with minimal side effects [42]. The importance of this technology is reflected by the discovery of siRNA in the late 1990s by Craig Mello and Andrew Fire for which they were awarded Nobel Prize for Medicine in 2006. They introduced an innovative approach to the relatively new field of gene therapy, allowing the turning off of single target genes without genomic integration [43]. RNA interference (RNAi) is a naturally occurring mechanism that cells use to turn down, or silence, the activity of specific genes in a sequence specific manner. The double-stranded RNA molecules are cut into 19-23 nucleotides small fragments, called as small interfering RNAs (siRNAs), upon entering a cell, by an enzyme called Dicer (RNase III family member). Each siRNA duplex consists of a guide strand and a passenger strand. The endonuclease Argonaute 2 (Ago 2) catalyzes the unwinding of the siRNA duplex. Once unwound, the passenger strand is cleaved and the guide strand is incorporated into the RNA Interference Specificity Complex (RISC). The guide strand then leads the cell’s RNAi machinery to mRNAs that match the genetic sequence of the fragments and cleaves the cellular mRNAs, effectively destroying their messages and shutting off the corresponding gene. As a consequence, the respective protein is no longer synthesized [44]. RNAi has been proposed as a new novel therapeutic strategy that offers important advantages over conventional treatments, with high specificity and potency and low toxicity. The most promising targets for RNAi-based therapies are the diseases that can be blocked by knocking down the activity of one or several genes that are deregulated in the disease; for treating viral infections; drug development; loss-of-function studies where a gene is specifically silenced and the impact of this loss is analyzed in cells or whole organisms [45].
1.6 Nanotechnology and IBD

Although siRNA is considerably more interesting as a therapeutic approach, the siRNA delivery to the target tissue with traditional agents such as lipofectamine is a challenge due to the lack of stability and poor pharmacokinetics of these agents [46-48]. Among the drug carriers, nanoparticle-based delivery systems show considerable potential for the treatment of IBD. For the purpose of drug delivery, nanoparticles (NPs) are defined as ultrafine colloidal particles with dimensions measured in nanometres (10-1000 nm). Depending upon the method of preparation, one can obtain nanoparticles, nanospheres or nanocapsules. In nanospheres, the drug is adsorbed, dissolved, or dispersed throughout the matrix, whereas in nanocapsules, the drug is confined to an aqueous or oily core surrounded by a unique polymer wall. The advantages of using NPs as a potential drug delivery system include the following: high stability, high carrier capacity, feasibility of incorporation of both hydrophilic and hydrophobic substances, and feasibility of variable routes of administration including oral, nasal, parenteral, and intra-ocular application. These advantages of nanoparticles enable improvement of drug bioavailability and reduction of the dosing frequency [49]. Previous studies have shown the significant potential of NPs not only in binding and delivering siRNA, but also in protecting siRNA against degradation in vitro as well as in vivo. Moreover, this protection markedly increases their pharmacological activity under both cell culture and physiological conditions [41, 50]. In the context of IBD, a non-cytotoxic and biodegradable system seems to be the most relevant. In recent years, NPs made up of biocompatible and biodegradable materials such as polymers, either natural (e.g., albumin) or synthetic (e.g., polylactides, polyethylene glycol) or solid lipids, have been used as potential drug delivery devices, because of their ability to circulate for a prolonged period of time, target a particular organ, and deliver proteins, peptides and genes [49, 51-54]. In the body, the
biodegradable polymeric envelope of NPs provides protection to the loaded drug (e.g., siRNA…) and transports the drug into the cytosol, usually by releasing the drug from the matrix by diffusion, swelling, erosion, or degradation. Moreover, this transport facilitates efficiency of siRNA activity in vivo [49, 55].

1.7 Administration of nanoparticle based drugs in IBD

Techniques to deliver drugs into the GI tract require the drugs to be supplied in solution. However, such drugs will be directly affected and degraded by the digestive enzymes or acidic pH of the stomach. To overcome this degradation, high drug doses are used or frequent administrations are performed which leads to side effects and may be problematic. Another problem is the fact that organs of the GI tract, particularly the colon, differ in drug absorption properties, and it is difficult to deliver the drug to the colon with minimal digestive enzyme degradation and/or systemic absorption. Thus there is an unmet need for targeted drug delivery to specific areas in the GI tract, particularly the colon. H. Laroui et al. has developed a double gavage technique and used polysaccharide hydrogels to target bioactive compounds to specific regions of the GI tract. The double gavage technique is based on the formation of a hydrogel by double linking of ions (Ca$^{2+}$ and SO$_4^{2-}$) that mediate cross-linking between two polymers, alginate and chitosan (Figure 1). By changing the polymer concentration, the drugs can be targeted to different regions of GI tract by this technique. Also, the technique significantly reduces degradation of the drug as well as its systemic side effects, as lower doses of drug can be efficiently loaded and delivered at specific target areas of the GI tract. The first step of the technique involves direct gavage of polysaccharides mixture containing drug into the mice stomach. Then a second gavage will be performed in the same manner as before but with an ionic solution of calcium and sulfate. As soon as the ions and the polysaccharides solution are
mixed, a hydrogel of the maximum possible size is formed by the double linking of ions (Figure 2). This technique can be used for encapsulation of either nanoparticles, liposomes, or drug molecules alone [56]. Recent studies have shown that biomaterial made up of alginate and chitosan (7/3 wt/wt ratio), collapses in intestinal solution at pH 5 or 6, which is the colonic pH under inflamed and non-inflamed conditions respectively. Thus, NPs from the hydrogel will be released mostly in the lumen of the colon as compared to the other parts of the GI tract [57, 58].

