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Fab'-bearing siRNA TNF α -loaded nanoparticles targeted to colonic macrophages offer an effective therapy for experimental colitis

Laroui Hamed^{2,*}, Viennois Emilie^{1,2,3}, Bo Xiao^{1,2}, Brandon S. Canup², Geem Duke^{1,4}, Timothy L. Denning^{1,4}, and Merlin Didier^{1,2,3,4}

¹Institute for Biomedical Sciences, Center Diagnostics and Therapeutics, Georgia State University, 100 Piedmont Avenue, Atlanta, GA 30303, USA

²Department Chemistry and Biology, Center Diagnostics and Therapeutics, Georgia State University, 100 Piedmont Avenue, Atlanta, GA 30303, USA

³ Veterans Affair Medical Center, 1670 Clairmont Rd, Decatur, GA 30033, USA

⁴ Center for Inflammation, Immunity, & Infection, Georgia State University, 100 Piedmont Avenue, Atlanta, GA 30303, USA

Abstract

Patients suffering from Inflammatory Bowel Disease (IBD) are currently treated by systemic drugs that can have significant side effects. Thus, it would be highly desirable to target TNF α siRNA (a therapeutic molecule) to the inflamed tissue. Here, we demonstrate that TNF α siRNA can be efficiently loaded into nanoparticles (NPs) made of poly (lactic acid) poly (ethylene glycol) block copolymer (PLA-PEG), and that grafting of the Fab' portion of the F4/80 Ab (Fab'-bearing) onto the NP surface via maleimide/thiol group-mediated covalent bonding improves the macrophage (MP)-targeting kinetics of the NPs to RAW264.7 cells *in vitro*. Direct binding was shown between MPs and the Fab'-bearing NPs. Next, we orally administered hydrogel (chitosan/alginate)-encapsulated Fab'-bearing TNF α -siRNA-loaded NPs to 3% dextran sodium sulfate (DSS)-treated mice and investigated the therapeutic effect on colitis. *In vivo*, the release of TNF α -siRNA-loaded NPs into the mouse colon attenuated colitis more efficiently when the NPs were covered with Fab'-bearing, compared to uncovered NPs. All DSS-induced parameters of colonic inflammation (*e.g.*, weight loss, myeloperoxidase activity, and Ikba accumulation) were more attenuated Fab'-bearing NPs loaded with TNF α siRNA than without the Fab'-bearing. Grafting the Fab'-bearing onto the NPs improved the kinetics of endocytosis as well as the MP-targeting ability, as indicated by flow cytometry. Collectively, our results show that Fab'-bearing PLA-PEG NPs are powerful and efficient nanosized tools for delivering siRNAs into colonic macrophages.

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*Corresponding author: hlaroui@gsu.edu.

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Keywords

TNF secretion; targeted siRNA release; DSS induced colitis; Ligand-mediated targeting

Introduction

Patients with inflammatory bowel disease (IBD) show defects in intestinal epithelial barrier function that can allow bacteria to colonize the colonic epithelia, secrete pro-inflammatory oligopeptides and penetrate into the tissue [1-3]. Bacterial antigens are presented to dendritic cells and macrophages (MPs), which secrete pro-inflammatory cytokines in the lamina propria, triggering the recruitment of circulating immune cells *via* the expression of adhesion molecules on endothelial and immune cells [4]. These pathogenic processes are the targets of modern research on therapeutic approaches for IBD, which can be divided into three categories: the development of inhibitors of inflammatory cytokines (*e.g.*, anti-TNF α) that induce T-lymphocyte apoptosis; the identification of anti-inflammatory cytokines that downregulate T-lymphocyte proliferation; and the synthesis of selective adhesion molecule (SAM) inhibitors that suppress the trafficking of T-lymphocytes into the gut epithelium [5-15]. However, the drugs that confer these effects are usually administered at high doses and/or systemically, leading to significant adverse events. A major drawback in the development of therapeutic strategies for diseases such as IBD has been the inability to target sufficient quantities of drugs to the site of inflammation, such that the local drug concentration is maximized while systemic side effects are minimized. Furthermore, the organs of the gastrointestinal tract, particularly the colon, differ in their drug-absorption properties, and it is difficult to deliver drugs to the colon while preventing degradation by digestive enzymes and/or systemic absorption.

We recently described an original technique for targeting the colon with anti-inflammatory peptide (KPV)-loaded nanoparticles (NPs) encapsulated in an alginate/chitosan hydrogel [16]. Our results showed that gavage of KPV-loaded encapsulated NPs to dextran sodium sulfate (DSS)-treated mice could overcome physiological barriers and target KPV to inflamed colonic regions at a 1200-fold lower concentration than that required to achieve the same efficacy when KPV was given in free solution [16].

Our optimized NP synthesis process allows encapsulation of many types of water-soluble molecules, including the prohibitin protein [17] and siRNA [18]. The discovery of siRNA by Fire and Mello [19] in the late 1990s introduced an innovative approach to the relatively new field of gene therapy, allowing single target genes to be turned off without genomic integration of exogenous DNA. The delivery of siRNA to target tissues via traditional agents (*e.g.*, Lipofectamine) are complicated because naked siRNA lacks stability and shows poor tissue penetration [20-22]. In the other hand, the pre-complexation of siRNA with low molecular weight polyethyleneimine (PEI) has been shown to protect against degradation, enhance drug loading, and increase siRNA lysosome-escape ability via the “proton sponge effect” [18, 23].

In the present study, we explored the therapeutic effect of colon-homing NPs with the ability to directly release specific siRNAs to target cells. This work utilized the advantages of NPs,

including their ability to easily pass through physiological barriers, evade phagocytosis, show rapid mixing kinetics, accept high loading concentrations, confer little or no toxicity, and resist degradation. Specifically, we orally administered intestinal-MP-targeting encapsulated Fab'-bearing TNF α -siRNA-loaded NPs and examined its efficacy in treating a mouse model of colitis.

Material and methods

Preparation of TNF α siRNA/PEI loaded NPs

NPs were synthesized via double emulsion/solvent evaporation, as described previously [16, 18]. Briefly, an internal phase (see details below) containing the drug was mixed with 20 g/L of PLAPEG or PLA-PEG-Mal in dichloromethane to generate a water-in-oil (W/O) emulsion after 2 min of vortexing (Maxi Mix II, Thermodyne, Dubuque, Iowa) and 1 min of sonication with 50% active cycles at 70% power ($P_{max}=400$ W) (Digital Sonifier 450, Branson, Danbury, CT). This first emulsion was dropped in a second water phase containing 0.3g/L of PVA to generate a water/oil/water emulsion (W/O/W).

The W/O/W emulsion was dropped in a dispersing phase of 0.1g/L polyvinyl alcohol (PVA), and stirred at 45°C under a vacuum to remove dichloromethane. NPs were centrifuged at 9953g and freeze-dried overnight at -50°C under 0.1 mbar pressure. As the second emulsion allowed PVA to be grafted on the surface by hydrophobic interaction with the PLA matrix, NPs were coated with PVA to prevent aggregations through electrostatic repulsions.

