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Phosphorothioated antisense oligodeoxynucleotide suppressing interleukin-10 is a safe and potent vaccine adjuvant

Jin Zhang [a], Ninghua Liu [a], Yang Lu [a], Zhen Huang [a], Yuhui Zang [a], Jiangning Chen [a], Junfeng Zhang [a,b,*], Zhi Ding [a,c,*]

[a] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, China
[b] Collaborative Innovation Center of Chemistry for Life Sciences, Nanjing University, Nanjing 210023, China
[c] Changzhou High-Tech Research Institute of Nanjing University, Changzhou 213164, China

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ABSTRACT

While vaccination is highly effective for the prevention of many infectious diseases, the number of adjuvants licensed for human use is currently very limited. The aim of this study was to evaluate the safety, efficacy, and to clarify the mechanism of a phosphorothioated interleukin (IL)-10-targeted antisense oligonucleotide (ASO) as an immune adjuvant in intradermal vaccination. The cytotoxicity of IL-10 ASO and its ability to promote T cell proliferation were assessed by Cell Counting Kit-8 (CCK-8) assay. The contents of IL-6, IL-8, TNF-α, IL-1β, and IL-10 in inoculated local tissue and the antigen-specific antibody titers in mouse serum samples were determined by ELISA. The target cells of IL-10 ASO were observed using immunofluorescent staining. The results showed that the specific antibody titer of ovalbumin (OVA), a model antigen, was increased 100-fold upon addition of IL-10 ASO as an adjuvant compared to that of OVA alone. IL-10 ASO showed an immunopotentiation efficacy similar to that of Freund's incomplete adjuvant, with no detectable cell or tissue toxicity. *In vitro* and *in vivo* experiments confirmed that IL-10 ASO enhances immune responses by temporarily suppressing IL-10 expression from local dendritic cells and consequently promoting T cell proliferation. In conclusion, IL-10 ASO significantly enhances immune responses against co-delivered vaccine antigens with high efficacy and low toxicity. It has the potential to be developed into a safe and efficient immune adjuvant.

1. Introduction

Vaccination is the most effective invention in human medical practice. It has eliminated smallpox globally, saved numerous lives, and reduced the threat of many infectious diseases over the past two centuries. However, despite this brilliant achievement, more than 3 million people die from vaccine-preventable diseases each year. Nearly half of these deaths are in children less than 5 years old [1]. One of the reasons for incomplete vaccination coverage is the lack of effective vaccine adjuvant. Administered prior to or together with a vaccine antigen, an ideal adjuvant can boost a vaccine's immunogenicity, prolong its protection duration, and modulate the type of immune response induced [2]. It can also decrease the minimum antigen dose required for sufficient protection to reduce costs, provide protection to a broader population, and induce a stronger and more enduring response, especially in children and the elderly [3,4]. For their significant medical and social benefits, novel adjuvants are a research focus worldwide, in both academia and industry.

Currently the number of adjuvants licensed for human use is very limited [5]. Among them, aluminum salts are the most widely used. Following the application of this adjuvant for over 80 years, its efficacy and safety have been confirmed, while its limitations

Abbreviations: IL-10, interleukin 10; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; ASO, antisense oligonucleotide; OVA, ovalbumin; CpG-ODN, short synthetic single-stranded DNA molecules containing unmethylated CpG motifs; TLR, Toll-like receptor; LPS, lipopolysaccharide; PEI, polyethyleneimine; FIA, Freund's incomplete adjuvant; GM-CSF, granulocyte-macrophage colony stimulating factor; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylbenzidine; IL-10 KO, *Il10* gene knockout; OCT, optimal cutting temperature compound; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; FBS, fetal bovine serum; APCs, antigen-presenting cells; DCs, dendritic cells; BMDCs, bone marrow-derived dendritic cells; Lipo, Lipofectamine™ 2000; PLGA, poly(lactic-co-glycolic) acid; hATTR, hereditary transthyretin-mediated amyloidosis; STAT3, signal transducer and activator of transcription 3; MFI, median fluorescent intensity; *i.d.*, intradermally.

* Corresponding authors at: State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Xianlin Avenue 163, Nanjing 210023, China.

E-mail addresses: jfzhang@nju.edu.cn (J. Zhang), dingzhi@nju.edu.cn (Z. Ding).

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are also well known. This adjuvant can only provoke a humoral immune response, leading to a Th2-type response with antibody production to eliminate extracellular pathogens. It cannot effectively induce a Th1-type response, involving the activation of cytotoxic T cells, to cope with intracellular viruses or parasites. Its efficacy is influenced by the administration route, and it also induces side effects at the injection site [6,7]. Cytokines are a family of signaling molecules secreted by immune-active cells to regulate the immune response. Interleukin (IL)-1, -2,- 6, and -12 and their downstream signaling molecules can all enhance the immune reaction and thus are being studied as adjuvants in preclinical studies with remarkable effects [8–10]. However, the quality, cost, and logistical chain required for proteins to be used as adjuvants are very demanding, limiting their application in vaccine formulations. Safe, stable, highly efficacious, water-soluble, and cheap adjuvants are still being pursued.