1.8 Goal of the proposed work

The present study proposes a nanoparticle based therapeutic approach, not only to deliver anti-TNF-α agents at the site of inflammation, specifically to dendritic cells/macrophages of colon (major sources of TNF-α secretion in IBD), but also to deliver at significantly lower therapeutic dose with higher therapeutic effect. Such a system is expected to be associated with minimal side effects. A targeted drug delivery system has been developed by synthesizing F4/80 Ab coated NPs that allows encapsulation of water-soluble siRNA molecule, which has great therapeutic potential (Figure 3). PEI, a cationic polymer, is complexed with TNF-α-siRNA (negative strand) by electrostatic interaction to form polyplex (an internal phase of NP) which protects siRNA against RNAse A digestion [59]. It carries siRNA across the cell membrane thus enhancing cytoplasmic drug delivery. NPs loaded with TNF-α siRNA are synthesized via double emulsion-solvent evaporation procedure. The first emulsion allows the polyplex to be coated with biocompatible and biodegradable PLA matrix. In the body, the lactide polymers are cleaved by hydrolysis to form natural metabolites (lactic acid), which enters the tricarboxylic acid cycle, metabolized and subsequently eliminated from the body as carbon dioxide and water [60]. The second emulsion allows NPs to be coated with the PVA that hydrophobically interacts with the PLA matrix and prevents the nanoparticle from aggregating through electrostatic repulsions. At
the end, the Fab’ parts of the F4/80 Ab have been grafted on NPs surface via maleimide/-SH functions covalent bonds. The attachment of antibodies to NPs helps in the specific and selective recognition of the target cells (Figure 4). In this study, the therapeutic effect of NPs with homing capabilities to the colon and their ability to directly release "molecularly-specific" siRNA to target cells has been explored. Further, the advantages of NPs including their ability to easily pass through physiological barriers, evasion of phagocytosis, high binding interactions, minimal or low cellular toxicity and their resistance to degradation will be utilized. Specifically, the effects of targeting intestinal macrophages by orally administering F4/80 Ab coated TNF-α-siRNA-loaded NPs in colitis mice model will be investigated.
Figure 1. Schematic representation of biomaterial encapsulation of NPs. (A) Hydrogel formation by cross linking of alginate and chitosan mediated by double linking of Ca$^{2+}$ and SO$_4^{2-}$ ions. NPs are suspended in the polysaccharide mixture to load the hydrogel with NPs. (B) Optical microscopy image of NPs encapsulated into a hydrogel bead [57].
Figure 2. Schematic representation of the double gavage method. (A) In the first step, alginate-chitosan polysaccharides mixture containing a homogenous suspension of NPs is gavaged directly into the stomach. Red Ponceau is added for visualization. (B) In the second step, the ionic solution is gavaged in the same manner, finally forming a hydrogel (C) by chelation [57].
Figure 3. Schematic representation of difference in localization of drug in targeted strategy (nanodrug) compared with systemic treatment (classical drugs). Upon oral or IV administration, the bioactive component of the classical drug disperses throughout the body without distinguishing healthy from inflamed tissue. Enema strategy can only target the distal part of colon. While, in targeting strategy, NPs are coated with antibodies whose ligands are over expressed in inflamed zone, target the inflamed zone, accumulate and release the drug in that specific area [61].
Figure 4. Schematic representation of the delivery of "molecularly specific" siRNA, loaded inside Ab coated NPs, to the target cells. NPs are packaged in polysaccharide hydrogel designed to home to the colon. Once in the colon, the hydrogel degrades and releases the NPs coated with Ab loaded with TNF-α-siRNA. The antibodies attached to the NPs helps in the specific and selective recognition of the target cells (dendritic cells/macrophages) [61].
2. EXPERIMENTAL DETAILS

2.1 Synthesis of PLA-PEG-Maleimide and PLA-PEG-OH polymers

Two copolymer types, maleimide-polyethyleneglycol-poly(lactic acid) and methoxypolyethyleneglycol-poly(lactic acid) (PLA used is of Mw=75–120 kg/mol, Aldrich Chemistry) were synthesized employing ring-opening polymerization in dry toluene under a moisture-free atmosphere of high-purity argon. PEG-OH and PEG-Mal were used as initiators to synthesize PLA-PEG-OH and PLA-PEG-Mal, respectively. Later, PLA-PEG-Mal polymer was used to graft mouse F4/80 Ab (Fab’ part) onto the surface of NPs by employing the functionality of Maleimide. Maleimide enables any molecule containing sulfur functions (-SH) to be covalently plugged to the copolymer. The second polymer served in construction of an antibody-free control (a naked NP). At the end of the reaction, the mixtures were purified by precipitation and recovered. The synthesis of both the copolymers was analyzed using H1NMR spectra.

2.2 Preparation of TNF-α siRNA/PEI NPs covered with PVA

NPs were synthesized using PLA-PEG-OH and PLA-PEG-Mal polymers via double emulsion/solvent evaporation procedure (Figure 5) [57]. An internal phase was prepared by mixing 36µL of 25µM TNF-α siRNA (Ambion, Austin, TX, USA) with 36 µL Polyethyleneimine (PEI, 5mM) (Aldrich Chemistry, St Louis, MO, USA). The mixture was incubated for 10 min at room temperature for complexation. After 10 min, 728 µL of Bovine Serum Albumin (BSA, 50g/L, Aldrich Chemistry) was added to the polyplex formed. The drug was mixed with 4 ml of PLA-PEG-Mal (20g/L) or PLA-PEG-OH (20g/L) dissolved in dichloromethane. This mixture was vortexed (Maxi Mix II, Thermodyne, Dubuque, Iowa, USA) for 2 min to generate a water-in-oil (W/O) emulsion followed by 1 min of sonication, in an ice bath, with 50% active cycles at 70% power (P_max=400 W) (Digital Sonifier 450, Branson,
Danbury, CT, USA). To this emulsion, a second water phase (8ml) containing an amphiphilic molecule, PVA (3g/L) was added (PVA, 86-89% hydrolyzed, low molecular weight, was purchased from Alfa Aesar, Ward Hill, MA, USA). The mixture was vortexed for 2 min and sonicated for 1 min respectively, to generate a water/oil/water emulsion (W/O/W). This W/O/W emulsion then was transferred to an aqueous dispersing phase (40 ml) of 0.1g/L PVA, and stirred at 45°C for 15 min under vacuum to evaporate the organic solvent (dichloromethane). The collected nanospheres were resuspended in water, and then centrifuged at 8000g for 45 min at 4°C to remove excess PVA. The final suspension was freeze-dried overnight at -50°C under 0.1 mbar pressure. Three independent rounds of nanoparticle synthesis were done to obtain around 150 mg of dry NPs. Nanoparticles containing FITC siRNA (Invitrogen, Eugene, OR, USA) or silencer negative control (Ambion, Austin, TX, USA) were prepared according to the procedure described above.

2.3 Designing targeted F4/80 Ab coated TNF-α siRNA-loaded NPs

2.3.1 Synthesis of Fab’ portion of F4/80 Ab coated NPs

In vivo, pegylated nanoparticles are not specifically targeted to the tissues as the attachment of a targeting ligand (such as monoclonal antibodies) to the tip of the PEG strands containing a terminal reactive group is required [62]. So, in order to prevent nonspecific interactions, Fab’ portion of F4/80 Ab was synthesized to target TNF-α siRNA-containing nanoparticles to MPs (F4/80 receptors are highly expressed on MPs). To create F(ab')2 (Fab’ is a single Fab portion deleted with the Fc part using pepsin digestion), Fc part of the F4/80 Ab was removed by pepsin digestion. The F(ab')2 fragment is composed of a pair of Fab' units connected by two disulfide bonds. The F(ab’)2 portion was then reduced by 2-Mercaptoethylamine-HCl (2-MEA, mild reduction) (Thermo Scientific Pierce, Pittsburg, PA) to cleave disulfide bonds into -SH
functions. The final result obtained was 2 Fab’ portions of the F4/80 Ab. Once the Fab’ portion is generated, it can be plugged on the NPs surface presenting maleimide functions. Finally Fab’ portion of F4/80 Ab was conjugated to pegylated nanoparticles by formation of a thioether bond between the thiol group of Fab’ portion and the Mal moiety located at the distal end of the PEG spacer present on the nanoparticle surface (Figure 6). Fab’ portion of F4/80 Ab was mixed with PLA-PEG-Mal NPs suspension at an Ab: maleimide molar ratio of 1:4 under stirring condition in an aqueous solution.