Preparation of the internal phase

The internal phase has a typical N/P ratio of the number of negative charges of siRNA (TNF α siRNA or FITC-tagged siRNA) (P as the phosphorous charge) and positive charges of PEI (N as the ammonium charge) (N/P ratios of 30 for PEI). A mixture of siRNA/PEI: 29 μ L TNF α siRNA (5 μ M) was combined with 18 μ L PEI (5mM), and incubated for 10 min at room temperature for complexation. After 10 min, a polyplex was formed, and 750 μ L bovine serum albumin (BSA, 50g/L) added, generating the first emulsion with dichloromethane.

Synthesis of Fab' portion of the F4/80 antibody—The F4/80 Ab is first digested by pepsin, a nonspecific endopeptidase, used to enzymatically digest the Fc portion of whole IgG to yield the fragment known as F(ab')₂. This fragment is composed of a pair of Fab' units connected by two disulfide bonds. As the pepsin protease is supplied in immobilized form as beaded agarose resin, the digestion reaction is stopped by removing the resin from the IgG solution. The resulting digestion products are enzyme-free. In order to covalently attach the Fab' fragment to the maleimide group of the NP matrix, -SH groups were generated. Thus, we performed a mild reduction using 2-Mercaptoethanol (Thermo Scientific Pierce, Pittsburg, PA) to cleave the Fab disulfide bonds. The final result was a Fab' fragment of the F4/80 antibody. Once the Fab' fragment is generated, it is suitable for covalent attachment onto the NP surface via maleimide.

Cell Culture—RAW 264.7 cells (mouse MPs) were cultured to confluence in 75-cm² flasks at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂. The culture medium was DMEM/Ham's F-12 medium (Invitrogen, Grand Island, NY) supplemented with l-glutamine (2 mm), penicillin (100 units/ml), streptomycin (100 µg/ml), and heat-inactivated fetal calf serum (10%, v/v) (Atlanta Biologicals, Atlanta, GA). For the fluorescent study, we placed a plate checker in the incubator (15 min, 200Hz).

WST-1—To assess the potential toxicity of NPs before (NP PEG-PLA-Mal) or after (NP PEGPLA-Ab) with Fab'-bearing, a WST-1 assay was performed. As described previously [54], RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^4 cells per well and exposed to 1 mg/mL of empty NPs (NP PEG-PLA-Ab empty), empty non Fab'-bearing NPs (NP PEG-PLAMal) and TNF α siRNA loaded NPs covered by Fab' (NP PEG-PLA-Ab with TNF α siRNA) for 48h. The WST-1 assay measures cleavage of the soluble red tetrazolium salt, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), by dehydrogenase present in intact mitochondria, which leads to the formation of dark red formazan crystals. WST-1 proliferation reagent (10 µL) was added to cells (10 µL/well) and incubated for 1–2 h at 37 °C. The wavelength for measuring absorbance of the formazan product was 440 nm.

Western Blotting—Cell lysates were resolved on polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with anti-IkBa (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).

ELISA—The ELISA detecting TNF α was performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

AFM Measurement—For atomic force microscopy (AFM) test, a drop of NPs made of PLAPEG-OMe suspension was deposited onto a freshly cleaved mica slide, followed by drying overnight at 25 °C. The images were taken using a SPA 400 AFM (Seiko instruments Inc., Japan) at tapping mode using high resonant frequency ($F_0 = 150$ kHz) pyramidal cantilevers with silicon probes at a scan frequency of 1 Hz.

Preparation of Gold Chips Used to Detect Surface Plasmon Resonance (SPR)

—For SPR experiments, we used gold films coated onto BK7 glass slides (Biosensing Instruments, Tempe, AZ). Each chip is a glass surface coated with a gold layer (47 nm thick) over an intermediate layer of chromium (2 nm in thickness). After each gold chip was cleaned with pure ethanol and dried under a stream of N₂, 15–20 µl of cystamine dihydrochloride (20 mm; Sigma, St Louis, MO) was cast onto each film overnight in a humidified reaction chamber. Cystamine dihydrochloride is light-sensitive, and the chamber was thus covered with a lid. Next, each chip surface was thoroughly rinsed with deionized water and dried by gentle blowing with a stream of N₂ [24]. We prepared a fresh mixture of 15 mm *N*-hydroxysuccinimide, 75 mm 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and 6 mg/ml carboxymethyl dextran (Sigma, St Louis, MO) and added this mixture to chips

modified with cystamine dihydrochloride followed by incubation 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 [24].

As shown in Figure 4A and Figure 5A, after placing a chip into the BI-2000 SPR machine, we coated PLA-PEG NPs covered with Fab' bearing onto a gold film after preactivation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide solution. This step was repeated until the NPs coating level permitted the commencement of SPR experiments (~60– 80 mDeg). Two fluidic channels can be used on this instrument, and the same sample plug flowed from the first to the second channel. When “serial mode” is selected, the BI-2000 machine simultaneously coats both channels with the same amount of NPs. This option is essential to permit comparison of the interaction between NPs and the two different types of cells (RAW 264.7 or CACO2BBE cells). Residual activated carboxyl groups were neutralized by a final injection of 1 mM ethanolamine (Sigma). For analysis, injection of RAW 264.7 cells was performed with “single mode” selected on channel 1. For comparison, the same protocol was used injecting CACO2BBE cells selecting single mode on channel 2.

Association and dissociation constants of interactions between NPS covered with Fab' fragments (coated molecules) and RAW 264.7 cells or CACO2BBE cells (circulating molecules) were obtained using a BI-2000 (Biosensing Instruments) using SPR theory. K_d values (expressed in mol/liter⁻¹), which measure the 50% adsorption levels of CMs onto gold chips covered with coated molecules, are commonly used to describe the affinity between two molecules, such as how tightly a ligand binds to a particular receptor. In our study, the software calculation model using 1:1 assumption as ligand: receptor ratio was not used. As cells expressed many receptors on their surface, attributing one interaction to only one cell would have been a mistake. Although the interaction between cells and NPs lead to a resonance deviation angle that can be used to compare quantitatively the interactions strength of the 2 types of cells with the NPs. These affinities are influenced by non-covalent intermolecular interactions between the two molecules, including hydrogen-bonding, electrostatic interactions, and hydrophobic and Van der Waals forces. Briefly, after coating a chip with NPs, increasing concentrations of cells solutions in PBS of were passed over the chip. A two-step interaction curve was obtained. The first step involved adsorption of cells to the maximal level. In the second step, when the flow of cells concentration returned to zero, nonspecific adsorbed cells are released with the running buffer. On the chip, only coated cells remained “attached.” The adsorption curve kinetics thus decreased to a plateau located at a level above the initial base line. The amplitude of cells binding to coated NPs was taken to be the difference between the initial and final levels. These different amplitudes obtained for each cells at different cells concentrations were then compared to each other.

DSS-induced colitis—Colitis was induced by 3% (w/v) dextran sodium sulfate (DSS; molecular weight 42 kDa; ICN Biochemicals, Aurora, OH) added to the drinking water. Colonic inflammation was assessed 7/8 days after DSS treatment [25, 26]. Eight mice were included in each group.