Oligodeoxynucleic acid with demethylated CpG motifs (CpG-ODN) is a ligand and activator of Toll-like receptor (TLR) 9. It interacts directly with antigen-presenting cells (APCs) and upregulates the co-stimulatory factors CD80 and CD86, thereby enhancing the maturation and differentiation of APCs and leading to the activation and proliferation of T cells. Although many of the reported immunostimulatory responses such as NF-κB stimulation to non-CpG ODNs 2137, 1982, and 2138 are mediated through TLR9, the magnitudes of TLR9-meidated stimulation were always inferior to those of CpG-ODNs [11–14]. Thus, some subtypes of CpG-ODN are currently being tested in clinic trials. However, CpG-ODN may induce high levels of pro-inflammatory cytokines, carrying the risk of impairing immune homeostasis and causing autoimmune diseases [15].

Logically, both positive and negative factors are involved in immune-regulation. In 1989, Mosmann and colleagues reported the discovery of IL-10, a typical immunosuppressive and anti-inflammatory cytokine [16]. It is secreted by APCs, regulatory T cells, and monocytes to counteract the effects of the abovementioned interleukins so that unnecessary immune responses and over-activation can be avoided. It is of great importance in maintaining immune homeostasis and modulating the type of immune response. Based on this, we propose to utilize an antisense oligonucleotide suppressing IL-10 (IL-10 ASO) as an immune potentiator. Reducing the level of IL-10 at the site of vaccination temporarily may increase the sensitivity of immune surveillance and boost the response against vaccines. In the current study, phosphorothioation was employed to increase the stability of the ASO. CpG motifs were avoided in the ASO and its control ODN to clarify the mechanism of function. We evaluated the IL-10-suppressing effects at the cellular and tissue levels, as well as its cellular and tissue toxicity. Its ability to stimulate the maturation of dendritic cells (DCs) and proliferation of T cells was determined, and its immunopotentiation efficacy was assessed *in vivo*.

2. Materials and Methods:

2.1. Materials

Ovalbumin (OVA), lipopolysaccharide (LPS), polyethyleneimine (PEI, branched, MW = 25,000 Da), and Freund's incomplete adjuvant (FIA) were acquired from Sigma-Aldrich (Shanghai, China). All enzyme-linked immunosorbent assay (ELISA) kits were purchased from 4A Biotech Co. Ltd. (Beijing, China), and the Cell Counting Kit-8 (CCK-8) was from Dojindo Co. Ltd (Shanghai, China). Recombinant mouse GM-CSF was purchased from Peprotech (Beijing, China). Pentobarbital sodium was obtained from Merck (Shanghai, China), and Tween 20 was from Sangon Biotech Co. Ltd (Shanghai, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ-chain specific) was purchased from Southern Biotech (Birmingham, AL, USA). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), 3,3′,5,5′-tetramethylbenzidine (TMB), antifade mounting medium, substrate buffer, and red blood cell lysis buffer were purchased from Beyotime B.V. (Shanghai, China). Skim milk powder (Yili, Hohhot, China) was obtained from a local supermarket. Purified rabbit anti-mouse CD11c mAb was purchased from Cell Signaling Technology (Shanghai, China). Alexa 488-conjugated donkey anti-rabbit IgG (H + L) antibodies was obtained from Invitrogen (Shanghai, China). All other chemicals used were of analytical grade, and all solutions were prepared with ddH_2O.

2.2. Animals

Female BALB/c mice (H-2^d; 6–8 weeks old at the start of the experiments) were purchased from the Experimental Animal Center of Nanjing Medical School (Nanjing, China). Female B6.129P2-Il10tm1Cgn/J [IL-10 knockout (KO)] mice and C57BL/6J mice were obtained from the Laboratory Animal Center of Nanjing University (Nanjing, China). All animals were maintained under standardized pathogen-free conditions in the animal facility of the State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University and housed in standard cages with free access to food and water. The animals were maintained at a constant temperature (20–21 °C) and humidity (55 ± 5%), under a 12-h light/dark cycle. All animals were handled in accordance with the Helsinki Declaration of 1975 (as revised in 2008) concerning animal rights, and the protocols were approved by the Institutional Animal Care and Use Committee of the Model Animal Research Center, Nanjing University.

2.3. Preparation of ASO and control ODN

The sequence of the ASO directed against the 3′-untranslated region (positions 637–654) of *Il10* mRNA was 5′-AGG TCC TGG AGT CCA GCA-3′. The control ODN, 5′-ACA CCC TTG GGA GTC AGG -3′, was designed to have the same GC content as the ASO without CpG motifs. The ASO and the control ODN were phosphorothioate-modified, purified by high-performance liquid chromatography, and synthesized by Invitrogen (Shanghai, China). All ODNs were dissolved in ddH_2O. Tamra-conjugated ASO and control ODN were purchased from SBS Genetech Co. Ltd. (Beijing, China). Vaccine formulations were prepared in an aseptic laminar hood, and their contents of endotoxins were monitored to be less than 0.57 EU/mL.

2.4. Cytotoxicity and tissue toxicity of ASO

Various concentrations of ASO, control ODN, and PEI were added to mouse bone marrow-derived dendritic cells (BMDCs), extracted as described previously [17] and cultured for 24, 48, and 72 h. Then, cell viability was measured with the CCK-8 assay. One hour after intradermal (*i.d.*) injection of 0.1 mg/mL, 1 mg/mL, or 10 mg/mL ASO, ASO control, or PEI, the mice were euthanized by cervical dislocation, and the local skin tissue was collected. Approximately 100 mg of skin sample from each mouse was excised and rinsed in cold PBS (at 100 mg/mL) and then homogenized on ice using Lysing Matrix D tubes and a Fast Prep-24 homogenizer (MP Biomedicals, Santa Ana, CA, USA). Levels of cytokines, including tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8, were quantified in the lysates using ELISA kits (4A Biotech Co. Ltd.), following the manufacturer's instructions.