2.3.2 Quantification of the number of Fab’F4/80 Ab fragments per nanoparticle

The Fab’ fragment (initial concentration=C_{i,Fab’}=201\mu g/mL) was added to 30mg of NPs PLA-PEG-Mal. Once the reaction was done, the NPs were centrifuged for 45 min at 5000g, and the supernatant consisting of non-attached Fab’ molecules (C_{f,Fab’}=139.45\mu g/mL) was collected. Knowing the initial amount of Fab’ fragments introduced and the non-adsorbed amount, the amount of Fab’ attached to the NPs was collected. Using a calculation model, Fab’ molecules per NP can be estimated:

\[ n_{NP} = \frac{6 m_{NP}}{\pi D^3 \rho} \]

\[ n_{NP} = \text{Number of NPs}, \quad m_{NP} = \text{mass of NPs}, \quad D = \text{diameter of NPs}, \quad \rho = \text{volumetric mass of NPs} \]

For 30mg of NPs, we calculate \( n_{NP} = 9.69 \times 10^{11} \) particles.

Then we estimate the number of Fab’ fragments adsorbed on 30 mg of NPs (300\mu L of Fab’):

\[ n_{Fab’} = V \left( C_{i,Fab’} - C_{f,Fab’} \right) / M_{w,Fab’} \]
$n_{\text{Fab}'} = \frac{3.3 \times 10^{14}}{55000}$

$N_{\text{Fab}'} = \frac{2.05 \times 10^{13}}{7.89 \times 10^{11}} = 2073$

Finally, the final amount of Fab’ molecules/NP ($N_{\text{Fab}'}$) was:

Our calculation indicated that 2073 Fab’ molecules per NP were present.

2.4 Cell culture

Raw 264.7 cells (mouse MPs) and Caco2–BBE cells were cultured to confluence in 75-cm² flasks at 37°C with 5% CO₂ (v/v) humidified atmosphere. The culture medium comprised Dulbecco's Modified Eagle Medium (Invitrogen, Grand Island, NY) supplemented with L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL), and heat-inactivated fetal calf serum (10%) (Atlanta Biologicals, Atlanta, GA).

2.5 Animal studies

Female C57BL/6 mice (age, 8 wk; weight, 18–22 g; Jackson Laboratories, Bar Harbor, ME) used for this study were group-housed under controlled temperature (25°C) and photoperiod (12:12-hour light–dark cycle) conditions, and given unrestricted access to standard diet and tap water. Mice were allowed to acclimate to these conditions for at least 7 days before inclusion in experiments.

2.6 TNF-α siRNA/PEI polyplex formation and protection from PEI

Formation of TNF-α siRNA/PEI polyplex was assessed via examination of electrophoretic mobility on a 4% (w/v) agarose gel for 45 min at 100V in 0.5X TBE buffer (40mM Tris-HCl,
445mM boric acid, 1mM ETDA). Ethidium bromide was used to stain siRNA. The polyplex was incubated with 4μL RNAse A (0.04 mg/mL) at 37°C for 2 h and 4 h. Polyplex and siRNA integrity was assessed via electrophoretic mobility analysis. To evaluate siRNA integrity after polyplex exposure to RNAse A, 4μL of concentrated NaOH solution was added.

**2.7 Particle size measurement**

**2.7.1 Photon correlation spectroscopy**

Diameters (nm) of NPs were measured using the principle of laser light scattering with a particle size analyzer (Model 90Plus, Brookhaven Instruments Corporation, Holtsville, NY, USA). The average and standard deviation of the diameters (nm) were calculated using 3 runs. Each run is an average of 10 measurements.

**2.7.2 Scanning electron microscopy**

Suspension of PLA-PEG-OH NPs was examined using a scanning electron microscope (Cambridge Instruments Stereoscan S) by the Georgia State University core facility.

**2.7.3 AFM Measurement**

For AFM, a drop of NPs made of PLA-PEG-OH suspension was deposited onto a freshly cleaved mica slide, followed by drying overnight at 25°C. The images were taken using SPA 400 AFM (Seiko instruments Inc., Japan) at tapping mode using high resonance frequency (F₀ = 150 kHz) pyramidal cantilevers with silicon probes at a scan frequency of 1 Hz.

**2.8 Cellular Toxicity measurement**

To assess the potential toxicity of F4/80 Ab coated TNF-α siRNA/PEI loaded NPs, WST-1 assay was performed. For WST-1 assay, MPs or Caco2-BBE cells were seeded in 96-well plates at a
density of $5 \times 10^4$ cells per well and exposed to 1 mg/mL of F4/80 Ab coated TNF-α siRNA/PEI loaded NPs, NPs loaded with siRNA or empty NPs coated with F4/80 Ab for 48 hrs. WST-1 assay (Roche Diagnostics, Indianapolis, IN, USA) measures the cleavage of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate), the soluble red tetrazolium salt, by dehydrogenase enzyme present in the intact mitochondria, which leads to the formation of dark red formazan crystals. After exposing for 48 hrs, 10 μL of WST-1 proliferation reagent was added to Caco2–BBE cells and MPs (10μL/well) and incubated for 1–2 hours at 37°C. The absorbance of the formazan product formed was measured at 450 nm.

2.9 Interaction force measurement between F4/80 Ab coated NPs and MPs using Surface Plasmon Resonance

The binding characteristics of F4/80 receptors (expressed on MPs) to the F4/80 Ab ligand bound to NPs was determined by using BI-2000 Biosensing Instrument (Tempe, AZ, USA), based on the surface plasmon resonance (SPR) theory. Ligand-protein affinities are influenced by non-covalent intermolecular interactions between the two molecules such as hydrogen bonding, electrostatic interactions, hydrophobic and Van Der Waals forces. Initially, the gold chip was prepared to detect SPR. The gold chip was first cleaned with 100% ethanol and dried under N$_2$ steam. 15–20 μl of cystamine dihydrochloride (20 mM; Sigma, St Louis, MO) was cast onto the film overnight in a humidified reaction chamber. The chip surface was then thoroughly rinsed with deionized water and dried by gentle blowing of N$_2$ steam. Next, the chip was covered with a layer of carboxydextran (6mg/mL in degassed water) and a fresh mixture of 15 mM N-hydroxysuccinimide and 75 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (NHS/EDC) (Sigma, St Louis, MO) in degassed water was added. The chip with the mixture was incubated in
a humidified chamber for at least 3–5 h (often overnight). We next rinsed and dried each chip surface under a stream of N₂ [63].

