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Targeting Suppressive Oligonucleotide to Lymph Nodes Inhibits Toll-like Receptor-9-Mediated Activation of Adaptive Immunity

Chunsong Yu¹, Myunggi An¹, Evan Jones², and Haipeng Liu^{1,3,4}

¹Department of Chemical Engineering and Materials Science, Wayne State University, Detroit, Michigan 48202, USA

²Department of Chemistry, Wayne State University, Detroit, Michigan 48202, USA

³Department of Oncology, Wayne State University, Detroit, Michigan 48201, USA

⁴Tumor Biology and Microenvironment Program, Barbara Ann Karmanos Cancer Institute, Detroit, Michigan 48201, USA

Abstract

Purpose—This paper aims to investigate the immunoinhibitory properties of a lymph nodes-targeting suppressive oligonucleotide (ODN) for the potential treatment of autoimmune diseases or chronic inflammation.

Methods—Synthetic suppressive ODN engineered with an albumin-binding diacyl lipid at the 5'-terminal (lipo-ODN) was synthesized. In *vitro* and *in vivo* experiments were designed to compare the immune suppressive properties of lipo-ODN and unmodified ODN. Cellular uptake and distribution, inhibition of Toll-like receptor (TLR) activation, lymph nodes (LN) draining, and the suppression of antigen-specific immune responses in an ovalbumin protein model was investigated.

Results—Compared to unmodified ODN, lipid functionalized suppressive ODN demonstrated enhanced cellular uptake and TLR-9 specific immune suppression in TLR reporter cells. Additionally, injection of a low dose of lipid-modified suppressive ODN, but not the unconjugated ODN, accumulated in the draining LNs and exhibited potent inhibition of antigen-specific CD8⁺ T cell and B cell responses *in vivo*.

Conclusions—Targeting suppressive ODN to antigen presenting cells (APCs) in the local LNs is an effective approach to amplify the immune modulation mediated by ODN containing repetitive TTAGGG motif. This approach might be broadly applicable to target molecular adjuvants to the key immune cells in the LNs draining from disease site, providing a simple strategy to improve the efficacy of many molecular immune modulators.

Keywords

albumin hitchhiking; CpG-induced activation; immune suppressive ODN; immunosuppression; lipid conjugation

INTRODUCTION

Toll-like receptors (TLRs) are highly conservative transmembrane receptors which play a key role in the early detection of pathogen and subsequent activation of the adaptive immune response (1,2). TLRs recognize specific pathogen-associated molecular patterns (PAMPs) associated with microbial pathogens, as well as a number of endogenous ligands such as selfprotein, lipids, and nucleic acids (1,2). In addition to pathogen sensing and clearance, accumulating evidence demonstrates that endogenous ligand-mediated signaling through TLR is involved in the pathogenesis of various autoimmune diseases (3–9), such as systemic lupus erythematosus (SLE) (6) and rheumatoid arthritis (RA) (8). In particular, intracellular nucleic acid sensing TLRs such as TLR-9 play an important role in the initiation of autoimmune pathology (7). For example, it was found that under certain conditions, TLR-9 recognized self-DNA, leading to the activation of B- and T-cells and production of proinflammatory cytokines (9). Such uncontrolled self or infectious pathogen DNA-driven immune activation can damage tissue, resulting in the development of autoimmune diseases such as RA (8,9). Targeting TLR receptors with antagonists which inhibit TLR-mediated immune responses may provide an effective approach for the prevention and therapeutic treatment of autoimmune diseases (3,5,10).

Synthetic oligonucleotides (ODNs) comprised of repetitive TTAGGG motif which mimics mammalian telomeric sequence can neutralize the bacterial DNA-induced immune activation (11,12). These suppressive ODNs (Sup-ODNs) act on dendritic cells and macrophages, inhibiting the activation and differentiation of B cells and multiple subsets of T cells (12). In several disease models, Sup-ODNs were shown to be able to attenuate the magnitude of autoimmune-mediated inflammation and alter the disease progression (12,13). These beneficial effects are associated with the inhibition of antigen-specific cellular and humoral immune responses, as well as downregulation of pro-inflammatory cytokines (3,12,14). However, the immune inhibition and therapeutic benefits were seen at relatively high ODN concentrations (14). Such high dose treatment can lead to serious side effects which have limited the suppressive ODN's clinic application (15,16). In chronic autoimmune inflammations such as RA, draining lymph nodes (LNs) are emerging as therapeutic targets for immune modulation (17). Housing a large portion of antigen presenting cells (APCs) and lymphocytes, LNs are the specialized organ for antigen presentation, defining the immunological fates of T- and B-cell responses (18). Since Sup-ODNs exert their activities through APCs, we hypothesize that approaches targeting Sup-ODNs to APCs in the draining LNs can amplify the Sup-ODN-mediated immune suppression, enabling possible dose sparing in the treatment of autoimmune diseases.