Gavage of scrambled and Fab'-bearing TNF α siRNA-loaded PLA-PEG NPs encapsulated in hydrogel

Chitosan powder was solubilized in acetic acid then neutralized by addition of NaOH (0.1 mol/L) to give a final chitosan concentration of 0.6% (wt/vol). Medium-viscosity sodium alginate was prepared in NaCl (0.15 mol/L) 1.4% (wt/vol). Before mixing with NaCl, or acetic acid, the polymer powders were weighed, placed in glass tubes, and autoclaved. Alginate solution and chitosan solutions were mixed at a 1:1 ratio for a final concentration of 7 and 3 g/L, respectively. The polymer suspension was homogenized for 24 hours.

Different NPs were added to obtain 5 mg/mL concentration of hydrogel solution and stirred to disperse NPs throughout the polymer solution. A chelation solution containing 70 mmol/L of calcium chloride and 30 mmol/L of sodium sulfate was prepared. Procedure for inclusion of loaded NPs into biomaterials and the gavage method are available at http://www.natureprotocols.com/2009/09/03/a_method_to_target_bioactive_c.php.

C57BL/6 mice (8 per group) were receiving daily gavages during the 7/8 days of DSS treatment with Fab'-bearing TNF α siRNA-loaded PLA-PEG NPs, Fab'-bearing scrambled siRNA-loaded PLA-PEG NPs, "naked" TNF α siRNA-loaded PLA-PEG NPs and "naked" scrambled siRNA-loaded PLA-PEG NPs. Then, mice were sacrificed and colons were collected for biological analysis.

Isolation of total colon cells—Isolation of colonic intestinal cells was performed as previously described with modifications [27]. Briefly, large intestines were removed, carefully cleaned and opened longitudinally, washed of fecal contents, cut into pieces 0.5 cm in length. The 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- μ m strainer and pelleted by centrifugation at 300 g.

Flow cytometry—Isolated intestinal colonic cells were resuspended in PBS containing 5% FBS. Live cells were identified using an Aqua Dead Cell Staining Kit accordingly to the manufacturer's instructions, and Fc receptors were blocked with the antibody anti-Fc γ RIII/II (2.4G2) for 15 minutes at 4°C. After incubation the cells were stained at 4°C for 30 minutes with labeled antibodies. Abs used for analysis were from eBioscience unless otherwise noted: 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, and eFluor 450-conjugated anti-mouse CD11b. Samples were then washed 2 times in PBS containing 5% FBS and analyzed immediately. Flow cytometric analysis was performed on a LSR II (BD).

Ly-6G staining—5- μ m paraffin-embedded 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. Epitope retrieval was performed by treating the tissues with 10 mM sodium citrate buffer (pH 6.0) with 0.05% Tween 20 at 100°C for 10 minutes in a pressure cooker. Ly-6G staining sections were blocked with 10% normal goat serum with 1% BSA in TBS for 2 h at room temperature followed by

incubation with rat monoclonal anti-Ly6g antibody (1: 500 dilution) (Abcam, Cambridge, MA, USA. ab25377) in TBS with 1% BSA at 4°C overnight. Tissue sections 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 reaction. Sections were then counterstained with hematoxylin, dehydrated in an ethanol and xylene. Images were acquired at 20X magnification using an Olympus microscope equipped with a D-26 color camera.

Statistical Analysis—Data are expressed as means $\pm$ S.E. Statistical analysis was performed using the unpaired two-tailed Student's *t* test featured in inStat version 3.06 (GraphPad) software. *p* < 0.05 was considered statistically significant.

Results

Synthesis and characterization of the polymer, PLA-PEG-Maleimide

Our NP matrix consisted of poly (lactic acid) poly (ethylene glycol) block copolymer (PLA-PEG). In order to covalently attach the Fab' portion of F4/80 (to get Fab'-bearing NPs) onto the NP surface, we added a reactive functional group (maleimide; Mal) to the PEG side of the PLA-PEG block. Mal enables any molecule containing thiol groups (–SH) to be covalently attached to the copolymer (Figure 1A).

The reaction of L-lactide with PEG-Mal was stoichiometric and total as all maleimide functional groups were conferred to the block polymer. The PLA-PEG linkage was synthesized by ring-opening polymerization in dry toluene, and the products were precipitated and recovered. We used methoxyPEG and PEG-Mal to initiate the polymerizations of methoxyPEG-PLA (PLAPEG-OMe) and maleimide-PEG-PLA (PLA-PEG-Mal), respectively. Performed in an organic solvent (toluene), this method allowed us to control the length of the PLA chain and ensure a low polydispersity of the polymer's molecular weight [28]. As shown in Figure 1B, the maleimide signal peak obtained by proton NMR (¹H NMR) did not appear at 6.7 ppm, indicating that maleimide functional groups were not conferred to the control copolymer (PLA-PEG) block, which we then used to synthesize control NPs with no Ab on the surface. For the PLA-PEG-Mal polymer, the spectrum showed a significant peak at 6.7 ppm, indicating that functional Mal groups had been connected to the (PLA-PEG) block (Figure 1C). Also, the ratio of the peak area for PLA-PEG-Mal methylene proton versus the peak area of the maleimide proton was 218.9 which indicated that the maleimide function was mostly preserved in the final product as the spectrum of PEG-Mal polymer showed the same ratio value (PEG-Mal methylene proton versus the peak area of the maleimide proton = 189, data not shown). This copolymer was used to synthesize Fab'-bearing NPs. The ¹H NMR spectra (Figures 1B and 1C) confirmed the syntheses of both copolymers and allowed us to calculate the ratio of maleimide/PEG. The Mal proton signals were observed at 6.7 (Figure 1C). This allowed us to conclude that the Mal functional groups were present and intact on the copolymer.

Generation and characterization of TNF α siRNA/PEI-loaded PLA-based nanoparticles

NPs were prepared with PLA-PEG-OMe and PLA-PEG-Mal by the double emulsion/solvent evaporation method [16, 18, 23, 29]. As previously described [18] and detailed in the Methods section, TNF α siRNA complexed with PEI (2kDa) (10 min at 4°C) can be loaded into NPs (36 μ L of 5m M PEI plus 36 μ L of 25 μ M siRNA). This inner aqueous phase was added to 4 ml of PLA-PEG-Mal or PLA-PEG-OMe dissolved in dichloromethane to form a first emulsion under sonication. This first emulsion was then mixed with a higher aqueous phase consisting of 8 mL of sodium cholate (0.3%). Sodium cholate was chosen as the surfactant because previous work showed that cholate reduced the hydrodynamic diameter and polydispersity index of NPs [30]. The hydrophilicity of PEG and the hydrophobicity of PLA result in a phase separation of the two blocks in water, whereupon the PEG chains orient themselves toward the aqueous phase to form a “corona” layer around the PLA NP matrix [31].