After placing the chip into the BI-2000 SPR machine, single layer of NPs covered with the Fab’ portion of F4/80 Ab was coated onto the gold chip by NPs injection. As the NHS/EDC mixture activates the carboxyl group that is able to react with any NH₂- function, NPs covered with the Fab’ portion of F4/80 Ab should only be able to covalently bound to the activated carboxydextran surface in comparison with other NPs (made of PLA-PEG-OH) that don’t have Fab’ part containing –NH₂ functions. The flow was optimized at 30µL/min to allow enough time for the Fab’ portion of F4/80 Ab to react with activated carboxydextran. After coating the gold chip with single layer of NPs coated with F4/80 Ab, the biological activity of the Fab’ part of the F4/80 Ab was assessed. Increasing concentration of RAW 264.7 cells (known to widely express F4/80 receptors) with a flow of 35µl/ml was successively injected onto the activated carboxydextran surface recovered with the Fab’ coated NPs. As a control, Caco2-BBE cells (a cell type that do not express the F4/80 mouse receptor) were used. Experiments have been performed in a PBS suspension of cells instead of regular cell medium to avoid any “parasite interactions” of -NH₂ containing molecules. Between each concentration of cells, a mild regeneration phase on the NP surface is performed with NaOH (0.01M). This step is required to remove all cells and debris from the surface of NPs. Ideally, at this step the surface of the gold chip should be formed only by a monolayer of NPs (covalently bound and thus not sensitive to mild NaOH treatment) and should be suitable for the next concentration of cells.

2.10 Intracellular NP uptake visualization

Fluorescence microscopy was employed to visualize the intracellular uptake of antibody coated NPs loaded with FITC-siRNA, at PLA concentration of 20g/L. MPs (10⁴) were seeded in four-
chamber tissue culture glass slides (BD Falcon, Bedford, MA, USA) and were exposed to F4/80 Ab coated NPs loaded with FITC-tagged siRNA (500μg/mL) or F4/80 Ab negative NPs loaded with FITC-siRNA (500μg/mL) or were left untreated (control). Culture slide chambers were placed in an incubator (5% CO₂ and 37°C) on an agitation plate and were agitated for short time (15 min) with frequency of motion set at 200Hz, as we wanted to observe the NPs phagocytosis only via interactions between the F4/80 receptors on the MPs and the Fab’ part of F4/80 Ab on the NPs. The mechanic agitation highly reduces/eliminates the part of the NPs usually phagocytosed by sedimentation on the cells. After 15 min mechanical agitation, MPs were washed 3 times with medium and 3 times with PBS to remove extracellular NPs, and subsequently fixed for 20 min in 4% paraformaldehyde. Alexa Fluor 568 Phalloidin and DAPI were successively added and diluted 60 and 10,000 times for staining cells for 45 min and 5 min, respectively. The slides were then visualized using Fluorescence microscopy.

2.11 In vitro anti-inflammation effect of TNF-α siRNA-loaded NPs coated with F4/80 Ab

To ensure the effectiveness of TNF-α siRNA loaded inside NPs, MPs (RAW 264.7, 10⁴ cells) were seeded in 24-well plate and exposed for 24 h with 500μg/mL of F4/80 Ab-NPs (empty) or F4/80 Ab-NPs loaded with TNF-α siRNA or F4/80 Ab-NPs loaded with scrambled siRNA. Lipofectamine with TNF-α siRNA was used as a control. After 24 hours, cells were stimulated with LPS (10 μg/mL, Aldrich Chemistry) for 1 h and the supernatants were analyzed for TNF-α secretion by ELISA. The ELISA was performed according to the manufacturer's protocol using an ELISA kit ((R&D Systems, Minneapolis, MN).
2.12 In vivo anti-inflammation effect of TNF-α siRNA-loaded NPs coated with F4/80 Ab

2.12.1 DSS induced colitis and oral administration of NPs

C57BL/6 mice were treated with 3% dextran sodium sulfate (DSS) during 8 days. Colonic inflammation was assessed 8 days after DSS treatment. Along with DSS in water, mice received a daily gavage of 10mg/ml of NPs encapsulated in hydrogel for 8 days to target the mice colon part. Hydrogel comprised of alginate and chitosan at a ratio of 7/3 (wt/wt). The dispersion of the NPs in a matrix made of alginate and chitosan is essential for in vivo studies [59, 64]. As described in previous studies [59, 64], the hydrogel encapsulated with NPs is able to specifically degrade in the colon, increasing the effect of NPs locally targeted. According to the protocol of NPs gavage for colon delivery [56], NPS (10mg/ml) were dispersed in the alginate/chitosan mixture (100ul) and were gavaged directly into the mice stomach. The COO⁻ functions of alginate and the NH₃⁺ of chitosan were chelated respectively by second gavage of Ca²⁺ and SO₄²⁻ ionic solution. One control group received water only with daily gavage of NPs covered with Fab’ part of F4/80 Ab (NPs-scrambled). Two groups were treated with 3% DSS and NPs covered with Fab’ part of F4/80 Ab and loaded either with TNF-A siRNA (NPs-F4/80-TNF si) or loaded with scrambled siRNA (NPs-F4/80-scrambled). Two groups were treated with 3% DSS and NPs not covered with Ab and loaded either with TNF-A siRNA (NPs-naked-TNF si) or loaded with scrambled siRNA (NPs-naked-scrambled). The gavage procedure is described in Figure 2.

2.12.2 Weight loss and Myeloperoxidase (MPO) activity measurement

Weight of mice was measured daily for 8 days. For MPO activity measurement, colonic tissue samples were homogenized in ice-cold potassium phosphate buffer (50 mM K₂HPO₄ and 50 mM KH₂PO₄, pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma). The
homogenates were then sonicated, freeze-thawed three times, and centrifuged at 17,500 rcf for 15 min. Supernatants (20 ml) or MPO standard were added to 1 mg/mL o-dianisidine hydrochloride (Sigma) and 0.0005% H₂O₂, and the change in absorbance was measured at 450 nm. One unit of MPO activity was defined as the amount that degraded 1 mmol peroxidase per minute. The results were expressed as absorbance per microgram of protein.

2.12.3 Western Blotting

For western blot analysis of IκBα and β-actin of total mouse colonic cells, cell lysates were resolved on polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with anti-IκBα (1:500 dilution, Santa Cruz) or anti-β-actin (1:5000 dilution, cell signaling) primary antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies (Amersham Biosciences). Blots were detected using the Enhanced Chemiluminescence Detection kit (Amersham Biosciences).

2.12.4 Ly6G Staining and Counterstaining with Hematoxylin

Ly6G immunostaining was performed to visualize the neutrophils infiltration respectively in mice receiving water only, mice treated with 3% DSS along with scrambled siRNA loaded Fab’ F4/80 Ab covered NPs, and mice treated with 3% DSS along with TNF-Å siRNA loaded Fab’ F4/80 Ab covered NPs. Tissue sections from mice were harvested and fixed in 10% neutral buffer formalin (Fisher Scientific, Tustin, CA). Fixed tissues were embedded in paraffin. These 5-µm tissue sections were deparaffinized in xylene and rehydrated using an ethanol gradient. Tissue sections were incubated with 3% hydrogen peroxide in PBS for 30 minutes at room temperature and then were heated in citrate buffer (10 mM sodium citrate and 0.05% Tween 20, pH 6.0) at 100°C for 10 minutes in a pressure cooker to retrieve epitope. Ly6G staining sections
were blocked with 10% normal goat serum with 1% BSA in TBS for 2 h at room temperature. Next, sections were incubated with rat monoclonal anti-Ly6G antibody (1: 500 dilution) (Abcam, Cambridge, MA, USA. ab25377) in TBS with 1% BSA overnight at 4°C and were treated with their respective biotinylated secondary antibodies for 45 minutes at room temperature (Vector laboratories PK-6101 and BA-9400). Color was developed using the Vectastain ABC kit (Vector Laboratories) followed by DAB (3, 3’-diaminobenzidine) reaction according to manufacturer’s protocol. Sections were then counterstained with hematoxylin, and dehydrated in ethanol and xylene. Images were acquired at 20X magnification using an Olympus microscope equipped with a D-26 color camera.