We have developed an 'albumin-hitchhiking' approach which uniquely targets synthetic ODNs to APCs in the LNs (19). In this approach, ODNs were conjugated to an albuminbinding diacyl lipid at the 5'-terminal (lipo-ODN) and following subcutaneous injection, accumulated in the draining LNs via binding and trafficking with endogenous albumin (19). The effectiveness of this approach relies on the molecular design that hijacks the traffic pathway of endogenous albumin in the interstitial fluid: following subcutaneous injection, ODNs bind avidly to albumin, transport to draining LNs via lymphatic capillary, and efficiently accumulate in LN-resident APCs. This approach simultaneously improves an

immunostimulatory ODN's adjuvant efficacy and safety by confining the ODN in the draining LNs, minimizing its systemic dissemination (19). We hypothesize that the efficacy of immune suppressive ODNs can be substantially improved by targeting them to the LNs, where a large portion of APCs is concentrated. In this paper, we evaluated the inhibitory properties of a lipid-modified suppressive ODN *in vitro* and *in vivo*. Our results revealed that *in vitro*, lipid modified Sup-ODN encoding repetitive TTAGGG motif enhanced cellular uptake and efficiently inhibited TLR-9 activation compared to unmodified ODN. *In vivo*, subcutaneous injection of a low dose of lipo-Sup-ODN led to enhanced accumulation in APCs in the draining LNs, and markedly suppressed the TLR-9 agonist-adjuvanted humoral and cellular immunity. Together, these findings suggested that LN-targeting of Sup-ODN via lipid modification is an effective approach to amplify the ODN's immunoinhibitory properties and thus might be applicable for the control of TLR-9-mediated immune activation.

MATERIALS AND METHODS

Materials

All reagents for DNA synthesis were purchased from Glenres (Sterling, VA) or Chemgenes (Wilmington, MA) and used following the manufacturer's instructions. 3'-Fluorescein amidite (FAM) labeled controlled pore glass was purchased from Allele Biotechnology (San Diego, CA). Fatty acid-free BSA was purchased from Sigma-Aldrich. Ovalbumin protein was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Murine MHC class I tetramer was obtained from MBL international Corporation (Woburn, MA). Antibodies were purchased from eBioscience (San Diego, CA) or BD Bioscience (San Jose, CA). All other reagents were from Sigma-Aldrich and used as received except where otherwise noted.

Animals and Cells

Animals were housed in the USDA-inspected WSU Animal Facility under federal, state, local and NIH guidelines for animal care. Female C57BL/6 mice (6–8 weeks) were obtained from the Jackson Laboratory. RAW-blue and HEK-Blue™-mTLR-9 reporter cell lines were purchased from invivogen (San Diego, California). Cells were cultured in complete medium (MEM, 10% fetal bovine serum (Greiner Bio-one), 100 U/mL penicillin G sodium and 100 µg/mL streptomycin (Pen/Strep), MEM sodium pyruvate (1 mM), NaH₂CO₃, MEM vitamins, MEM non-essential amino acids (all from Invitrogen), and 20 µM β-mercaptoethanol (β-ME)).

Synthesis of Diacyl Lipid Phosphoramidite

The diacyl lipid phosphoramidite was synthesized as previously described (19,20). A solution of stearoyl chloride (6.789 g, 22.41 mmol) in 1,2-dichloroethane (50 mL) was added dropwise to 1,3-diamino-2-hydroxypropane (1.0 g, 11.10 mmol) dissolved in 1,2-dichloroethane (100 mL) and triethylamine (2.896 g, 22.41 mmol). The reaction mixture was stirred for 2 h at 25°C and then heated at 70°C for 12 h. The reaction mixture was then cooled to 25°C, filtered, and the solid was sequentially washed with 100 mL CH₂Cl₂CH₃OH, 5% NaHCO₃ and diethyl ether. The product was dried under vacuum to