The mean hydrodynamic diameter of the TNF- α siRNA/PEI-loaded NPs was found to be 609 nm ($\pm$ 37 nm) for those recovered by PLA-PEG-OMe using dynamic light scattering (DLS). This size calculation was confirmed by atomic force microscopy AFM (Figure 2A) and SEM (Figure 2B). The Figure 2A showed that the size of NPs PLA-PEG-OMe (1 mg/mL) calculated by DLS was correct as AFM diameter average was 582 nm. In figure 2B, SEM picture shows the distribution of NPs PLA-PEH-OH size (1 mg/mL). The NPs size distribution was sharp as the polydispersity index (PDI) was equal to 0.11, considered as monodisperse, as the PDI is below 0.3.

Synthesis of NPs covered with the Fab'-bearing

Various specific antibodies, ligands, and peptide ligand mimetics have been grafted on nanocarriers surface to target cells and tissues [32, 33]. Active targeting is intended to cause NPs to accumulate in close proximity to the target cell and actively cross the cell membrane, facilitating the transport of siRNAs into the cytoplasm, where they activate the RNAi pathways. Here, we aimed to use the Fab'-bearing to target our TNF- α siRNA-containing NPs to MPs. The Fab'-bearing part is a single half of Fab portion. Basically, Fab part is an antibody deleted with the Fc part using pepsin digestion. The removal of the Fc portion of an antibody significantly eliminates its interaction with immune cells and decreases non-specific binding [34]. The separation of the dimeric antigen binding site fragment, F(ab)₂, into Fab was achieved by incubation with a reducing agent 2-mercaptoethanol to yield the Fab portion of F4/80 Ab. We then cut this Fab to obtain two Fab' portions of F4/80, as described in the Methods section (Fab' portion synthesis). Finally, the Fab' portion of F4/80 was conjugated to pegylated NPs via the formation of a thioether bond between the thiol groups of the Fab' portion and the Mal moiety at the distal end of PEG-PLA-Mal polymer. A reproducible coupling was achieved with a Fab'-to-Mal ratio of 1:4. Using this strategy, we successfully generated NPs made of PLA-PEG coated with the Fab' part of the F4/80 Ab (Figure 2C).

The covalent bonding of the Fab'-bearing with PLA-PEG stabilizes the colloidal dispersion, size and repulsion force of the NPs, and enhances their biocompatibility

Physical and biological characterizations of new NPs are needed to optimize them as therapeutic vectors. In the context of colloidal stability, surface repulsion forces are essential to obtaining a homogenous dispersion in an aqueous phase (*e.g.*, PBS or a biological medium). As the addition of the Fab'-bearing was the final step after freeze drying the NPs, we were able to test the PLAPEG-Mal NPs before and after the addition of the Fab'-bearing. As shown in Figure 3A shows, the PLA-PEG-Mal NPs aggregated significantly in the aqueous phase; this yielded particles larger than 2 μm in diameter with the potential to have deleterious biological effects, such as heterogeneous dispersion, inconsistent concentrations, and cell cytotoxicity. However, adding the Fab'-bearing to the surface of the NPs completely blocked this unwanted aggregation, likely reflecting the increased hydrophilicity of the coated NPs. This disaggregation increased the degree of maleimide function that could be loaded onto the surfaces of the NPs, which became saturated by the slight excess of the Fab' portion of the F4/80 Ab. As shown in Figure 3B, SEM confirmed the lack of aggregation among NPs that were coated with the Fab' portion of the F4/80 Ab. The average diameter estimated from the SEM pictures was around 400 nm, whereas the exact measurement by DLS indicated that the Fab'-bearing PLAPEG NPs had a diameter of 376 nm (± 19 nm). SEM also showed a homogenous distribution and no aggregation of these particles, indicating that the repulsion force occurring between Fab' portions of the F4/80 Ab was higher than the force of attraction between the Mal functional groups (Figure 3B).

Next, we examined the cytotoxicity of the generated NPs on RAW 264.7 cells utilizing the WST-1 test (mitochondrial dehydrogenase activity), with the viability of RAW 264.7 cells cultured in DMEM medium alone (control) set to 100%. As shown in Figure 3C, the aggregation of uncoated NPs had a dramatic cytotoxic effect on MPs. Only 16.5% survival was seen among MPs that were treated with aggregated NPs for 24h, whereas 112% survival was seen among MPs treated with the Fab'-coated, non-aggregated NPs. NPs covered with the Fab'-bearing but lacking siRNA (another control) did not aggregate and did not exert any cytotoxicity (viability of 104%). Beside mouse cells, we also tested human macrophages cells lines such as U937 and THP-1 cells. Fab'-bearing siRNA TNF α -loaded nanoparticles were exposed to human macrophages such as U937 or THP-1. U937 are mature and differentiate cells that response to a number of soluble stimuli, adopting the morphology and characteristics of mature macrophages. THP1 is a human monocytic cell line derived from an acute monocytic leukemia patient. We thus performed further cytotoxicity tests with the indicated human macrophage cell lines.

We first checked that the human cells described above can uptake the NPs in a significant amount. We performed this experiment using Fab'-bearing PLA-PEG NPs loaded with FITC-siRNA (green). We performed a short exposure time (15 min) experiment in a dynamic system wherein cells were placed in an incubator (5% CO₂ and 37°C) and subjected to mechanical agitation (200 Hz) to prevent the phagocytosis of NPs induced by sedimentation. As shown in Figure 1A and 1B, NPs loaded with FITC-tagged siRNA and covered with the Fab' portion of F4/80 Ab (500 $\mu\text{g}/\text{mL}$) showed a high uptake by human MPs (similarly to mice MPs described in Figure 6A) at the same time of exposure (15min).

Once the endocytosis of the NPs by human MPs has been demonstrated, we have performed the cytotoxicity test for human MPs. As for mice MPs shown in Figure 3C, we found that the exposure of NPs to the two types of human MPs maintained the cells viability for a high concentration of NPs (500 µg/mL) at 48h and 72h (supplementary Figure 1C). Together, the results from WST-1 analyses indicated that Fab'-bearing NPs were biocompatible with eukaryotic cells and candidates for potential therapeutic use.

Finally, we examined the release kinetics of siRNA loaded into the NPs. The results confirmed our previous observations that siRNA complexed with PEI (siRNA/PEI) avoid the early release of siRNA [18]. As shown in Figure 3D, the siRNA release kinetics were better for NPs loaded with PEI-complexed siRNA versus naked siRNA. Interestingly, there was no significant difference in the release of siRNA/PEI between PLA NPs and Fab'-bearing PLAPEG NPs. These kinetic curves demonstrated that our NPs avoided the so-called "burst effect" (*i.e.*, the deleterious early release of the loaded drug).