2.13 Flow cytometry analysis

Antibodies and reagents The following antibodies were used: CD45-PerCP (30F11; BD), CD98-PE (RL388; eBioscience), CD11c-allophycocyanin (N418; eBioscience), MHC II (I-Ab)–Alexa Fluor 700 (M5/114.15.2; eBioscience), CD11b–eFluor 450 (M1/70; eBioscience), and F4/80–PE-Cy7 (BM8; eBioscience). Dead cells were identified using the fixable Aqua Dead Cell Staining Kit (Invitrogen).

Isolation of total colonic cells Total colonic cells isolation was performed as previously described with modifications [65]. Briefly, small and large intestines were removed and carefully cleaned off their mesentery. Small and large intestines were opened longitudinally, washed of fecal contents, cut into pieces 0.5 cm in length, and subjected to two sequential 20-minute incubations in HBSS with 5% FBS and 2 mM EDTA at 37°C with agitation to remove epithelial cells. After each incubation step, media containing epithelial cells and debris was discarded. The remaining tissue was minced and incubated for 11 minutes in HBSS with 5% FBS, 1.5 mg/ml collagenase VIII (Sigma-Aldrich), and 40 U/ml DNase I (Roche) at 37°C in agitation. Cell
suspensions were collected and passed through a 100-μm strainer and pelleted by centrifugation at 300 g.

Flow cytometry Isolated total colonic cells were resuspended in PBS containing 5% FBS. Live cells were identified using an Aqua Dead Cell Staining Kit according to the manufacturer’s instructions, and Fc receptors were blocked with the antibody anti-FcγRIII/II (2.4G2) for 15 minutes at 4°C. After incubation the cells were stained at 4°C for 30 minutes with labeled antibodies. Antibodies used for analysis were from eBioscience unless otherwise noted: FITC-siRNA NPs, PE-conjugated anti-mouse CD103 (BD Pharmingen), PerCP-conjugated anti-mouse CD45 (BD Pharmingen), PE-Cy7-conjugated anti-mouse F4/80, Allophycocyanin-conjugated anti-mouse CD11c, Pacific blue-conjugated anti-mouse CD11b, and Alexa Fluor 430 (live/dead stain; Invitrogen). Samples were then washed 2 times in PBS containing 5% FBS and analyzed immediately. Flow cytometric analysis was performed on LSR II (BD).

2.14 Statistical Analysis

Data are expressed as means ± S.E. Statistical analysis was performed using the unpaired two-tailed Student's t test featured in In Stat version 3.06 (Graph Pad) software. p < 0.05 was considered statistically significant.
Figure 5. Schematic representation of NP coated with F4/80 Ab. Fab’ portion of Ab is grafted on PLA matrix via PEG spacer. Maleimide enables any molecule (ex- Ab) containing sulfur functions (-SH) to be covalently plugged to the PLA-PEG-Mal copolymer. The inside of the NPs is loaded with TNF-α siRNA complexed with polyethylenimine, a cationic polymer.
Figure 6. Schematic of NPs synthesis by double emulsion technique. (A) A hydrophilic drug is encapsulated by water in oil in water emulsion (W/O/W). (B) Schematic representation of PLA NPs loaded with a hydrophilic drug, and coated with PVA [57].
3. RESULTS

3.1 Complexation/protection of TNF-α siRNA by PEI

PEI (positively charged) was used to condense negatively charged TNF-α siRNA by forming electrostatic interactions. The N/P ratio, between the number of negative charges of siRNA (P is the negative phosphorus charge) and positive charges of PEI (N is the positive ammonium charge), was set at 30 for PEI. TNF-α siRNA/PEI polyplex formation and its protection from RNAse A was assessed by electrophoretic mobility analysis of 0.4μg/mL (w/v) of free TNF-α siRNA (lane 1) and complex formed with PEI (lane 2) on a 4% agarose gel for 45 min under 100 V. The free siRNA after complexation with PEI showed strong affinity for PEI at the N/P ratio analyzed. Protection study was performed by electrophoretic migration (100V) of a free TNF-α siRNA alone (lane 3) or with RNAse A during 2h digestion at 37°C (lane 4) or a complex formed with PEI (lane 5 during 2h of RNAse A exposure, lane 6 during 4h of RNAse A exposure). Decomplexation of polyplex of lane 6 (PEI) shown in lane 7 (PEI) with a concentrated NaOH (0.1M, 1min) solution confirmed that the degree of protection of siRNA with PEI within the polyplex was high and siRNA maintained its integrity after RNAse A exposure (Figure 7) [59].

3.2 Characterization of TNF-α siRNA-loaded NPs

The particle size (diameter in nm) was measured using the principle of laser light scanning with a particle size analyzer (Brookhaven Instruments Corporation) (data not shown). The TNF-loaded NPs size average was 609nm for the NPs recovered by PLA-PEG-OH. The size calculation was then confirmed by AFM in figure 8A and SEM in figure 8B. Figure 8A and figure 8B showed that the size of NPs PLA-PEG-OH (1mg/mL) calculated by light scattering was correct as AFM diameter average was 612nm. In figure 8B, SEM picture also shows the distribution of NPs PLA-PEH-OH size (1mg/mL). It was sharp as the polydispersity index was
equal to 0.11. The distribution of NPs is generally considered as monodisperse when the polydispersity index is below 0.3.

3.3 **TNF-α siRNA-loaded NPs coated with F4/80 Ab do not affect cell viability and may be used to safely transfect MPs.**

A simple cytotoxicity test, WST-1 assay, was performed in order to assess the potential toxicity of F4/80 Ab coated TNF-α siRNA/PEI loaded NPs on MPs and Caco2-BBE cells. As shown in Figure 9, at 450 nm, it was observed that F4/80 Ab coated TNF-α siRNA/PEI loaded NPs at a concentration of 1 mg/mL did not affect cell viability of MPs and Caco2-BBE cells over 48-h period of exposure time, when compared with the controls (empty NPs with F4/80 Ab and NPs with siRNA). This non-cytotoxic behavior of F4/80 Ab coated TNF-α siRNA/PEI loaded NPs suggests that it may be used to safely transfect MPs and Caco2-BBE cells.