give the intermediate product as a white solid (yield: 90%). ^1H NMR (55°C, 300 MHz, CDCl_3 , ppm): δ 6.3 (m, 2H), 3.8 (m, 1H), 3.43.2 (m, 4H), 2.2 (t, 4H), 1.6 (m, 4H), 1.3–1.2 (m, 60H), 0.9 (t, 6H). The intermediate compound (5.8 g, 9.31 mmol) and N,N-Diisopropylethylamine (DIPEA, 4.2 mL, 18.62 mmol) were suspended in anhydrous CH_2Cl_2 (100 mL). The mixture was cooled on an ice bath and 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (8.6 mL, 0.47 mmol) was added dropwise under dry nitrogen. After stirring at 25°C for 1 h, the solution was heated to 60°C for 90 min. A clear solution was formed at the end of reaction. The solution was cooled to room temperature and washed with 5% NaHCO_3 and brine, dried over Na_2SO_4 and concentrated under vacuum. The final product was isolated by precipitating from cold acetone to afford 4 g (55% yield) lipid phosphoramidite as a white solid. ^1H NMR (300 MHz, CDCl_3): δ 6.4 (m, 2H), 3.9 (m, 2H), 3.8 (m, 2H), 3.6 (m, 2H), 3.0–2.9 (m, 2H), 2.6 (t, 2H), 2.2 (m, 4H), 1.6 (m, 6H), 1.3–1.2 (m, 72H), 0.9 (t, 6H). ^{31}P NMR (CDCl_3): 154 ppm.

Synthesis and Purification of Oligonucleotides

Both lipid-modified and free Sup-ODN were synthesized on a 1.0 micromole scale using an ABI 394 synthesizer. Diacyl lipid phosphoramidite was conjugated as a final ‘base’ on the 5’ end of oligos. Lipid phosphoramidite was coupled using the DNA synthesizer as previously described (20). After the synthesis, ODNs were cleaved from the solid support, deprotected, and purified by reverse phase HPLC using a C4 column (BioBasic-4, 200 mm x 4.6 mm, Thermo Scientific). A gradient of 20–60% (buffer B) in 10 min, was used for the unmodified ODN purification and for lipid-modified ODN, the gradient was set at 50–80% (Buffer B) for 10 mins and 80–100% for 5 mins. Buffer A: triethylammonium acetate (TEAA, 0.1 M, pH 7.0), buffer B: Methanol. Lipophilic ODNs typically eluted at 12 min while unconjugated oligos eluted at 5 min. Fluorescein label ODNs were synthesized using 3’-(6-Fluorescein) tagged controlled pore glass purchased from Chemgenes. Lipid-conjugated Sup-ODN (ODN A151: 5’- ttagggtagggtagggtaggg –3’) (11) and CpG ODN 1826 (CpG B ODN: 5’-tccatgacgttctctgacgtt-3’) (21) were synthesized by the above method and characterized by Mass Spec. All ODN sequences were modified by phosphothiolation to improve stability against nuclease degradation.

In Vitro DC Uptake and Confocal Imaging

DC 2.4 cells were cultured with RPMI-1640 supplemented with 10% FBS and 1% P/S; and were incubated with 1 μM FAM-labeled free or lipid-modified Sup-ODN at 37°C for 12 h. After incubation, cells were washed twice with 1 x PBS by centrifuge at 3000 rpm for 5 mins prior to flow cytometry quantification. To visualize *in vitro* cellular uptake, DC 2.4 cells were cultured and incubated again under the same experimental conditions and were subjected to confocal imaging by Zeiss LSM 510 microscope.

In Vitro TLR Reporter Cells Stimulation

HEK-Blue™-mTLR-9 and RAW-Blue™ cells were purchased from InvivoGen and were used to evaluate Sup- ODN’s inhibitory activities *in vitro*. In a typical procedure, 500 nM CpG 1826 ODN and 1.5 μM Sup-ODN were added to InvivoGen HEKBlue™ murine TLR-9 or RAW-Blue™ mouse macrophage reporter cells, both of which are engineered with secreted embryonic alkaline phosphatase (SEAP) reporter system. After incubating for 24 h,

SEAP levels were quantified by developing supernatants with QuantiBlue™ substrate for 1 h and reading absorption at 620 nm, following manufacturer's instructions.

LNs Imaging and Cellular Uptake

Groups of C57BL/6 mice ($n = 4$ LNs/group) were injected subcutaneously at the tail base with 3.3 nmol of FAM- labeled free or lipid-modified Sup-ODN. After 24 h, animals were sacrificed and inguinal and axillary LNs were excised and imaged using *In-Vivo Xtreme* (Bruker) imaging system. LNs were then digested with 1.5 mL freshly prepared enzyme mixture comprised of RPMI-1640, 0.8 mg/mL Collagenase/ Dispase (Roche Diagnostics) and 0.1 mg/mL DNase (Roche Diagnostics). LNs cells were stained with antibodies against F4/80 and CD11c *versus* ODN fluorescence in viable cells. Percentages of ODN positive cells in the LNs were determined by flow cytometry.