2.5. Confocal observation of tissue distribution and cellular uptake of ASO after i.d. injection

5 μg Tamra-conjugated ASO was injected into the skin tissues of mice. One hour later, the mice were euthanized, and the skin samples at the injection site were extracted and embedded in optimal cutting temperature compound (OCT). Samples were stained with rabbit anti-mouse CD11c mAb, Alexa 488-conjugated donkey anti-rabbit IgG (H + L), and DAPI after frozen sectioning. Samples were then imaged and analyzed by confocal microscopy (Ti-C2, Nikon, Tokyo, Japan).

2.6. Cellular ASO uptake assessment by confocal microscopy and flow cytometry

RAW264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) at 37 °C and 5% CO_2. Before the addition of Tarma-ASO, the cells were incubated at 4 °C or 37 °C for 15 min to achieve temperature balance. Then, the cells were seeded at 10^6 cells/mL into each well of a 24-well plate containing pre-placed coverslips and were treated with Tamra-ASO (5 μg/mL). After co-culture for 2 h, the coverslips were incubated with DAPI for 5 min and washed with PBS. Then, antifade mounting medium was added, and the coverslips were sealed with nail polish. Images were taken and processed by confocal microscopy (Ti-C2, Nikon, Tokyo, Japan).

RAW264.7 cells were incubated with Tamra-ASO (5 μg/mL) at 37 °C and 5% CO_2 and collected at different time points for fluorescence-activated cell sorting (FACS) analysis. The median fluorescent intensities (MFI) of RAW264.7 cells upon uptake of Tamra-ASO were recorded by a BD FACSCalibur™ (Franklin Lakes, NJ, USA) and compared among different groups.

2.7. Determination of the efficacy of ASO in vitro and in vivo

For *in vitro* assessment, BMDCs were extracted as described previously and seeded in 6-well plates. Six days later, BMDCs were transfected with 1.2 μg/mL IL-10 ASO or its control ODN with or without Lipofectamine™ 2000 (Lipo) as an agent for 6 h. Then, 1 μg/mL LPS was added to the renewed cell culture medium and incubated overnight. The content of IL-10 in the supernatant was determined by ELISA. For *in vivo* assessment, PBS, OVA, OVA + ASO, and OVA + ASO control were administered to mice *via i.d.* injection. Six hours later, the mice were euthanized by cervical dislocation, and the levels of IL-10 in the treated skin tissues were measured by ELISA.

2.8. Co-culture of DCs and T cells

BMDCs and spleen T cells from BALB/c, C57BL/6J or IL-10 KO mice were obtained as described previously. 1.2 μg/mL ASO or ASO control were added to immature BMDCs with Lipo on day 6 for transfection. After transfection for 6 h, 1 μg/mL LPS was added as a stimulus for 24 h. Then, the supernatants were collected, and the IL-12p70 contents were determined by ELISA. BMDCs were collected and seeded in 48-well plates at densities of 2×10^2, 2×10^3, 2×10^4, and 2×10^5 cells/mL and co-cultured with 2×10^5 T cells for 96 h. BMDCs from wild-type or IL-10 KO mice were seeded at the density of 2×10^5 and co-cultured with 2×10^5 T cells from C57BL/6J mice for 96 h. T cell proliferation rates were determined by CCK-8 assay, and interferon (IFN)-γ contents in the supernatant of the co-cultures were measured by ELISA.

2.9. Immunization and serum antibody assays

Three immunization studies were carried out. In the first study, mice were immunized *i.d.* using hypodermal 31G needles with 50 μL PBS containing 5 μg of OVA and 5 μg ASO or control ODN under anesthesia (0.1 mg/g pentobarbital sodium) on day 1, 21, and 42 at a site between the back and thigh and were euthanized on day 56. 5 μg OVA alone, 5 μg OVA + 50 μL FIA and 5 μg OVA + 5 μg PEI delivered *i.d.* were included as negative and positive controls, respectively. Blood samples were drawn from the tail vein 1 day before each immunization or from the retro-orbital venous sinus during euthanasia under systemic anesthesia. Cell-free serum was obtained in collecting tubes with coagulation agent and separation gel (Gongdong, Taizhou, China) after centrifugation (4 °C, 3000 rpm, 10 min) and clot formation and was stored at −80 °C.

In the second study, IL-10 KO C57BL/6 mice were immunized *i.d.* with 5 μg of OVA and compared with C57BL/6 mice immunized with 5 μg of OVA with or without IL-10 ASO to confirm the mechanism of action of ASO. The dose of ASO as an adjuvant was optimized in the third study. OVA-specific IgG titers in the sera were determined as previously described [18]. Briefly, ELISA plates (Greiner Bio-one high binding, Tianjin, China) were coated with OVA at 4 °C overnight. Twofold serial sample dilution was applied. Antibodies were detected with HRP-conjugated goat anti-mouse IgG using TMB as a substrate. Titers are expressed as the calculated dilution times corresponding to half of the maximum absorbance at 450 nm of a complete sigmoid absorbance-log dilution curve. If the samples were not diluted in the optimal range, additional measurements with more diluted or concentrated samples were carried out to complete the S-shaped curve. Mice whose serum samples did not reach the half-saturated absorbance value at the lowest (tenfold) dilution were considered non-responders, and their titers were arbitrarily considered to be 10.