3.4 **Nanoparticles coated with F4/80 Ab interact preferentially with RAW 264.7 cells than Caco2-BBE cells.**

The binding characteristics of F4/80 receptor (expressed on MPs) to the F4/80 Ab ligand (bound to NPs) was determined by using BI-2000 Biosensing Instrument (Tempe, AZ, USA) based on the surface plasmon resonance (SPR) theory. It was found that the interaction between the layer of NPs coated with the Fab’ portion of F4/80 Ab on the chip is significantly higher with the RAW 264.7 cells (binding coefficient of ~50) compared to Caco2-BBE cells (binding coefficient ~0.5) (respectively 13, 17, 33 and 42 mDeg of resonance angle deviation versus 2, 5, 7 and 10 mDeg for the Caco2-BBE for the injected cells concentrations of 2.5k, 5k, 7.5k and 10k cells/mL) (Figure 10). This significant interaction indicates the higher specificity of NPs (coated with Fab’ of F4/80 Ab) towards MPs (cells expressing F4/80 receptors) compared to intestinal
epithelial cells (cells negative for F4/80 receptors) and suggests that Fab’ portion of F4/80 Ab coated NPs are suitable and efficient for MPs targeting.

3.5 Intracellular uptake of FITC-tagged siRNA by MPs is increased by using NPs coated with Fab’ portion of F4/80 Ab

It has been shown previously that kinetics of NPs uptake by MPs is fast and within an hour, MPs are already saturated with NPs [59]. We wanted to visualize the intracellular uptake of NPs only via interactions between the F4/80 receptors on the cells and the Fab’ part of F4/80 Ab on the NPs loaded with FITC-siRNA. For visualization, MPs in slide chambers were exposed to F4/80 Ab coated NPs loaded with FITC-tagged siRNA (7.3 ng), at PLA concentration of 20g/L. Also MPs were also exposed to F4/80 Ab negative NPs loaded with FITC-tagged siRNA or were left untreated (control) and a dynamic system (mechanical agitation, frequency of motion set at 200Hz) was employed for short exposure time (15min) in an incubator (5% CO2 and 37ºC). Upon fluorescence microscopy, it was found that the NPs loaded with FITC tagged siRNA and covered with the Fab’ portion of F4/80 Ab (500µg/mL) (Figure 11A) were highly taken up by MPs compared to NPs not recovered with Ab (500µg/mL) (Figure 11B). In order to confirm these observations, the quantification of the fluorescence intensity was performed by selecting more than 50 regions of interest (ROI) (data not shown). The intensity of fluorescence per cell surface was significantly higher for the cells exposed to NPs covered with F4/80 Ab Fab’ portion versus cells exposed to non-covered NPs. The high fluorescence signal of F4/80 Ab-NPs treated RAW 264.7 cells correlates with the maximal fluorescent NP uptake by MPs (Figure 11A, B & C) as compared to F4/80 Ab negative NPs treated cells. This is because of the high interaction between F4/80 Ab coated NPs and RAW 264.7 cells, thus indicating not only specific binding but also the higher uptake of these NPs by MPs.
3.6 F4/80 Ab coated TNFα siRNA-loaded NPs reduce TNF-α expression in inflamed macrophages and shows in vitro anti-inflammation effect

Once it was assessed that siRNA loaded NPs coated with F4/80 Ab Fab’ portion were effectively taken up by MPs, ELISA was performed to check if NPs were able to deliver loaded TNF-α siRNA and downregulate TNFα expression. The MPs were pre-treated overnight with F4/80 Ab-NPs (empty) or F4/80 Ab-NPs loaded with TNF-α siRNA or F4/80 Ab-NPs loaded with a scrambled siRNA (NPs concentration of 500µg/mL of medium). MPs were transfected with the same concentration of siRNA TNFα as in NPs using lipofectamine as a control, followed by 1 h LPS (10 µg/mL) treatment to induce in vitro inflammation. Then, to ensure the effectiveness of TNF-α siRNA, TNFα secreted in the medium was measured by ELISA and it was found that TNFα siRNA transfected by lipofectamine showed no anti-inflammatory effect upon LPS stimulation on MPs. The scrambled siRNA loaded NPs and the empty NPs also did not show any anti-inflammatory effect after LPS treatment. The MPs treated with TNFα siRNA loaded NPs covered with Fab’ portion of F4/80 Ab induced a significant decrease in TNF-α secretion upon LPS stimulation (Figure 12). This justifies the use of TNFα siRNA loaded NPs versus directly using siRNA transfected by Lipofectamine and thus indicates that the anti-inflammatory effect of the TNFα siRNA was maintained and amplified by loading it in NPs as already mentioned in previous studies [59]. This shows that the high uptake of F4/80 Ab coated siRNA TNF-α/PEI loaded NPs into cells effectively releases TNF-α siRNA and reduces TNFα expression in inflamed macrophages.
3.7 F4/80 Ab covered NPs loaded with TNFα siRNA attenuate DSS-induced colitis in mice and thus shows in vivo anti-inflammation effect

3.7.1 Weight loss and MPO activity analysis

To investigate the in vivo potential of TNF-α siRNA, C57BL/6 mice were treated with 3% DSS and gavaged for 8 days with hydrogel [mixture of alginate and chitosan at a ratio of 7/3 (wt/wt)] containing F4/80 Ab-NPs loaded either with TNF-α siRNA or scrambled siRNA, or naked NPs loaded either with TNF-α siRNA or scrambled siRNA as described in methods section. Untreated mice received water only and were gavaged with NPs coated with F4/80 Ab. Hydrogel delivers the NPs in the mice colonic lumen. As weight loss and MPO activity are the markers of inflammation, a daily gavage of NPs encapsulated in hydrogel showed gradual weight loss of DSS treated mice. Interestingly, the DSS treated mice gavaged with F4/80 Ab-NPs loaded with TNF-α siRNA showed significant reduction in weight loss and less MPO activity when compared to others (Figure 13). Weight loss for this group was 6% of the initial weight (MPO=0.07 unit/µg of total colon protein) compared to mice treated with naked NPs loaded with TNFα siRNA loosing 9% of the mice initial weight (MPO=0.1 unit/µg of total colon protein). Mice treated with 3% DSS and NPs loaded with scrambled siRNA (NPs-F4/80 Ab scrambled siRNA or NPs naked-scrambled) did not show reduction in weight loss (respectively 15% and 25% weight loss compared to initial weight) or myeloperoxidase activity (respectively 22 and 23 unit of MPO/µg of total colon protein) reduction. These measurements show that TNFα siRNA loaded NPs covered with Fab’ portion of F4/80 Ab is efficient to attenuate DSS-induced colitis. This attenuation might be because of TNF-α siRNA release from the NPs which is efficiently inhibiting TNF-α secretion by MPs in the colon and thus reducing inflammation.
3.7.2 Western Blotting analysis

Western blot of IκBα and β actin of total mouse colonic cells was performed to assess anti-inflammatory effect of TNFα siRNA. In figure 14, we demonstrated that IκBα accumulation was lower in mice treated with 3% DSS and receiving scrambled siRNA loaded NPs. On the other hand, in mice receiving TNFα siRNA loaded NPs covered with Fab’ part of F4/80 Ab, the IκBα unit is accumulated in higher level. These results suggest that the IκBα unit was protected from degradation in colon treated with TNFα siRNA loaded NPs covered with Fab’ part of the F4/80 Ab. This protection leads to an inhibition of NFκB pathway. To confirm further, the colon of these 2 mice groups were processed and it was observed that the mice colon treated with TNFα siRNA loaded NPs coated with F4/80 Ab was less short as compared to the control NPs mice (data not shown).