***In Vivo* Tolerization with Lipo-Sup-ODN**

C57BL/6 mice (6–8 weeks; $n = 3$ per group) were tolerized with PBS, 1.24 nmol of free or lipo-Sup ODN on day 0. On day 3, mice were immunized with 3.72 nmol CpG 1862 ODN plus 10 μ g OVA. On day 10, blood was collected from mice and OVA-specific immune responses were evaluated. The same mice were tolerized once again as previously on day 14 and three days later, a boost injection of 1.24 nmol lipo-G₂- CpG ODN 1826 (19) plus 10 μ g OVA was injected into each animal. Blood samples were collected on day 24 to analyze the cellular and humoral anti-OVA immune responses. The volume of all vaccine injections was 100 μ L/animal. All injections were performed s.c. (subcutaneously) at the base of the tail.

Statistical Analysis

All plots represent mean values and error bars represent the standard error of the mean (SEM). Comparisons of mean values of two groups were performed using unpaired Student's t-tests. One-way analysis of variance (ANOVA), followed by a Bonferroni post-test was used to compare >2 groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 6 software (San Diego, CA).

RESULTS

Lipid Functionalization of A151 Oligonucleotide Enhances the Cellular Uptake *in Vitro*

Synthetic ODN A151 which contains the immunosuppressive motif TTAGGG has been shown to block TLR-9 signaling and attenuate a variety of inflammatory responses (11). To target A151 to the APCs in the LNs through "albuminhitchhiking", we functionalized A151 at the 5'-terminal with a diacyl lipid (lipo-A151) as an albumin binding domain. Hydrophobic modification of ODNs such as antisense oligos and siRNA has been shown to be an effective approach to enhance their cellular uptake (22,23). To test whether lipid modification alters the uptake and intracellular distribution of A151, we incubated DC2.4 cells (a mouse dendritic cell line) with fluorescein-labeled lipo-A151, using unmodified A151 as a control. DC2.4 cells treated with lipo-A151 exhibited significantly enhanced cellular uptake when compared with unmodified A151 (Fig. 1), as demonstrated by flow cytometry and confocal microscopy analysis. Similar to our previous observations, lipid-

modified ODN preferentially accumulated in intracellular membrane structures (19,23). *In vitro*, the lipid-modification enables quick membrane association and subsequent cellular internalization by a mechanism similar to endocytic pathways. In contrast, without the lipid conjugation, A151 ODN alone showed less uptake in DC2.4 cells, suggesting the plasma membrane permeability of the unmodified A151 was limited by the negatively charged nature of oligonucleotide following exposure to dendritic cells.

Lipid-Modified Immune Suppressive ODN Inhibits CpG-Induced Immune Activation *in Vitro*

Chemical modification of immunostimulatory ODNs often affects their biological functions (19,24). To test whether lipid modification on A151 compromises its biological activity, the immune suppressive properties of both lipo-A151 and unmodified A151 were first evaluated in murine TLR-9 reporter

Tetramer Staining of Cytotoxic CD8⁺ T Cells

Seven days after challenge, blood samples were collected and red blood cells were lysed by ACK lysing buffer. Cells were then blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody) and stained with SIINFEKL loaded phycoerythrin-labeled tetramer (Beckman Coulter) and anti-CD8-APC (ebioscience) for 30 min at room temperature. Cells were washed twice, resuspended in FACS buffer, and analyzed on Attune Focus flow cytometer. Analysis typically gated on live, CD8⁺, Tetramer positive cells.

Intracellular Cytokine Staining

Cells were plated in 96-well round-bottomed plates and pulsed with OVA protein for 6 h at 37°C in T-cell media (RPMI 1640, 10%FBS, 50 µM β-mecaptoethanol, 100 U/ mL Penn/Strep, 1x Gibco® MEM NonEssential Amino Acids Solution (Life Technologies), 1 mM Sodium pyruvate, 1 mM HEPES), followed by the addition of brefeldin A for 4 h. Cells were stained with anti-CD4-APC and then fixed using Cytofix (BD biosciences) according to the manufacturer's instructions. Cells were then washed and permeabilized. Intracellular staining for anti-IFN-γ-PE was performed according to the manufacturer's instructions.