Quantifying the number of Fab' F4/80 antibody fragments per NP

The above results indicate that the linkage of the Fab'-bearing is key to obtaining a homogenous suspension of NPs and maintaining cellular integrity (Figure 3C). The Fab' fragment provides positive charges that can generate repulsion between the NPs (zeta potential $\approx +2.56$ mV) and be a factor to avoid the aggregation phenomenon. Next, we wanted to estimate the number of Fab' fragment of F4/80 Ab grafted on each NP. Thus, we mixed the Fab' fragment [initial concentration ($C_{iFab'}$) = 201 µg/mL] with 30 mg of PLA-PEG-Mal NPs (total volume of 300 µL). Once the reaction was complete, we centrifuged the NPs (5000 g for 45 min) and collected the supernatant containing the non-attached Fab' molecules ($C_{fFab'} = 139.45$ µg/mL). Using the initial amount of Fab' fragments introduced and the amount of non-adsorbed fragments, we estimated the amount of Fab' attached to the NPs as:

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

where n_{NP} is the number of NPs; m_{NP} is the mass of the NPs; D is the diameter of the NPs; and ρ is the volumetric mass of the NPs. For 30 mg of NPs, we calculated $n_{NP} = 9.89 * 10^{11}$ particles. Next, we estimated the number of Fab' fragments adsorbed on 30 mg of NPs (300 µL of Fab') as:

$$\begin{aligned} n_{Fab'}' &= V (C_{iFab'}' - C_{fFab'}') / M_{wFab'}, \\ n_{Fab'}' &= 3.3 * 10^{11} / 55000 \\ n_{Fab'}' &= 2.05 * 10^{15} \text{ Fab' fragments} \end{aligned}$$

Finally, we calculated the final amount of Fab' fragments per NP ($N_{Fab'}$) as:

$$F_{Fab'}' = \frac{2.05 * 10^{15}}{9.89 * 10^{11}} = 2073$$

This calculation revealed that there were approximately 2073 Fab' fragments per NP.

PLA-PEG NPs coated with the F4/80 antibody preferentially interact with RAW 264.7 cells over Caco2 BBE cells

The above experiments showed that the NPs were significantly covered with the Fab' portion of F4/80 Ab, which increased the repulsive force between NPs. To confirm this and check the integrity of Fab' portion, we used surface plasmon resonance (SPR) experiments in which the gold chip was coated with a single layer of NPs.

Briefly, the gold chip was covered with a layer of carboxydextran (6 mg/mL in degassed water), and the carboxyl group was activated with EDC/NHS (15 mM/75 mM in degassed water), so it could then react to any circulating molecule injected into the system with an amine group. Thus, the NPs covered with the Fab'-bearing portion of the F4/80 Ab (containing amine groups) should covalently bind to the activated carboxydextran surface, whereas the PLA-PEG-OMe NPs should not. To avoid any interference with NP-released products containing -NH₂ functional groups (PEI, siRNA, etc.), we worked exclusively with empty NPs. Figure 4A shows the design of our SPR experiments. The flow was optimized at 30 μ L/min to allow enough time for the Fab'-bearing to react with the activated carboxydextran. The SPR device allows injections of two separate solutions respectively in two channels of the same gold chip (respectively channel 1 and channel 2). Control NPs composed of PLA-PEG-OMe and lacking amine groups did not shift the resonance angle (data not shown in channel 2). In contrast, a resonance angle shift of 91 mDeg was observed in channel 1 when we applied two successive injections of Fab'-bearing NPs (Figure 4B), indicating that the Fab' fragments were covalently attached to the gold chip surface.

Next, we assessed whether the Fab' fragments retained their tridimensional conformation and proper biological activity upon binding to the NPs. To test this, we performed a second SPR experiment using RAW 264.7 cells, which are known to widely express the F4/80 antigens [35-37]. As a control, we used Caco2 BBE (human epithelial) cells that do not express the mouse F4/80 antigen. The experimental strategy is shown in Figure 5A. The SPR experiments were performed in a PBS cell suspension instead of cell media to avoid interference from molecules with amine groups. First, we covalently attached the Fab'-bearing NPs onto the surface of the gold chip (data not shown). At the end of the first step, both channels were equally covered with Fab'-bearing NPs. Then, a second step was performed using the "single" injection mode. In this second step, increasing concentrations of RAW 264.7 cells (channel 1) and Caco2 BBE cells (channel 2) were successively injected onto the activated carboxydextran surface coated with Fab'-bearing NPs. The flow rate (35 μ L/min) and cell concentrations were optimized with respect to the adsorption kinetics of the cells and NPs, allowing the cells to "roll" across the NP-bound surface and interact with any potential surface receptors. The sensorgram presented in Figure 5B shows the results from two different concentrations of RAW 264.7 cells (2,500 and 7,500 cells/mL); the absolute values of resonance angle deviation were 13.66 and 36.70 mDeg, respectively. As the resonance angle deviation mDeg is directly correlated to the binding between the RAW 264.7 cells and the NPs, this indicates that Raw 264.7 cells expressing F4/80 bound directly to the Fab'-bearing NPs in a dose-dependent manner. Between each injection of specific cell

concentrations, the NPs were subjected to NaOH (0.01 M) in order to remove cells and debris that only weakly interacted with the NPs (*e.g.*, via hydrogen bonds, hydrophobic interactions, weak electrostatic interactions, *etc.*) compared to the strong covalent interaction between the NPs and the gold chip. Figure 5C shows the resonance angle deviations (mDeg) for each NP type and cell concentration. The Fab'-bearing NPs showed significantly higher interactions with the RAW 264.7 cells compared to the Caco2 BBE cells (13.66, 18.15, 36.70 and 46.34 mDeg versus 0.00, 0.68, 1.06 and 1.23 mDeg, respectively, for the specified injected cells concentrations of 2.5k, 5k, 7.5k and 10k cells/mL). As the receptor/antibody interaction is strictly correlated to tridimensional requirements, these results indicate that the integrity of Fab' fragments was preserved following covalent attachment to the NP surface. As the reaction between maleimide and sulfhydryl (-SH) groups (the Fab' portion of F4/80 Ab) is not site-specific, these experiments were required to verify that the random binding of the Fab' fragment did not attenuate the specificity or efficiency of this interaction.

Our *in vitro* experiments also showed that the Fab'-bearing NPs had a significantly higher interaction with RAW 264.7 cells compared to Caco2 BBE cells. As the kinetic modeling software used in the present work assumed a basic 1:1 stoichiometric interaction between the ligand and receptor, we were not able to calculate the absolute K (kinetic of interaction) between the Fab'-bearing NPs and the F4/80 antigens on the RAW 264.7 cells. Altogether, the SPR experiments validated one of the main objectives of this study by demonstrating that Fab'-bearing NPs are suitable and efficient for MP targeting.

Fab'-bearing NPs show increased endocytosis into RAW 264.7 cells

Next, we studied the kinetics of NP phagocytosis by RAW 264.7 cells *in vitro*. As shown previously [18], the phagocytosis of NPs by MPs occurs quickly, and MPs may be saturated with FITC-tagged siRNA/PEI-loaded NPs within one hour [18].

