Prevention of Experimental Autoimmune Uveoretinitis by Blockade of Osteopontin with Small Interfering RNA

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Footnote

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Abstract

Osteopontin (OPN) is elevated during the progression of experimental autoimmune uveoretinitis (EAU) in C57BL/6 (B6) mice. Furthermore, EAU symptoms are ameliorated in OPN knockout mice or in B6 mice treated with anti-OPN antibody (M5). Recently, OPN has been shown to promote the Th1 response not only in the extracellular space as a secretory protein but also in cytosol as a signaling component. Thus, we attempted to reduce OPN in both compartments by using a small interfering RNA (siRNA) targeting the OPN coding sequence (OPN-siRNA). EAU was induced in B6 mice by immunization with human interphotoreceptor retinoid-binding protein (hIRBP) peptide sequence 1–20. The OPN- or control-siRNA was administered with hydrodynamic methods 24 hours before and simultaneously with immunization (prevention regimen). When plasma OPN levels were quantified following siRNA administration with the prevention regimen, the level in the OPN-siRNA-treated group was significantly lower than that in the control-siRNA-treated group. Accordingly, the clinical and histopathological scores of EAU were significantly reduced in B6 mice when siRNA caused OPN blockade. Furthermore, TNF-α, IFN-γ, IL-2, GM-CSF and IL-17 levels in the culture supernatants were markedly suppressed in the OPN-siRNA treated group, whereas the proliferative responses of T lymphocytes from regional lymph nodes against immunogenic peptides was not significantly reduced. On the other hand, the protection was not significant if the mice received the OPN-siRNA treatment on day 7 and day 8 after immunization when the clinical symptoms appeared overt (reversal regimen). Our results suggest that OPN-blockade with OPN-siRNA can be an alternative choice for the usage of anti-OPN antibody and controlling uveoretinitis in the preventive regimen. (Word count: 261 words)

1. Introduction

Experimental autoimmune uveoretinitis (EAU) is an animal model of human endogenous uveoretinitis, including sympathetic ophthalmia, birdshot retinochoroidopathy, Vogt-Koyanagi-Harada's disease, and Behçet's disease (Caspi et al. 1988). EAU is induced by immunization with a retinal antigen (Ag), e. g., interphotoreceptor retinoid-binding protein (IRBP), or by the adoptive transfer of retinal Ag-specific T lymphocytes (Mochizuki et al. 1985; Caspi et al. 1986; Gregerson et al. 1986). EAU induced with immunization of retinal Ag now represents not only Th1 but also Th17-cell-mediated ocular diseases. A massive inflammatory infiltration composed primarily of mononuclear cells causes a rapid and irreversible destruction of photoreceptor cells (Jiang et al. 1999; Silver et al. 1999; Caspi 2003; Amadi-Obi et al. 2007; Luger et al. 2008). It has been demonstrated that the augmentation of the Th2 response and T regulatory cytokine production and down-regulation of the Th1 response can mitigate inflammation and protect against the development of EAU (Saoudi et al. 1993; Kezuka et al. 1996; Caspi 2002).

Osteopontin (OPN), also known as early T lymphocyte activation 1 (Eta-1), a secreted phosphoglycoprotein (SPP), contains the arginine-glycine-aspartic acid (RGD) integrin-binding sequence that is found in many extracellular matrix (ECM) proteins (O'Regan and Berman 2000). OPN mediates adhesion and migration of a number of different cells types (O'Regan and Berman 2000). OPN is widely produced by a variety of inflammatory cells, including T cells, macrophages, NK cells, and NKT cells (Denhardt et al. 2001; Diao et al. 2004; Diao et al. 2008) and induces interleukin-12 (IL-12) and IFN-γ production and inhibits IL-10 expression (Ashkar et al. 2000). Moreover, the intracellular isoform, OPN-i is now considered to promote the Th1

response through type I interferon production (Shinohara et al. 2006, Cantor and Shinohara 2009). OPN has therefore been considered to act as a cytokine contributing to the development of Th1-mediated immunity and diseases and is anticipated to be a therapeutic target for controlling these diseases.

Indeed, recent studies, including ours, indicate that OPN possesses an aggravating role in EAU (Hikita et al. 2006; Kitamura et al. 2007), as had already been demonstrated in experimental autoimmune encephalomyelitis (EAE) (Ashkar et al. 2000; Chabas et al. 2001), anti-type II collagen antibody-induced arthritis (Yumoto et al. 2002; Yamamoto et al. 2003), and autoimmune hepatitis (Diao et al. 2004; Saito et al. 2007). Furthermore, we demonstrated that a specific antibody (M5) reacting to the SLAYGLR sequence, a newly exposed binding site, within OPN by thrombin cleavage, significantly suppressed clinical and histopathological scores of EAU in mice (Kitamura et al. 2007).

RNA interference (RNAi) is a powerful tool for silencing gene expression, by which double-stranded RNA (dsRNA) triggers the sequence-specific degradation of messenger RNA. In particular, small interfering RNAs (siRNAs), 21-23 nucleotide-length fragments (Elbashir et al. 2001; Hannon 2002; Xie et al. 2006), have been shown to be of great value in decreasing the expression of the target gene by *in vivo* administration (Song et al. 2003; Nakamura et al. 2004; Schiffelers et al. 2005; Khoury et al. 2006)

In the present study, we applied siRNA targeting to an OPN coding sequence (OPN-siRNA) to inhibit OPN function by reducing OPN expression. We demonstrate the remarkable efficacy of OPN-siRNA to prevent EAU in mice.