ELISA for OVA-Specific IgG

Mice were bled and blood samples were collected. Serum anti-OVA IgG levels were determined by ELISA: 96-well plates were coated overnight with 10 µg/ml OVA in PBS and blocked with 1% BSA in PBS. After incubation of serum samples for 1 h at a series of dilutions, plates were washed with PBS/1% Tween 20. Goat anti-mouse IgG conjugated to Horseradish peroxidase (HRP) was added at 1 µg/ml for 30 min. Plates were washed with PBS/1% Tween 20 and ELISA was developed by (3,3',5,5'-Tetramethylbenzidine) (TMB, ebioscience). The reaction was stopped by 1 M H₂SO₄ and the absorbance was read at 450 and 570 nm using a plate reader.

cells.—HEK-Blue cells transfected with murine TLR-9 and an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene were incubated with lipo-A151 or soluble A151 in the presence of CpG ODN (a single-stranded synthetic ODN with cytosine-phosphate-guanine (CpG) motifs that stimulate the TLR-9) and the activation of NF-κB was

quantified by measuring the SEAP concentrations in the supernatant. TLR-9 reporter cells were activated when stimulated with a B type of CpG-containing ODN (CpG-1826, 500 nM) which specifically binds to mouse TLR-9 (Fig. 2a). CpG-dependent TLR-9 stimulation was inhibited by up to 75% following incubation with A151 (1.5 μ M). Residual TLR-9 activation was retained in cells blocked with soluble A151, showing a small but statistically significant increase in SEAP production as compared with no treatment group (Fig. 2a). However, the addition of same concentration of lipo- A151 completely inhibited the CpG ODN induced TLR-9 activation in HEK cells (Fig. 2 a), lowering the SEAP production to the basal level that was statistically indistinguishable from control levels (Fig. 2a). The successful inhibition of CpG-induced HEK cells activation requires the treatment of TLR-9 cells with suppressive ODN before or during the CpG stimulation, as both lipo-A151 and soluble A151 were unable to block the SEAP production when HEK cells were stimulated with CpG prior to the addition of suppressive ODN (Fig. 2b). However, when HEK-TLR-9 cells were treated with suppressive ODN for 3 h, washed, and then stimulated with CpG ODN, both A151 and lipo-A151 were partially effective in the suppression of TLR-9 activation (Fig. 2c). In this case, statistically significant inhibition persisted for lipo-A151, showing a retention of 50% of its inhibitory capacity compared to unmodified A151 (Fig. 2c, d). These findings suggest that instead of compromising the suppressive properties, lipid-modification on A151 enhances the inhibitory efficacy of TLR-9 stimulation. To test whether lipo-A151 can act broadly to suppress the immune activation elicited by other TLR stimulants (25,26), Raw- Blue cells were used to replace HEK-TLR-9 cells as reporter cells in the above experiments. Raw-Blue cells are stably transfected with SEAP gene inducible by NF-KB expressing many pattern-recognition receptors, including Toll-like receptors (except TLR5) and NOD-like (nucleotide-binding oligomerization domain-like) receptors. In contrast to the inhibition of NF-KB activation by TLR-9 stimulation, both lipo-A151 and unmodified A151 showed little suppressive effect on Raw-Blue cells stimulated by imiquimod (TLR7 agonist) and LPS (TLR4 agonist) (Fig. 2e, f), respectively, suggesting in these reporter cells, ODN A151 appeared to be a TLR-9 specific inhibitor.

Lipid Modified Sup-ODN Accumulates in Draining LNs

in Vivo—Our previous study demonstrated the lipid-modified ODNs accumulated in the LNs following injection by binding and trafficking with endogenous albumin (19). To test whether lipid modification leads to efficient trafficking of A151 ODN to draining LNs, we assessed LN accumulation of A151 ODN following subcutaneous injection. C56BL/6 mice were injected subcutaneously with 3.3 nmol FAM-labeled free A151 or lipo-A151 at the tail base and 24 h later, inguinal and axillary LNs were isolated and imaged. Consistent with our previous findings, lipid-modified A151 exhibited 10- and 18 fold increases in inguinal and axillary nodes than unmodified A151, respectively (Fig. 3a, b). To identify the cells in the LNs that take up A151, the LNs were digested and stained with antibodies against F4/80 and CD11c. By flow cytometry, uptake of lipo-A151 in the draining LNs was highest in APCs, with the majority of lipo-A151 accumulated in CD11c+ DCs and F4/80+ macrophages (Fig. 3c, d). These cells are key APCs that specialized in antigen presentation and immune activation. Consistent with the imaging analysis, lipo-A151 exhibited significantly more uptake in both DCs and macrophages than soluble A151. Together, these data demonstrated

that *in vivo*, lipid functionalized A151 efficiently accumulated in the APCs in the draining LNs.