Here, we examined the effect of the Fab' portion of the F4/80 Ab on the uptake of NPs by MPs, using a short exposure time (15 min) in a dynamic system wherein cells were placed in an incubator (5% CO₂ and 37°C) and subjected to mechanical agitation (200 Hz) to prevent the phagocytosis of NPs induced by sedimentation. Thus, our fluorescent microscopic studies focused on the NPs that were phagocytosed after interacting with the F4/80 antigens on the MPs. As shown in Figure 6A, NPs loaded with FITC-tagged siRNA and covered with the Fab' portion of F4/80 Ab (500 µg/mL) showed a higher uptake by MPs compared to uncoated NPs (500 µg/mL). To confirm these observations, we selected more than 50 regions of interest (ROIs) and quantified the fluorescent intensity. Figure 6C shows the results from three representative ROIs (ROI-001, -002 and -003 for covered and non-covered NPs). To enable comparison across different conditions, we report the average intensity by cell surface area. The calculations were first verified by comparing the values obtained for the cell surface and perimeter. The cell surface and perimeter were determined to be 319 µm² and 114 µm, respectively, for cells in contact with Fab'-bearing NPs, while those for cells in contact with uncoated NPs were 388 µm² and 142 µm, respectively. These values are within an acceptable size range for RAW 264.7 cells as eukariotic cells. Figure 6D shows the average fluorescent intensity per unit of surface area. The fluorescence intensity (arbitrary units, AU) per surface was significantly higher for RAW 264.7 cells

exposed to Fab'-bearing NPs (51325 AU/ μm^2) compared to those exposed to uncoated NPs (10279 AU/ μm^2). These results indicate that the Fab' fragments can boost the phagocytosis of NPs by MPs via a direct interaction between Fab'-bearing and the F4/80 antigens on RAW 264.7 cells.

Fab'-bearing TNF α siRNA-loaded NPs reduce TNF expression in inflamed macrophages

Having observed that Fab'-bearing siRNA-loaded NPs were more efficiently taken up by MPs, we next tested whether these siRNA-loaded NPs could downregulate TNF α expression. The MPs were pre-treated overnight with different NPs conditions (250 $\mu\text{g/mL}$) and then stimulated with LPS (10 $\mu\text{g/mL}$ for 1 h) to induce inflammation *in vitro*, and the secretion of TNF α to the medium was measured by ELISA.

As shown in Figure 7A, Lipofectamine-mediated transfection of TNF α siRNA (at the same concentration used in the NPs; Lipofectamine TNF α siRNA) did not have any anti-inflammatory effect in LPS-stimulated cells. This finding was consistent with our previous report [18] and emphasizes the potential value of TNF α siRNA-loaded NPs compared to the direct transfection of siRNA. The controls, which consisted of scrambled siRNA-loaded NPs and empty NPs, also failed to show any anti-inflammatory effect. In contrast, the Fab'-bearing TNF α siRNA-loaded NPs (denoted as NP-F4/80-TNF α siRNA in Figure 7A) significantly decreased the level of TNF α secreted from the MPs. Together, our *in vitro* results show that the coating of NPs with the Fab'-bearing enhanced the kinetics of uptake by MPs, and loading of the NPs with PEI-complexed TNF α siRNA decreased the amount siRNA required to obtain results similar to those obtained using the conventional method of oligonucleotide transfection.

Hydrogel encapsulation of Fab'-bearing TNF α siRNA-loaded NPs

To deliver the Fab'-bearing-coated TNF α siRNA-loaded NPs to the colonic lumen, we encapsulated them into a biomaterial comprised of alginate and chitosan at a ratio of 7:3 (wt/wt). We previously showed that this biomaterial collapses in intestinal solutions at pH 5 or 6, which reflect the colonic pH under inflamed and non-inflamed states, respectively [16, 17]. Thus, the release of NPs from the hydrogel will occur mostly in the colonic lumen rather than other parts of the gastrointestinal tract, such as the stomach or small intestine [16, 17]. First, we needed to confirm that our Fab'-bearing NPs could be evenly dispersed in the alginate/chitosan hydrogel, as this is essential for *in vivo* applications [18, 23]. We dispersed the NPs in the alginate/chitosan matrix and then chelated the COO⁻ of the alginate and the NH⁺₃ of the chitosan with a mixture of Ca²⁺ and SO²⁻₄, according to the protocol of NP gavage for colon delivery [38]. After encapsulating the NPs with the polysaccharides mix, we cut the material transversely and processed it for SEM observations. As shown in Figure 7B, the PLA-PEG NPs did not cluster or agglomerate; instead they were homogenously dispersed in the alginate/chitosan matrix. This finding validated the potential value of the PLA-PEG copolymer as a matrix for a new therapeutic delivery system.

Fab'-bearing NPs loaded with TNF α siRNA can attenuate DSS-induced colitis in mice

Next, we investigated the therapeutic potential used of Fab'-bearing TNF α siRNA-loaded PLAPEG NPs *in vivo*. C57BL/6 mice were treated with 3% DSS to induce colonic

inflammation. As shown in Figure 7C, daily gavages of hydrogel-encapsulated Fab'-bearing TNF α siRNA-loaded NPs (10 mg/mL) [16] attenuated the weight loss induced by 3% DSS treatment, whereas NPs loaded with scrambled siRNA (NPs-F4/80 scrambled siRNA or NPs naked-scrambled) did not. DSS-treated mice that received Fab'-bearing TNF α siRNA-loaded NPs showed an average weight loss of 6%, compared to the 15% and 25% observed in mice that received NPs-F4/80 scrambled siRNA or NPs naked-scrambled, respectively. Similar results were also seen for myeloperoxidase (MPO) activity. MPO, a hydrogen peroxide oxidoreductase, is specifically found in mammalia granulocytic leukocytes, including polymorphonuclear leukocytes (PMNs), monocytes, basophils, and eosinophils [39, 40]. MPO activity was 0.07 unit/ μ g of total colon protein for mice that received Fab'-bearing TNF α siRNA-loaded NPs, compared to 22 and 23 units/ μ g of total colon protein in mice that received NPs-F4/80 scrambled siRNA or NPs naked-scrambled, respectively. Furthermore, we observed that the Fab'-bearing TNF α siRNA-loaded more effectively attenuated DSS-induced colitis compared to the corresponding non-coated NPs, as DSS-treated mice that received these NPs showed 6% and 9% weight reductions, respectively, and MPO activities of 0.07 and 0.1 unit/ μ g of total colon protein, respectively. These measurements showed that TNF α siRNA-loaded NPs covered with the Fab' part of the F4/80 Ab efficiently attenuated DSS-induced colitis. Thus, the improvements made by the Fab'-bearing grafting were significant and meaningful.

As TNF α is a major upstream activator of the NF κ b pathway, we analyzed the accumulation of the IK β α protein (an inhibitor of NF κ b activity) in the colon of DSS-treated mice that received the various NPs. Figure 7D shows that IK β α accumulation was lower in DSS-treated mice that received Fab'-bearing scrambled siRNA-loaded NPs, whereas it was much higher in mice that received Fab'-bearing TNF α siRNA-loaded NPs. Thus, the Fab'-bearing TNF α siRNA-loaded NPs appear to protect IK β α from colonic degradation. The accumulation of IK β α leads to a significant inhibition of the NF κ b pathway.