2.10. Statistical analysis

Data were analyzed using Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Comparisons were performed as indicated using the Student's *t*-test or one-way ANOVA where suitable to assess differences between experimental groups and control groups. Antibody titers were logarithmically transformed to improve normality. Differences with a p value of <0.05 were considered statistically significant.

3. Results

3.1. Cytotoxicity and tissue toxicity of ASO

The cytotoxicity of ASO was assessed in primary mouse BMDCs after co-culture for 24, 48, and 72 h. PEI, with known cytotoxicity, served as a positive control, and the data are shown in Fig. S1. With 0.6 μg/mL of ASO, control ODN, or PEI, no significant cytotoxicity was observed, while at a concentration of 4.8 μg/mL, cell survival rates started to decrease in the PEI group. The cytotoxicity increased with higher PEI concentrations. In contrast, IL-10 ASO and its control ODN were not cytotoxic for up to 72 h to BMDCs at up to 153.6 μg/mL.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.05.076.

The tissue toxicity of ASO was evaluated by measuring the concentrations of related cytokines in the treated skin area after tissue homogenization (Fig. 1). The concentrations of TNF-α, IL-1β, IL-6, and IL-8 increased with higher amounts of PEI injected, started

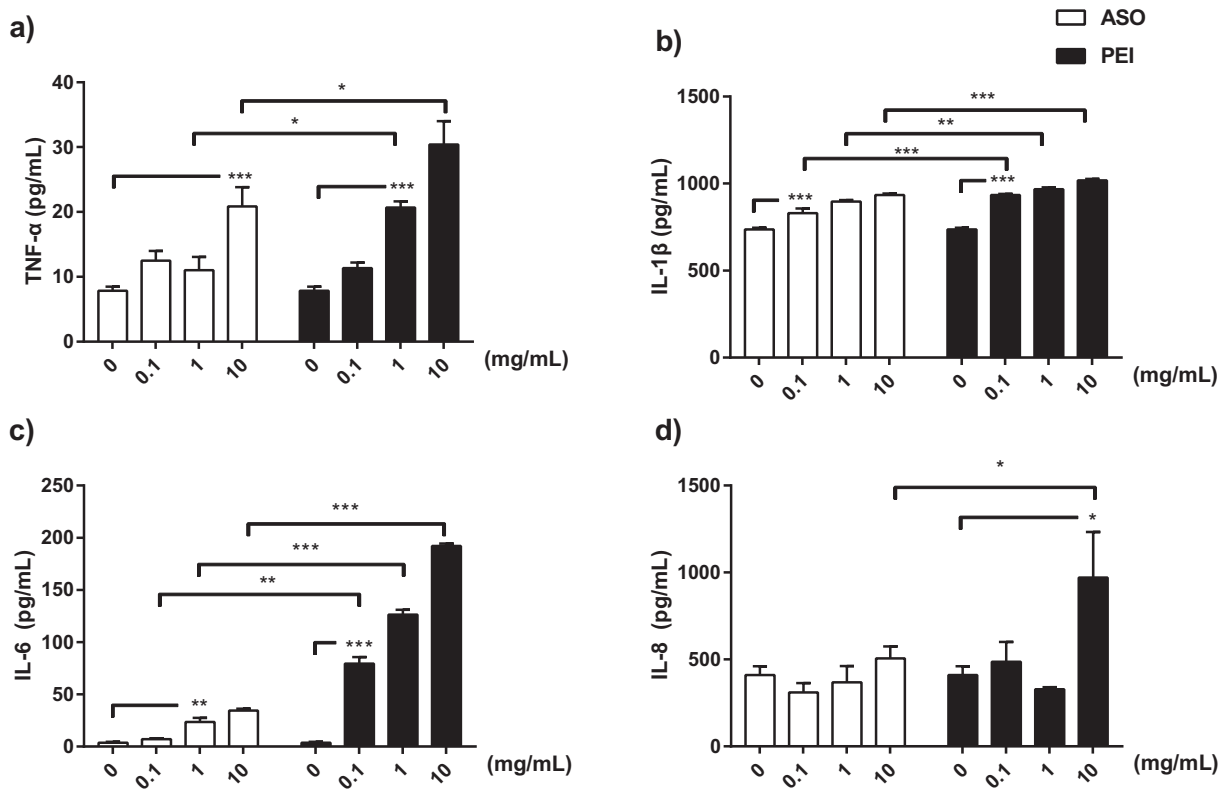

Fig. 1. Local cytokine levels after intradermal injection of adjuvants. Cytokine levels in skin tissues were determined using ELISA at 1 h after intradermal injection of ASO or other adjuvants. Data shown as mean + SEM, n = 3; one-way ANOVA with Tukey's multiple comparison test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

from the concentration of 0.1 mg/mL for IL-6 and IL-1β, 1 mg/mL for TNF-α, and 10 mg/mL for IL-8, indicating the occurrence of cell damage and immunostimulatory effects in the local tissue. In the groups treated with ASO, the levels of these cytokines increased at the concentration of 0.1 mg/mL for IL-1β, 1 mg/mL for IL-6, and 10 mg/mL for TNF-α, indicating a superior safety of ASO compared to that of PEI and a lower immunostimulatory effect without the presence of antigens.