Lipid Modified Suppressive A151 ODN Inhibits CpG DNA Induced Antigen-Specific Cellular and Humoral Responses *in Vivo*

To determine whether LNs targeting suppressive A151 ODN can suppress the CpG-induced immune responses *in vivo*, the inhibitory effects of lipo-A151 v.s. soluble A151 on vaccine-elicited T- and B-cell responses were examined. Naïve C57BL/6 mice were tolerized with a low dose (1.24 nmol) of lipo-A 151, or the same dose of soluble A151, injected s.c. at the tail base. Three days later, all the animals were challenged with a large dose of CpG (3.72 nmol) plus 10 µg of ovalbumin (OVA). OVA-specific immune responses were determined in the blood on day 10 (Fig. 4a). A151 at this dosage had little impact on the OVA-specific CD8⁺ T cells, as comparable frequencies of OVA tetramer⁺ CD8⁺ T cells were observed in mice tolerized with A151, or PBS (Fig. 4b, c). In contrast, lipo-A151 treatment significantly lowered the frequency of antigen-specific CD8⁺ T cells in the blood (Fig. 4b, c). An identical tolerization injection was administered on day 14 and three days later, mice were challenged with 1.24 nmol LNs-targeting CpG (lipo-CpG) mixed with 10 µg OVA. As shown in Fig. 4b and d, the CD8 T cell responses after boost were high in mice tolerized with PBS or unmodified A151, reaching 17.5% and 14% tetramer positive CD8⁺ T cells in the blood, respectively. However, in mice tolerized with lipo- A151, no more than 5% of the CD8⁺ T cells in blood were OVA-specific. To our knowledge, this is the first example where the priming of CD8⁺ T cells is suppressed to such a low level without using a global immunosuppressant. Interestingly, at this low (1.24 nmol) dose used, both A151 and lipo-A151 significantly reduced Th1-leaning CD4⁺ T cells capable of secreting IFN-γ after antigen restimulation (Fig. 4e). Additionally, we measured the levels of OVA-specific IgG in the sera of immunized mice to access the lipo-A151's ability to impact the humoral immunity. Sera from immunized mice were collected on day 27 following the initial treatment. ELISA measurements of serum titers of OVA-specific IgG showed a significantly reduced level of antiOVA IgG in mice tolerized with lipo-A151 but not soluble A151 (Fig. 4f). Together, these data demonstrated LNs targeting immune suppressive ODN greatly improved the immune suppressive activities that controlled both the CD8⁺ T cell- and B-cell-mediated immunity.

DISCUSSION

Strengthening activation of innate immune system and thereby modifying the adaptive immune responses has long been considered in clinic in the treatment of cancer, allergy and infections (1,2). In parallel with the discovery of CpG ODN in stimulating TLR-9 and eliciting potent antigen-specific immunity, it was found activation of the innate immunity through TLR such as TLR-9 could lead to deleterious immune responses that resulted in destruction of self-tissues. (3—9) This has sparked the search for specific inhibitors which can inhibit CpG-mediated activation (3—9). Studies have revealed several structurally distinct ODNs can differently modulate TLR-9-mediated immune stimulation, and suppress the functional outcomes of TLR-9 ligation (11,12,14,25). In particular, ODNs containing the immunosuppressive motif TTAGGG were recognized for their ability to attenuate

inflammatory responses *in vivo* by blocking TLR-9 signal pathways (12,25). Administration of A151 (a representative suppressive ODN with TTAGGG motif) has been shown to inhibit dendritic cell activation, suppress Th1 differentiation and support the regulatory T cell induction, prevent STAT1 and STAT4 phosphorylation, block cytosolic nucleic acid sensing pathways (12). However, systemic administration of phosphorothioate ODN is known to induce side effects including platelet activation, which has limited the clinic translation of suppressive ODN in humans (15,16).

Emerging evidence supports the contribution of LNs in the generation and perpetuation of autoimmunity resulted from chronic inflammation (17,18). During the progression of autoimmune diseases such as RA, antigens and inflammatory signals (e.g., DNA) resulted from the immune destruction drain into the LNs and aggravate the pathogenic inflammation (17). Thus, approach that targets and modulates the immune cell cross-talk and reciprocal interactions within the LNs microenvironment represents a viable strategy for the treatment of autoimmune inflammation.