Next, the beneficial effects of Fab'-bearing TNF α siRNA-loaded NPs on DSS-induced colitis was demonstrated by histological examinations of mouse colon tissues from these two groups. We observed that mice treated with Fab'-bearing TNF α siRNA-loaded NPs had less shortening of the colon compared to mice that received control NPs (data not shown). Hematoxylin counterstaining can reveal many of the hallmarks of DSS-induced colitis, such as crypt destruction, mucosal ulceration, erosion, and infiltration of lymphocytes into the mucosal tissue (Figure 7Eb). The colons of DSS-treated mice that received Fab'-bearing NPs loaded with scrambled siRNA demonstrated multifocal inflammatory cell infiltration into the submucosa, severe denudation of the surface epithelium (erosion), and mucodepletion of glands (Figure 7Eb). In contrast, DSS-treated mice that received Fab'-bearing TNF α siRNA-loaded NPs (Figure 7Ec) showed near-normal colonic histology with intact tridimensional organization of colonic epithelial cells and the mucosa, which resembled those seen in water-control mice (Figure 7Ea). Furthermore, Ly6g immunostaining (arrows in Figure 7E a, b and c) showed that Fab'-bearing TNF α siRNA-loaded NPs (Figure 7Ec) dramatically reduced the level of neutrophil infiltration compared to that seen in DSS-treated mice that received Fab'-bearing scrambled siRNA-loaded NPs (Figure 7Eb).

Fab'-bearing NPs loaded with TNF α siRNA target intestinal MPs

Finally, we examined whether the therapeutic effect of Fab'-bearing NPs was mediated via interactions with F4/80 antigens by flow cytometric analysis. We examined the pool of colonic MPs phagocytizing the FITC tagged siRNA when loaded in NPs with or without the Fab'-bearing. Uncoated (Figure 8A) or Fab'-bearing (Figure 8B) NPs were loaded with FITC-tagged siRNA (as described for TNF α siRNA loading), encapsulated in hydrogel, and used to gavage DSS-treated mice for one week. We defined several subsets of cells and analyzed colonic MPs, which were identified as MHCII+CD11b+F4/80+CD11c-. Interestingly, 28% of MPs showed positive FITC signals in cultures treated with Fab'-bearing NPs (Figure 8B), whereas only 19% of cells treated with uncoated NPs showed FITC signals (Figure 8A). These results confirmed that the overall attenuation of DSS-induced colitis occurred due to increased specific uptake by MPs allowing more siRNA release and subsequent attenuation of inflammation.

Collectively, our results show that TNF α siRNA/PEI-loaded NPs covered with the Fab' fragment of the F4/80 Ab may be a useful new tool for specifically delivering siRNA to MPs. As the Fab' linkage is the final step of our synthesis, our design strategy could also be used for any cell-type-specific ligand to coat NPs.

Discussion

TNF- α plays a crucial role as the central pro-inflammatory mediator in the pathogenesis of IBD, and patients with IBD have been successfully treated with anti-TNF- α antibodies in multiple clinical trials [41-43]. Such antibodies have been demonstrated to reduce intestinal inflammation in patients and in various animal models, including DSS-induced colitis in mice [41, 44, 45]. However, nearly 25% of patients taking the monoclonal antibody, infliximab, experienced at least one serious adverse effect, such as pneumonia, cancer, or acute inflammation [42, 46]. The observed adverse effects are mainly due to the lack of targeted treatments and the drug "over dosage" usually inherent to systemic drug administration. Furthermore, the action mechanism of antibodies against TNF- α remains poorly understood [47]. The efficacy of anti-TNF α antibodies in IBD has been attributed to multiple effects, such as regulating the expression of genes implicated in cell adhesion, triggering intestinal fibroblast motility, and/or inducing regulatory macrophages in an Fc region-dependent manner [48-50].

More recently, high-dose systemic administration of mice with TNF- α antisense oligonucleotides was found to ameliorate acute and chronic colitis in DSS-treated and IL-10-deficient mice [43]. The intravenous injection of these siRNA formulations required doses ranging from 50 to 125 mg kg⁻¹ in mice [44, 45, 51-54] and 1 mg kg⁻¹ in nonhuman primates [55]. Here, we demonstrated that the effective dose can be significantly decreased by NP-mediated targeting and increased cellular uptake. Our experiments revealed that 60 μ g kg⁻¹ TNF- α siRNA could silence TNF- α expression by 60% in intestinal MPs of NP-treated mice *in vivo*. We also demonstrated that ~ 30% of the intestinal MPs were able to take up the NP-delivered TNF- α siRNA.

The high potency of such low doses of TNF- α siRNA is likely to result from the PEI-mediated protection of siRNAs against nuclease and the proton sponge effect associated with such PEI complexes [18, 56, 57]. In addition, coating the TNF- α siRNA-loaded NPs with the Fab'-bearing increased the specificity of their binding to intestinal MPs. Colitis is characterized by stricture (a narrowing of the intestinal wall), perforation, hemorrhage, abscesses, fistula and diarrhea requiring drug uptake to be rapid in order for efficient and optimal dosing. This requirement influenced our strategy for NP design, and our results emphasize the beneficial effect of the Fab' fragments on the kinetics of uptake and the recognition of NPs by MPs. We demonstrate that oral administration of our MP-homing, siRNA-loaded vehicle can lead to the direct release of TNF α siRNA in intestinal MPs and reduce colitis.

Conclusions

Our delivery system has several advantages compared to the traditional method. First, our encapsulated NPs can overcome physiological barriers and target the siRNA to inflamed areas of the colon. Second, the TNF- α siRNAs may be loaded into the NPs at very low concentrations for administration to mice ($60 \mu\text{g kg}^{-1}$). Third, by designing the NPs according to the physiological consequences of colitis, and covering the NPs with an MP-specific ligand (the Fab'-bearing), we increased the kinetics of uptake. Finally, we demonstrated that the TNF- α siRNAs are preferentially targeted to colonic MPs (which are the major source of TNF- α) compared to other colonic cells (mainly epithelial cells). Together, our results show that we have developed a novel IBD treatment option by combining the positive aspects of siRNA with the safety of a biodegradable polymeric delivery system to facilitate specific targeting of colonic MPs via oral administration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of poly (lactic) acid – poly (ethylene glycol) (PLA-PEG) and PLA-PEG-Mal polymers

A. Semi-developed formula of poly (lactic) acid – poly (ethylene glycol) grafted with maleimide (Mal). The schematic representation shows the Fab' fragments covalently bound to the –SH functional groups of Mal. B. ¹H NMR spectrum of PLA-PEG at 298K in deuterated toluene. C. ¹H NMR spectrum of PLA-PEG-Mal at 298K (Mal peak at 6.7 ppm).

Figure 2. Characterization of nanoparticles (NPs) made of PLA-PEG and PLA-PEG-Mal

A. Atomic force microscopy (AFM) of NPs made of PLA-PEG-OMe (1 mg/mL, scale bar = 10 μ m). B. Scanning electron microscopy (SEM) of NPs made of PLA-PEG-OMe (1 mg/mL, scale bar = 1 μ m). C. Tridimensional schematic of a TNF- α siRNA/PEI-loaded NP made of PLA-PEG grafted with maleimide functional groups (Mal). The schematic representation shows the Fab' fragments covalently bound to the -SH functional groups of Mal on the NP surface.