3.7.3 Histology analysis

One of the hallmarks of DSS-induced colitis is profound colonic inflammation characterized by crypt destruction, mucosal ulceration, erosion, and infiltration of lymphocytes into the mucosal tissue. Hematoxylin counterstaining was performed to visualize the histological damage of mice colon respectively in mice receiving water only (Figure 15A), mice treated with 3% DSS along with scrambled siRNA loaded Fab’ F4/80 Ab covered NPs, and mice treated with 3% DSS along with TNFα siRNA loaded Fab’ F4/80 Ab covered NPs. The colon of DSS-treated mice, gavaged with scrambled siRNA loaded NPs covered with Fab’ part of F4/80 Ab (Figure 15B) showed multifocal inflammatory cell infiltration into the submucosa, severe denudation of the surface epithelium (erosion), and mucodepletion of glands. In contrast, DSS mice treated with TNFα
siRNA loaded NPs covered with Fab’ part of F4/80 Ab (Figure 15C) showed near-normal colonic histology.

Ly6G immunostaining was performed to visualize the neutrophils infiltration. The neutrophil marker immunostaining showed that TNFα siRNA loaded NPs coated with F4/80 Ab dramatically reduced the level of neutrophils infiltration (Figure 15C) compared to DSS treated mice receiving scrambled siRNA loaded NP covered with Fab’ part of F4/80 Ab (infiltration of immune cells including neutrophils and lymphocytes were seen in the lamina propria) (Figure 15B). Even with DSS, TNFα siRNA loaded NPs maintained the colonic epithelial cell tridimensional organization and mucosa structure similar to the water control (Figure 15A). These results thus show that TNFα siRNA loaded NPs covered with Fab’ part of F4/80 Ab is efficient to reduce DSS-induced colitis.

3.8 Fab’ part of F4/80 Ab covered NPs loaded with TNFα siRNA target intestinal macrophages

Finally, we wanted to check if the beneficial effect of the NPs covered with Fab’ part of F4/80 Ab was mediated via F4/80 interactions. Thus, we checked if the major type of cells phagocytizing the NPs was the MPs. Mice were treated with 3% DSS and gavaged for one week with FITC tagged siRNA loaded NPs coated without (Figure 16A) or with Fab’ portion of F4/80 Ab (Figure 16B). Several subset of cells were defined and analyzed resident colonic macrophages as CD11c(-)CD11b(+)F4/80 (+). The results confirmed that the overall attenuation of the DSS-induced colitis was done via NPs interaction and uptake by MPs. Interestingly, 28% of mice MPs treated with FITC siRNA loaded NPs covered with F4/80 Ab showed a positive
signal to FITC (Figure 16B). In Figure 16A, mice treated with FITC siRNA loaded NPS non-covered showed only 19% of positive cells to FITC.
Figure 7. Complexation/protection of TNF-α siRNA by PEI. Electrophoretic migration in a 4% agarose gel of 0.4μg/mL (w/v) free TNF-α siRNA (1), complex formed with PEI (2), of free TNF-α siRNA alone (3) or at 37°C with RNAse A during 2h digestion (4), and complex formed with PEI (lane 5 during 2h of RNAse, lane 6 during 4h of RNAse). Decomplexation of polyplexes of lane 6 (PEI) showed in lane 7 (PEI) with a concentrated NaOH (0.1M, 1min) solution [59].
Figure 8. Characterization of TNF-α siRNA-loaded NPs. (A) Atomic force microscopy (AFM) picture of NPs made of PLA-PEG-OH (1mg/mL, scale bar=2μm). B. Scanning electron microscopy (SEM) picture of NPs made of PLA-PEG-OH (1mg/mL, scale bar=1μm).
Figure 9. Cytotoxicity assessment on MPs and Caco2-BBE cells of F4/80 Ab coated TNF-α siRNA-loaded NPs. WST-1 assay to determine the cytotoxicity of F4/80 Ab coated TNF-α siRNA NPs (1 mg/ml) compared to F4/80 Ab coated empty NPs (1mg/mL) and siRNA NPs (1 mg/ml) on RAW 264.7 cells (mouse macrophages) and CaCo2-BBE cells after 48h of exposure.

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Figure 10. Measurement of RAW 264.7 cells and IECs (intestinal epithelial cells) interaction with NPs coated with Fab’ portion of F4/80 Ab using SPR binding experiment. Nanoparticles coated with Fab’ of F4/80 Ab significantly interact with MPs compared to IECs as shown by amplitude of resonance angle deviation (mDeg) for each type and increasing concentration of cells (2500, 5000, 7500 and 10000 cells/mL).
Figure 11. Fluorescence microscopy picture of uptake of F4/80 Ab-coated FITC-tagged siRNA/PEI loaded NPs (500μg/mL) by MPs after 15min mechanic agitation, at 200Hz. (red: cell cytosol, purple: cell nucleus and green: NPs). (A) MPs exposed to F4/80 Ab coated NPs loaded with FITC-tagged siRNA (green). (B) MPs exposed to F4/80 Ab negative NPs loaded with FITC-tagged siRNA (green). (C) Untreated control cells.
Figure 12. In vitro anti-inflammation effect of TNF-α siRNA-loaded NPs coated with Fab’ portion of F4/80 Ab on MPs. MPs were exposed for 24h with 500µg/mL of NPs covered with Fab’ part of F4/80 Ab loaded with a scrambled siRNA (NP-F4/80-scrambled siRNA) or empty NPs (NP-F4/80-empty) or loaded with TNFα siRNA (NP-F4/80-TNFα siRNA). After NPs exposure, all samples were treated with LPS (10µg/mL) for 1h and the supernatants were analyzed by ELISA.
Figure 13. In vivo anti-inflammation effect of TNF-α siRNA-loaded NPs coated with F4/80 Ab assessed by weight loss and MPO activity in mice treated by 3% DSS during 8 days. Along with DSS in water, mice received a daily gavage of NPS encapsulated in hydrogel to target the mice colon part. One control group received water only with daily gavage of NPs covered with Fab’ part of F4/80 Ab (NPs-scrambled). Two groups were treated with 3% DSS and NPs covered with Fab’ part of F4/80 Ab and loaded either with TNFα siRNA (NPs-F4/80-TNF si) or loaded with scrambled siRNA (NPs-F4/80-scrambled). Two groups were treated with 3% DSS and NPs none covered with Ab and loaded either with TNFα siRNA (NPs-naked-TNF si) or loaded with scrambled siRNA (NPs-naked-scrambled).
Figure 14. Western blot analysis of IκBα and β-actin of total mouse colonic cells. Water control group received water only with daily gavage of NPs covered with Fab’ part of F4/80 Ab (Water control). Two others groups received NPs-F4/80 Ab-TNF-α siRNA or NPs-F4/80 Ab-scrambled and were respectively treated with 3% DSS.
Figure 15. Histology Analysis. Hematoxylin/Eosin staining was performed to visualize histological damage of mice colon. Additionally Ly6g Immunostaining was performed to visualize the neutrophils infiltration respectively in mice receiving water only (A), mice treated with 3% DSS along with scrambled siRNA loaded Fab’ F4/80 Ab covered NPs (B) and mice treated with 3% DSS along with TNFα siRNA loaded Fab’ F4/80 Ab covered NPs (C). Arrows indicate the neutrophil infiltration staining.
Figure 16. Attenuation of DSS-induced colitis in mice using TNFα siRNA loaded NPs and covered with Fab’ of F4/80 Ab is mediated via interaction of NPs with F4/80 receptors of MPs. (A) Flow cytometry analysis (FACS) of the total colon cells for mice treated with 3% DSS and a daily gavage of non-covered FITC tagged siRNA loaded NPs (endocytosis of the NPs occurred in 19% of the colonic MPs). (B) Flow cytometry analysis (FACS) of the total colon cells for mice treated with 3% DSS and a daily gavage of Fab’ part of the F4/80 Ab covered FITC tagged siRNA loaded NPs (endocytosis of the NPs occurred in 28% of the colonic MPs).
4. DISCUSSIONS