3.2. Confocal observation of tissue distribution and cellular uptake of ASO after i.d. injection

Representative fluorescent images of cryo-sectioned specimens from Tamra-conjugated ASO-treated mouse skin are shown in Fig. 2a. Nearly all of the $CD11c^{+}$ cells (green) contained the red label of ASO, indicating that these cells actively uptake and accumulate ASO. This is likely to affect the secretion of IL-10 by $CD11c^{+}$ cells, with subsequent consequences for the immune response. Tamra-conjugated ASO was co-cultured with RAW264.7 cells at 37 and 4 °C for 2 h, and then the cell nuclei were labeled with DAPI for confocal observation (Fig. 2b). ASO uptake was found to be significantly inhibited at 4 °C, indicating that uptake mainly occurs *via* pinocytosis or endocytosis (Fig. 2c). During 24 h of co-culture at 37 °C, the concentration of ASO in cells was found to increase over time and reach saturation after 6 h. The mean fluorescence intensity decreased after 8 h (Fig. 2d & e).

3.3. Determination of the efficacy of ASO in vitro and in vivo

Lipo was utilized to enhance the transfection of maturing BMDCs with ASO or ASO control. IL-10 secretion was monitored after 6 h of co-culture, and the resulting data are shown in Fig. 3a. The ASO + Lipo group exhibited significantly lower levels of IL-10 as compared to the other groups tested, while the ASO group, ASO control group, and ASO control + Lipo group showed similar levels of IL-10 as the untreated group. In the *in vivo* test, mice were injected *i.d.* with OVA, OVA + ASO, or OVA + ASO control. The mice in the ASO group showed lower IL-10 levels than those in the other groups, indicating that ASO suppresses IL-10 secretion locally in the skin tissue, even in the absence of Lipo (Fig. 3b).

3.4. ASO enhances the influence of BMDCs on T cell proliferation and differentiation

The production of IL-12 by matured DCs is triggered by LPS and it is crucial for the differentiation of the naïve Th cells subsequently in contact with these DCs [19]. We found ASO treatment significantly increased the secretion of IL-12p70 by DCs, which promoted T cell proliferation and differentiation (Fig. 4a). Concentrations of IFN-γ in the co-culture medium were increased after co-culture for 96 h (Fig. 4b). The viability of LPS-treated and ASO-transfected DCs will drop to around 65% after cultured alone for 96 h. Therefore, the T cell proliferation ratio was non-specifically enhanced by co-culture with ASO-transfected DCs at densities of more than 2×10^4 cells/mL (Fig. 4c). In order to confirm that T cell proliferation is caused by down-regulation of IL-10 expression of DCs by IL-10 ASO, 2×10^5/mL DCs from IL-10 KO mice were co-cultured with the T cells from corresponding wild type mice and compared with IL-10 ASO-treated DCs from wild type mice. IL-10 ASO-treated wild type DCs promoted T cell proliferation as compared to the ASO control-treated DCs. The DCs from IL-10 KO mice can promote T cell proliferation to about 200% no matter if they were treated with IL-10 ASO or ASO control (Fig. 4d).