Figure 3. Beneficial effect of coating PLA-PEG-Mal NPs with the Fab' portion of F4/80

A. Scanning electron microscopy (SEM) of NPs made of PLA-PEG-Mal (1 mg/mL, scale bar = 1 μ M). The free Mal functional groups on the surfaces of the NPs lead to their aggregation. B. Scanning electron microscopy (SEM) of PLA-PEG-Mal (1 mg/mL) Fab'-bearing NPs (scale bar = 1 μ M). Coating with the Fab' fragments avoids the aggregation of NPs. C. Cytotoxicity test (WST-1) were used to assess the viability of RAW 264.7 cells (MPs) after 24 h incubation with PLA-PEG-Mal NPs, Fab'-bearing TNF α siRNA-loaded PLA-PEG NPs (PLA-PEG-Ab with TNF α siRNA), and Fab'-bearing NPs without siRNA (PLA-PEG-Ab empty). D. Kinetics of the release of TNF α siRNA from PLA-PEI-siRNA NPs, PLA-PEG-Fab'-PEI-siRNA NPs, or PLA-siRNA.

Figure 4. Surface plasmon resonance (SPR)-based assessment of the binding between the NPs and the Fab' portion of F4/80

A. Schematic representation of the SPR experiments. Fab'-bearing (channel 1) or uncoated (channel 2) NPs were injected onto the activated-carboxydextran-bearing chip surface. B. Sensorgram of Fab'-bearing NPs (5 mg/mL) on the activated carboxydextran surface. The two successive injections of NPs deviated the resonance angle by 74 mDeg and 17 mDeg (flow = 35 μ L/min; ① and ② show the deviations of the resonance angle due to injections 1 and 2).

Figure 5. SPR-based assessment of binding between RAW 264.7 cells and Fab'-bearing NPs

A. Schematic representation of the experimental design. RAW 264.7 cells (MPs; which express F4/80) or CaCo2 BBE cells (which do not express F4/80) were injected onto an activated carboxydextran surface covered with Fab'-bearing NPs. Channel 1 contained MPs and channel 2 contained CaCo2 BBE cells. B. Sensorgram of the binding of MPs (2,500 and 7,500 cells/mL) to the Fab'-bearing NPs. The two successive injections of MPs deviated the resonance angle by 13 mDeg and 33 mDeg, respectively (flow = 35 μ L/min). All injections were followed by regeneration with NaOH (0.01 M) treatment to remove bound cells. Symbols: ③, deviation of the resonance angle due to adsorption of MPs (2,500 cells/mL) on the Fab'-bearing NPs; ①, regeneration step; and ②, deviation of the resonance angle due to adsorption of MPs (7,500 cells/mL) on the Fab'-bearing NPs. C. Amplitude of the resonance angle deviations (mDeg) observed after the passages of increasing concentration of MPs and CaCo2 BBE cells (2500, 5000, 7500 and 10,000 cells/mL).

Figure 6. The Fab'-bearing on NPs increases the endocytosis of FITC-tagged siRNAs

A. Fluorescent microscopy of MPs mixed with Fab'-bearing PLA-PEG NPs loaded with FITC-tagged siRNA (500 µg/mL) for 15 min with mechanical agitation at 200 Hz (red, cell cytosol; purple, cell nucleus; and green, NPs). B. Fluorescent microscopy of MPs mixed with FITC-tagged siRNA loaded into PLA-PEG NPs (500 µg/mL) for 15 min with mechanical agitation at 200 Hz (red, cell cytosol; purple, cell nucleus; and green, NPs). C. MPs were mixed with uncoated NPs (NPs-OH) or Fab'-bearing NPs (NPs-F4/80), and three regions of interest (ROI) were randomly selected and analyzed. D. Quantification of the average fluorescent intensity per cell surface unit (AU/µm²) in MPs mixed with coated (NP-F4/80) or uncoated (NP-OH) NPs for 15 min with mechanical agitation at 200 Hz.

Figure 7. Attenuation of LPS-mediated inflammation *in vitro* and DSS-induced colitis in mice using Fab'-bearing TNFα siRNA loaded PLA-PEG NPs

A. TNFα secretion was examined from RAW 264.7 cells (MPs) treated with 500 μg/mL of Fab'-bearing scrambled siRNA loaded NPs (NP-F4/80-scrambled siRNA), empty Fab'-

bearing NPs (NP-F4/80-empty), or Fab'-bearing TNF α siRNA loaded NPs (NP-F4/80-TNF α siRNA). B. Scanning electron microscopy (SEM) of the final hydrogel (alginate 7g/L and chitosan 3g/L) beads encapsulating PLA-PEG-OMe NPs (1 mg/mL). C. Weight loss and myeloperoxidase (MPO) activity in mice treated with 3% dextran sodium sulfate (DSS) for 8 days, with or without daily gavages of hydrogel-encapsulated NPs. The groups were as follows: water only with daily gavage of Fab'-bearing scrambled siRNA loaded NPs (control; NPs-scrambled); 3% DSS and Fab'-bearing TNF α siRNA loaded NPs (NPs-F4/80-TNF si); 3% DSS and Fab'-bearing TNF α siRNA loaded NPs (NPs-F4/80-scrambled); 3% DSS and non-coated NPs loaded with TNF α siRNA (NPs-naked-TNF si); or 3% DSS and non-coated NPs loaded with scrambled siRNA (NPs-naked-scrambled). D. Western blot analysis of IkB α and β actin in total mouse colonic cells. The groups received: water with daily gavage of Fab'-bearing scrambled siRNA loaded NPs (water control); 3% DSS with Fab'-bearing TNF α siRNA loaded NPs (NPs-F4/80-TNF si); and 3% DSS with Fab'-bearing scrambled siRNA loaded NPs (NPs-F4/80-scrambled). E. Ly6g immunostaining was used to visualize neutrophil infiltration in mice that received water only (a), 3% DSS with Fab'-bearing scrambled siRNA loaded NPs (NPs-F4/80-scrambled) (b), and 3% DSS with Fab'-bearing TNF α siRNA loaded NPs (NPs-F4/80-TNF si) (c). Scale bars: 500 μ m. Arrows indicate neutrophil infiltration (hematoxylin counterstaining was used to visualize histological damage in the mouse colon).

Figure 8. Increased attenuation of DSS-induced colitis in mice by Fab'-bearing NPs loaded with TNF α siRNA is mainly by targeting MPs

Flow cytometric analysis of MPs (MHCII+ F4/80+ CD11c- CD11b+ FITC+ cells) from mice treated with 3% DSS and daily gavages of uncoated PLA-PEG NPs loaded with FITC-tagged siRNA (A) or Fab'-bearing PLA-PEG NPs loaded with FITC-tagged siRNA (B). Endocytosis of uncoated NPs was observed in 19% of colonic MPs and in 28% of the colonic MPs with Fab'-bearing NPs.