IBD includes Crohn’s disease and ulcerative colitis and is characterized as a chronic inflammatory disorder of the gastrointestinal tract. TNF-α is one of the central cytokines in IBD, produced chiefly by macrophages, and is the proinflammatory mediator involved in the pathogenesis of IBD. Therefore TNF-α has been the primary target of biologic therapies [66]. As TNFα is mainly secreted by MPs, our goal was to design a nanoparticle based therapeutic approach to target this specific cell type using both in vitro (RAW264.7 cells) and in vivo models (3% DSS colitis mice model). In this study, NPs were synthesized using PLA-PEG-Mal and PLA-PEG-OH copolymers. Once the NPs were synthesized, the Ab (F4/80, well characterized specific receptors on MPs) was modified, as Fc part of the Ab is known to be a specific site for complement attachment and MPs binding that may lead to some nonspecific interactions. So, to prevent any immune response or reaction, Fc part of the Ab was pepsin digested and Fab’ portions were generated by mild reduction so that they present –SH functions. Interestingly, the Fab part is the only part that provides the receptor/ligand specificity and characterizes the Ab family. PLA-PEG-Mal polymer then permitted grafting of mouse F4/80 Ab (Fab’ portion) onto the surface of NPs by employing the functionality of Maleimide. Maleimide enables any molecule containing sulfur functions (-SH) to be covalently plugged to the copolymer. As mentioned earlier, our strategy of targeting mostly MPs was driven by the fact that MPs are the main TNFα secreting cells. Therefore, the MPs were treated with F4/80 Ab NPs and it was assessed that uptake in MPs was enhanced after NPs showed high interaction with MPs receptors F4/80. Moreover, the TNFαsiRNA was efficiently released not only in vitro in MPs but also in vivo in mice. The ELISA, MPO assay and histology analysis showed that F4/80 Ab -NPs loaded with TNF-α siRNA effectively and specifically delivered siRNA-TNF-α into the
cytoplasm and induced efficient gene silencing of endogenous TNF-α in inflamed MPs. Moreover, the cell sorting (FACS analysis) showed enhanced signal of NPs (FITC⁺ cells) when NPs were covered with the Fab’ part of F4/80 Ab. This study is a suitable strategy to target any cell type as in the NPs synthesis process the attachment of the Fab’ part of Ab is performed at the extreme end of the synthesis protocol. This strategy could be applied to target any specific receptor of any type of cell. This study is of interest due to various applications in different diseases and organs. In addition to targeting several other organs or cells, one can also imagine using other drugs or components that are needed to be protected and forwarded to their target cells (siRNA, miRNA, aptamers…).
5. CONCLUSIONS

Today, treatments available for IBD consists mainly of immunomodulators and immunosuppressants that are administered systemically or orally. But due to the limitations associated with safety and efficacy of these conventional IBD treatments, biological therapies are becoming increasingly attractive. Biological therapies treating IBD involve directing anti-inflammatory cytokines or inhibiting proinflammatory genes to the actual place of the inflammation. In this aspect, strategies have been proposed using anti TNF-α agents such as monoclonal antibodies, which are highly effective in general. However, few patients showed at least one of the adverse effects such as pneumonia, cancer, or acute inflammation which is largely due to systemic TNF-α suppression [41]. To minimize the systemic side effects, current NP approaches based on TNF-α siRNA have been proposed for drug delivery, but still several factors limit the utility of siRNA. The most important factor limiting siRNA utility as therapeutics is the difficulty to deliver siRNA to its intracellular target site because of its physicochemical properties (negative charges, large molecule weight, and size) and its instability with plasma half-lives of about 10 min [48, 67, 68]. Moreover, siRNA after endocytosis is transported to lysosomes where siRNA is degraded [69]. In our study, we have proposed a new way to target and deliver siRNA. We complexed siRNA TNF-α with PEI that improves the stability of siRNA without affecting its RNAi efficiency, protects from degradation, and enhances its intracellular delivery [69, 70]. And thus PEI consequently induces gene silencing of endogenous TNF-α in inflamed MPs. The next step was to specifically target siRNA TNF-α/PEI-loaded NPs to the inflamed MPs. The conjugation of surface pegylated polymeric nanoparticles with cell-specific targeting ligands may improve the pharmacokinetics, biodistribution, and selectivity of siRNA therapeutics and reduce their toxic side effects. With this study, we have
made progress towards treating IBD locally by combining positive aspects of RNA interference with the safety of biodegradable polymeric nanoparticulate systems and specificity of cell specific targeting ligands, enabling a localized treatment through oral administration of siRNA rather than using the traditional systemic approach as reported previously [71, 72]. Our results demonstrated that TNF-α siRNA loaded NPs coated with F4/80 Ab are non-cytotoxic and thus can be used for therapeutic purposes. In vitro studies revealed the internalization of these NPs via endocytosis and that TNF-α siRNA reduced TNF-α secretion by inflamed MPs. Moreover, in vivo studies involving oral administration of hydrogel encapsulated TNF-α siRNA/PEI-loaded NPs coated with F4/80 Ab demonstrated their potential in effectively delivering drug into the mice colon and specifically targeting colon MPs. TNF-α siRNA treatment in murine DSS colitis resulted in the reduction of the mice weight, colon length, MPO activity, the extent of neutrophil infiltration and the microscopic evidence of inflammation; findings which are in agreement with the silencing ability of TNF-α. Altogether, these results demonstrated that the efficacy of TNF-α loaded inside the antibody coated NP is greater, both in vivo and in vitro, compared to that of the classic carriers (e.g., liposomes, cell-penetrating peptides, polymer nanocarriers, dendrimers, siRNA bioconjugates….) [69, 73-75]. The safety, effectiveness, and ease of manufacturing set TNFα siRNA loaded NPs covered with Fab’ part of F4/80 Ab as an appropriate carrier and a new tool to specifically deliver anti-TNF-α agents to MPs with minimum systemic toxicity for the treatment of colon inflammation. This protocol can be applied as a generic tool for any type of cells. One can imagine generating any specific ligand for one type of cell and plugging it on the NPs.
REFERENCES