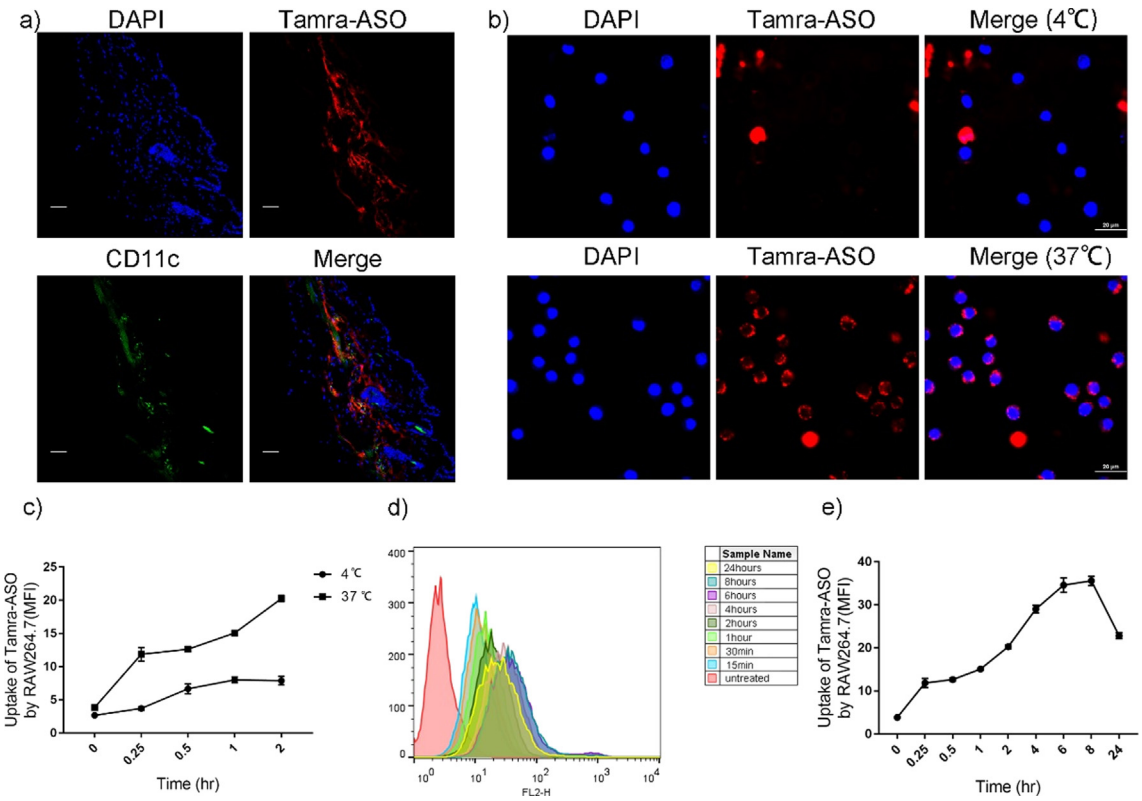

Fig. 2. Tissue distribution and cellular uptake of ASO. (a) 5 μg Tamra-labeled ASO was injected into the mouse skin, and the local skin was removed after 1 h. Immunofluorescent staining was performed and photographed using confocal fluorescence microscope (scale bar = 50 μm). (b) 5 μg/mL Tamra-conjugated ASO was co-cultured with RAW264.7 cells for 2 h at 4 and 37 °C, and then cell nuclei were stained with DAPI. Blue: DAPI-labeled nuclei, Red: Tamra-labeled ASO; representative pictures from 3 independent experiments (scale bar = 20 μm). (c) MFI of Tamra was recorded using flow cytometry. (d) & (e) Uptake of Tamra-ASO by RAW264.7 cells in 24 h. Data shown as mean ± SD, n = 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

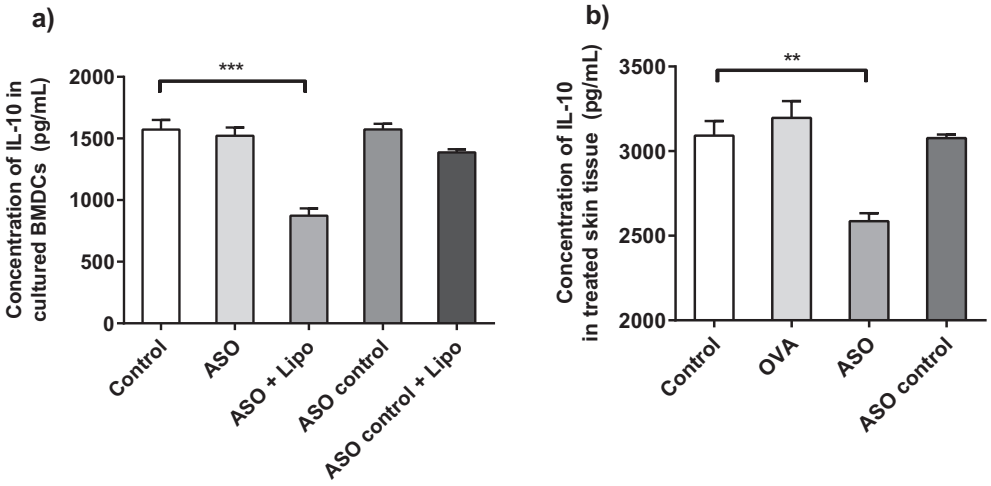

Fig. 3. IL-10 secretion from maturing BMDCs and skin tissue treated with ASO. (a) IL-10 secretion from maturing BMDCs when co-cultured with ASO (n = 3). (b) IL-10 secretion in ASO-treated skin tissue Lipo: Lipofectamine™ 2000 (n = 3). Data shown as mean + SEM, one-way ANOVA with Tukey's multiple comparison test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.5. Immune enhancing effect and dose optimization of ASO as an adjuvant

The OVA-specific antibody titers in the serum samples after the priming, first booster, and second booster injections were determined using ELISA and are shown in Fig. 5. In the first immunization study, the antibody titers of the groups administered ASO were 100-fold higher than those of the OVA group and reached levels similar to those of the FIA and PEI groups after the first and second boosters (Fig. 5a). Meanwhile, the ASO control group was not significantly different from the OVA group at all three time points, indicating that ASO is an effective adjuvant. To confirm the immunopotentiation effect of ASO, a second immunization study was performed using IL-10 KO mice, with C57BL/6 mice with the same genetic background used as a control. The results showed that the IL-10 KO mice produced antibody titers comparable to those produced by control mice immunized with OVA + ASO after the second booster, both of which were significantly higher than