We present here the results of our attempt to design and implement an ‘albumin-hitchhiking’ approach to locally deliver an immune suppressive ODN to the draining LNs to suppress the TLR-9-activation-induced adaptive immune responses. We previously developed a diacyl lipid-ODN conjugation which uniquely targets the ODN to APCs in the LNs via an albumin-mediated transportation (19). Albuminbinding increases the hydrodynamic size of ODN and prevents it from diffusing into blood circulation, re-targeting it to the lymphatics where a large portion of APCs reside. In addition to LNs targeting, lipo-ODN exhibited high affinity toward membrane and accumulated in the endosome (19,23). We assessed the *in vitro* and *in vivo* properties of diacyl lipid- modified suppressive ODN A151. *In vitro*, lipo-A151 significantly enhanced the cellular uptake compared with unmodified A151. Our data are consistent with previous observation where hydrophobic conjugation on siRNA resulted in the enhancement of cellular uptake (22). Lipo-A151 exhibited potent suppressive capability in TLR-9 reporter cells. We observed although the co-presence of lipo-A151 and CpG was most effective at blocking TLR-9 activation in HEK-TLR-9 cells, lipo-A151 still resulted in partial inhibition when it was incubated with cells for 3 h and subjected to CpG stimulation, showing a significantly better suppression than unmodified A151. Our results suggested that *in vitro*, lipid modification of A151 not only retained its suppressive ability toward TLR-9 stimulation but also resulted in prolonged suppression when CpG ODN was added at a late time point.

In vivo, diacyl lipid-modified A151 accumulated in the draining LNs following subcutaneous injection. The LNs enrichment was consistent with our previous observations, where lipid-modified ODNs bound endogenous albumin and transported to the LNs (19). In contrast, unmodified ODN quickly diffused into blood circulation due to its small molecular size and exhibited minimal LNs accumulation (19,27). Flow cytometry analysis of the cells in the LNs revealed that CD11c⁺ dendritic cells and F4/80⁺ macrophages were the major cells responsible for the uptake of lipo-A151. These cells are key APCs in the LNs that actively bind and internalize macromolecules such as albumin (28).

Using the well-studied ovalbumin as a model antigen, we also assessed the ability of LNs targeting lipo-A151 to suppress the OVA-specific immunity *in vivo*. Subcutaneously injected OVA combined with TLR-9 ligand CpG ODN elicited a strong CD8+ T cell and humoral responses, mimicking the inflammatory responses of autoimmune diseases. Coinjection of LNs targeting lipo-A151, but not the soluble A151 effectively suppressed the OVA elicited CD8+ T cell and B cell immune responses, demonstrating our LNs targeting strategy was superior in the immune inhibition. In a real situation, autoimmune diseases can be triggered by multiple TLR stimulations (3). In our experiments, the immune suppression by lipo-A151 appeared to be TLR-9 specific, as no suppressive effect was observed for lipo-A151 when Raw-Blue cells were stimulated with TLR-7 (imiquimod) or TLR-4 (Lipopolysaccharide, LPS) agonists. However, broad spectrum ODN-based antagonists have been recently demonstrated. For example, IMO-3100 (29) and IMO-8400 (30) were reported to act as antagonists against multiple TLRs, including TLR-7, TLR-8, and TLR-9. Thus, it is possible to extend our approach to other endosomal TLR antagonists with broad suppressive capacity. Although clinical safety data of phosphorothioate ODN were well documented, the longterm toxicity effects are lacking. For example, recent study has shown the development of antibodies against phosphorothioate ODN in patients (31). As our method targets lipo-A151 in the LNs, it is possible that there may exist a potent anti-lipo-A151 antibody response.

Our approach takes advantage of albumin to target LNs, which accommodates a wide variety of immune cells including APCs, T cells and B cells. Unlike the nanoparticles which require sophisticated encapsulation design or modification, this delivery system exhibits a natural, simple and efficient way to target LNs and modulate the immune response. Our results may pave the way to amplify the induction of immunosuppression in the immunomodulation that would target autoimmune conditions.

CONCLUSION

In summary, the data presented in this study suggest that targeted delivery of immunosuppressive ODN to the LNs by “albumin-hitchhiking” improves its inhibitory activities *in vitro* and *in vivo*. This finding underlines the potential of targeting immunomodulators to the LNs in the treatment of autoimmune diseases. Further work is required to assess the effectiveness in the suppression of human TLR-driven inflammation by lipid-modified ODN.