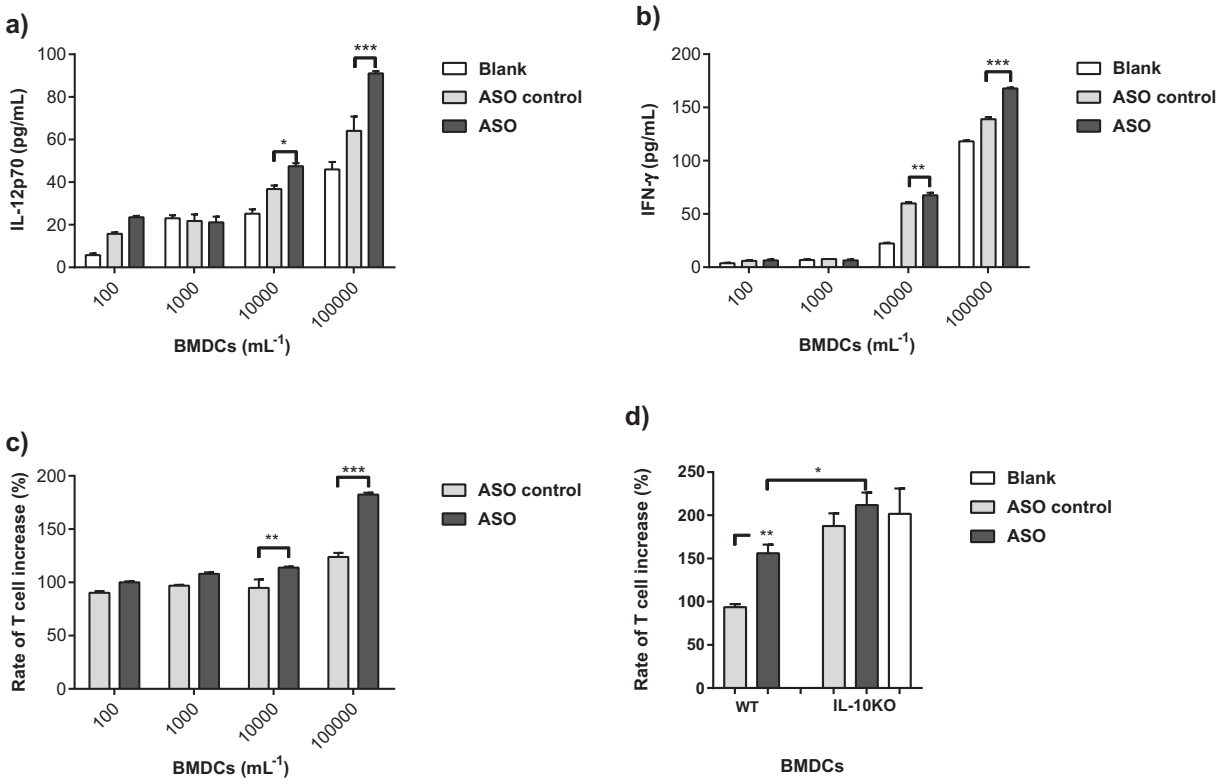

Fig. 4. Influence of BMDCs co-cultured with ASO on the proliferation and differentiation of naïve T cells. (a) Contents of IL-12p70 in the supernatant of ASO- and ASO control-transfected BMDCs on day 7. (b) Concentrations of IFN-γ in the supernatant of BMDCs and T cells co-cultured on day 11. (c) T cell proliferation ratio determined using CCK-8 after co-culture with ASO- or ASO control-transfected BMDCs for 96 h. (d) T cell proliferation ratio determined using CCK-8 after co-culture with ASO- or ASO control-transfected BMDCs from IL-10 KO mice for 96 h. Data shown as mean + SEM, n = 3; two-way ANOVA with Tukey's multiple comparison test for (a), (b) and (c); student's *t*-test for (d): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

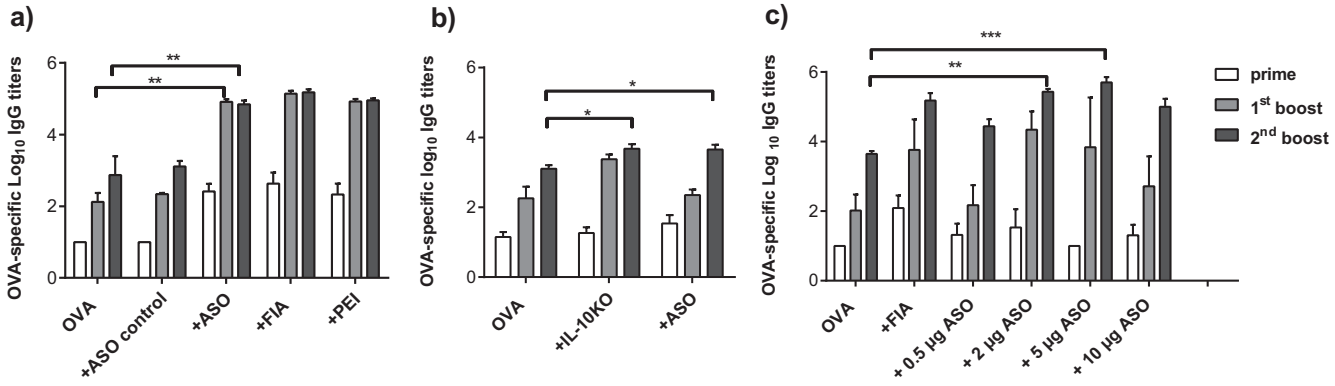

Fig. 5. OVA-specific IgG titers induced by OVA + ASO formulations. 50 μL formulations were administered *i.d.* on day 0, 21, and 42, with 5 μg OVA alone, 5 μg OVA + 50 μL FIA, and 5 μg OVA + 5 μg PEI as controls. Sera were collected after priming, first booster, and second booster injections (day 20, 41, and 55), and IgG titers were determined by ELISA. Non-responders were given an arbitrary log-value of 1. (a) Immunization with OVA + ASO in BALB/c mice. (b) Immunization with OVA + ASO in C57BL/6 mice and IL-10 KO mice from the same background. (c) ASO dose optimization in BALB/c mice. Data shown as mean + SEM, n = 6; one-way ANOVA with Dunnett's multiple comparison test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the titers of OVA-immunized wild-type mice (Fig. 5b). The third immunization study was again performed in BALB/c mice to optimize the dose of ASO as an adjuvant. The highest antibody titers were induced with a dose of ASO of 2–5 μg per mouse. Higher doses of ASO did not induce higher IgG titers (Fig. 5c).

4. Discussion and conclusion

Cytokines are protein molecules involved in signal transduction and expressed by various types of cells in response to immunogens or other stimuli. IL-10 exhibits a bidirectional immune regulation function but mainly plays an immunosuppressive role [20,21]. In 1998, Arima *et al.* designed and synthesized an IL-10 ASO that suppresses the expression of IL-10 in RAW264.7 cells [22]. In 2000, Igietseme *et al.* revealed that both Ag-pulsed DCs from IL-10 KO mice and IL-10 ASO-treated DCs from wild-type mice were efficient cellular vaccines in adoptive immunotherapeutic vaccination against genital chlamydial infection [23]. IL-10 ASO was delivered through the skin using an iontophoresis system and showed a therapeutic effect on the established atopic dermatitis model of NC/Nga mice in 2004 [24]. In 2012, Huang *et al.* reported that IL-10 ASO could

enhance the anti-tumor function of tumor-associated macrophages [25]. These studies, using different ODN antisense sequences of *Il10* mRNA, confirmed their function to down-regulate of IL-10 expression, and demonstrated the immunopotentiation and immunomodulation properties. In our study, we assessed the immune enhancing effect of IL-10 ASO as an adjuvant. The cytotoxicity and tissue toxicity of ASO were assessed to be minimal, while it induced a considerable upregulation in IL-12 and IFN-γ production with elevated T cell proliferation when co-cultured with BMDCs. When the IL-10 ASO was inoculated *i.d.* with OVA, antibody titers were 100-fold higher compared with those of OVA alone, showing the potential of ASO as a vaccine adjuvant.

For the selection of a candidate ASO, we chose an IL-10 ASO without CpG motifs and enhanced its stability using a fully phosphorothioated backbone [22]. A control ODN with the same GC content and no homology to the genes of other interleukins or their receptors was designed. 25 kDa branched PEI, a potent experimental adjuvant with high cytotoxicity by disrupting mitochondria and inducing apoptosis, was used as a positive control [26–28]. IL-10 ASO induced weaker immunostimulatory effects locally when injected into the skin. The same amount of PEI and IL-10 ASO induced similar levels of antibody titers when delivered with OVA as an antigen, indicating that IL-10 ASO may possess better safety profile.

DCs derived from monocytes polarize T cells *via* the secretion of IL-12 and other Th1 cytokines such as TNF-α and IL-6 [29–32]. We found that an optimal density of BMDCs treated with ASO and Lipo could increase IL-12p70 secretion, promote DC differentiation and maturation, and in turn promote T cell proliferation with IFN-γ secretion. These findings indicate that ASO can non-specifically promote T cell proliferation *via* immature DCs and enhance innate immunity during the antigen-presenting process, which was further confirmed using IL-10 KO mice. Such mice developed a stronger response than that observed in the ASO group after the first booster, probably due to their systemic IL-10 deletion, although this difference disappeared after the second booster [22]. These data support the idea that IL-10 ASO increases the sensitivity of the immune system by down-regulating IL-10 expression.

There are two possible approaches for further optimizing the IL-10 ASO as an adjuvant. Chemical structure modifications could prolong its half-life *in vivo* and increase its affinity to the targeted mRNA sequence. For example, the sugar ring of pegaptanib sodium (Macugen®), an RNA aptamer that targets blood vessel endothelial growth factor-165, was modified with 2′-F and 2′-OMe, which increased its resistance to RNases by several orders of magnitude [33]. In addition, 8′ nitrine-modified adenine and 2′ sulfo-modified thymidine can increase the stability of the double strands formed by ASO and its targeted mRNA, thereby enhancing ASO-induced inhibitory effects [34]. As a second approach, non-viral carrier systems could be recruited to increase the ASO uptake efficiency of cells. These delivery systems include liposomes, such as Lipofectamine® and TurboFect™; cationic polymers, such as PEI and chitosan; dendritic polymers; organic nano-particles; hyaluronic acid; and peptides rich in arginine and lysine [35]. They can be produced on a large scale, as the raw material is readily available, and their chemical structure is well defined. In addition, some of them also possess adjuvant properties, such as PEI, a potent systemic adjuvant with a different mechanism, which may provide synergistic effects when combined with ASO in vaccine formulations [28].

Pradhan et al. reported that poly(lactic-co-glycolic) acid (PLGA)-PEI particles loaded with IL-10 siRNA and CpG-ODN induced a more balanced secretion of Th1/Th2 cytokines in lab animals [36]. A safe and efficient adjuvant platform including IL-10 ASO may be established as a further strategy. For example, it could be combined with CpG-ODN or form nano-complex using a cationic polymer as a carrier. The resulting formulation may not only possess better immunopotentiation properties with lower toxicity than that of CpG-ODN alone but may also exhibit more versatile immune regulatory features following adjustment of the component ratios.

In August 2018, Alnylam's Onpattro® (patisiran), the world's first siRNA drug, was approved by the FDA for the treatment of polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR) in adult patients. Currently, many siRNA drugs have entered the clinical stage. Compared with siRNA, ASOs are more specific and stable in circulation. They can be readily synthesized on a large scale at a low cost [37,38]. Moreira *et al.* has described new bifunctional CpG- signal transducer and activator of transcription 3 (STAT3) ASO molecules, which could effectively target $TLR9^{+}$ myeloid cells in bone-localized prostate tumors, resulting in immune-mediated eradication of tumors [39].

In summary, IL-10 ASO is a safe, stable, highly effective, water-soluble adjuvant with a low cost of production. Its concentration is readily adjustable, and it is suitable for administration *via* various vaccination routes. It can also be combined with other adjuvants for a synergetic effect, so that the most favorable reaction type and intensity for disease prevention can be acquired. Thus, IL-10 ASO as an adjuvant has the potential for further development and application.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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