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ABBREVIATIONS

BSA	Bovine serum albumin
CpG	ODN CpG oligodeoxynucleotides
DAPI	4',6-diamidino-2-phenylindole

DC 2.4	Dendritic cell 2.4
ELISA	Enzyme-linked immunosorbent assay
FAM	Fluorescein amidite
IgG	Immunoglobulin G
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
OVA	Ovalbumin
PBS	Phosphate buffered saline
SEAP	Secreted embryonic alkaline phosphatase
Sup-ODN	Suppressive oligodeoxynucleotides
TLR-9	Toll-like receptor 9

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Fig. 1.

Lipid modification promotes the uptake of suppressive ODN *in vitro*. (a) DC 2.4 cells were incubated with 1 μM free A151 or lipo-A 151 for 12 h at 37°C. Cells were washed and then subjected to laser scanning confocal microscopy imaging for intracellular distribution. Representative confocal images of cells treated with A151 (top panels) and lipo-A 151 (bottom panels). Scale bar: 20 μm . (b) Flow cytometry analysis for quantification of cellular uptake. Data show the mean values $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by unpaired two-tailed t-test.

Fig. 2.

Lipid-modified AI5I blocks TLR-9 activation *in vitro*. (a) Free AI5I or lipo-AI5I (1.5 μ M) with or without CpG B ODN (500 nM) were co-incubated for 24 h in HEK-TLR-9 reporter cells. Cell activation was quantified by measuring the SEAP levels in the supernatant. (b) HEK-TLR-9 cells were stimulated with 500 nM CpG for 3 h first, after which 1.5 μ M of free AI5I or lipo-AI5I were added to the cells. Cell activation was determined. (c and d) HEK-TLR-9 cells were incubated with AI5I, or lipo-AI5I for 12 h, after which cells were washed (c), or without wash (d) before CpG ODN (500 nM) was added for the TLR-9 stimulation. (e and f) AI5I and lipo-AI5I were co-incubated with imiquimod (TLR-7 agonist) (e) or lipopolysaccharide (LPS) (TLR-4 agonist) (f) for 24 h in RawBlue cells. SEAP levels were quantified by incubating supernatant with QuantiBlue substrate for 1 h and reading absorption at 620 nm. Data show the mean values $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant by one-way ANOVA with Bonferroni post-test.

Fig. 3.

Lipid-modified A15I accumulates in draining LNs following s.c. injection *in vivo*. Mice (n = 4 LNs/group) were injected s.c. at the tail base with 3.3 nmol FAM-labeled A151 or lipo-A151. Inguinal LNs (proximal nodes) and axillary LNs (distal nodes) were isolated 24 h post injection and were imaged (**a**) and quantified (**b**) by using *In-Vivo Xtreme* (Bruker) imaging system. (**c** and **d**) LNs were digested and LNs cells were stained with antibodies against F4/80, CD11c. Percentages of A15I ODN positive cells in inguinal nodes (**c**) and axillary nodes (**d**) were determined by flow cytometry at 24 h. Data show the mean values $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by unpaired two-tailed t-test

Fig. 4.

Lipid-modified suppressive A151 ODN inhibits CpG induced antigen-specific cellular and humoral responses *in vivo*. **(a)** Tolerizing model displays experimental protocol. C57Bl/6 mice (n = 3/group) were tolerized with PBS, 1.24 nmol of A151, or lipo-A 15 1 on day 0 and day 14. Mice were challenged with 3.72 nmol CpG ODN plus 10 Ug OVA on day 3 and 1.24 nmol lipo-CpG ODN plus 10 UgOVA on day 17. One week after each challenge (day 10 and day 24), blood samples were collected and antigen-specific CD8⁺ T-cell responses in peripheral blood were evaluated by SIINFEKL tetramer assay. **(b)** Representative flow

cytometric plots of H-2K^b/SIINFEKL tetramer staining of CD8⁺ cells post-prime (upper panels) and post-boost (lower panels). (c and d) Quantification of SIINFEKL tetramer-positive CD8⁺ cells in the blood after CpG and OVA antigen prime (c) and boost (d). (e) Stimulation of white blood cells ex vivo for 6 h in the presence of OVA showed reduced response in CD4⁺ T cells after tolerization using A15.1 or lipo-A 15.1 compared with PBS. IFN- γ -secreting CD4⁺ T cells frequencies were determined by intracellular cytokine staining. (f) On day 24, serum samples were collected and assayed by ELISA for anti-OVA IgG production. Data show the mean values $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant by one-way ANOVA with Bonferroni post-test.