2. Materials and Methods

2.1 Experimental animals

6- to 8-week old female C57BL/6 (H-2^b; B6) mice were obtained from Japan SLC (Shizuoka, Japan). All studies were conducted in compliance with the Statement for the Use of Animals in Ophthalmic and Vision Research by the Association for Research in Vision and Ophthalmology (ARVO, Rockville, MD) and with the Hokkaido University Committee for Animal Use and Care.

2.2 Reagent

hIRBP (human interphotoreceptor retionoid-binding protein) peptide sequence 1–20 (GPTHLF QPSLVLDMAKVLLD) was purchased from Sigma-Genosys Japan (Ishikari City, Hokkaido, Japan). Purified *Bordetella pertussis* toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO) and complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* strain H37Ra were purchased from Difco (Detroit, MI).

2.3 Immunization

To analyze the cell proliferative response, $hIRBP_{1–20}$ (100 μg) was emulsified in CFA (1:1 v/v) and a total of 50 μl of the emulsion was injected subcutaneously (s.c.). To induce EAU, $hIRBP_{1–20}$ (200 μg) was emulsified in CFA (1:1 v/v) containing 2.5 mg/ml *Mycobacterium tuberculosis* H37Ra. A total of 200 μl of the emulsion was injected s.c. concurrent with immunization, 0.1 μg of PTX in 100 μl phosphate-buffered saline (PBS) was injected intraperitoneally (i.p.) as an additional adjuvant (Kezuka et al. 1996).

2.4 Evaluation of EAU

Clinical assessment by funduscopic examination of the chorioretinal inflammation was carried out every 3 or 4 days from day 7 after immunization (Namba et al. 2000). The severity of EAU was graded 0-4 as described previously (Thurau et al. 1997). Briefly, the clinical scoring was based on vessel dilatation, the number of vessel white focal lesions, vessel white linear lesions and hemorrhages, and the extent of retinal detachment.

For the histological assessment of EAU, eyes were enucleated on day 21 after immunization. Removed eyes were fixed in 4 % paraformaldehyde for an hour and transferred into 10 % phosphate-buffered formaldehyde until processing. Fixed samples were embedded in paraffin and 5 μm sagittal sections were cut near the optic nerve head and stained with hematoxylin and eosin. The severity of EAU in each eye was scored on a scale of 0-4 as described previously (Caspi et al. 1988). In brief, no change was scored as 0. Focal non-granulomatous, monocytic infiltrations in the choroid, ciliary body, and retina were scored as 0.5. Retinal perivascular infiltration and monocytic infiltration in the vitreous were scored as 1. Granuloma formation in the uvea and retina, occluded retinal vasculitis, along with photoreceptor folds, serous retinal detachment, and loss of photoreceptors were scored as 2. In addition, the formation of granuloma at the level of the retinal pigmented epithelium and the development of subretinal neovascularization were scored as 3 and 4 according to the number and the size of the lesions.

2. 5 Preparation of OPN- and control-siRNA

OPN- and control-siRNAs were purchased from B-Bridge (Sunnyvale, CA, USA). The

sequences of sense and anti-sense strands of each siRNA were as follows: mouse OPN-RNAi-5/239: 5'-GCCAUGACCACAUGGACGAdTdT-3' (sense), 5'-UCGUCCAUGUGGUCAUGGCdTdT-3' (anti-sense), control-siRNA pair, as designed to avoid specific sequences in mice; 5'-ACUCUAUCUGCACGCUGACUU-3' (sense), 5'-GUCAGCGUGCAGAUAGAGUUU-3' (anti-sense) (Saito et al. 2007). The siRNAs were deprotected, annealed and desalted.

2.6 In vivo treatment of mice with siRNA

Synthetic siRNAs were delivered *in vivo* using a modified hydrodynamic transfection method (Song et al. 2003), in which 50 μg of either OPN- or control-siRNA dissolved in 1 ml PBS was rapidly injected into the tail vein. Mice were treated with two injections of either OPN- or control-siRNA, 24 hours before and simultaneously with the immunization (prevention regimen). Another group of mice was treated with two injections of either OPN- or control-siRNA on day 7 and day 8 after the immunization (reversal regimen).

2.7 Plasma OPN level

To quantify OPN concentration in plasma from EAU mice treated with either OPN-siRNA or control-siRNA, mice were deeply anesthetized with ether, and then blood was collected transcardially before immunization and on days 3, 7, 10, 14, 21, and 28 after immunization. All blood samples were collected in the presence of EDTA to avoid cleavage by thrombin *in vitro*, centrifuged to remove cells and debris, and stored at –80°C. OPN concentration in plasma samples (n=24 mice) was quantified by an enzyme-linked immunosorbent assay (ELISA) kit for

mouse OPN (Immuno-Biological Laboratories Co. Ltd., Takasaki, Japan) according to the manufacturer's protocol.

2.8 T cell proliferative responses

T cells obtained from B6 mice that had been primed with $hIRBP_{1-20}$ were cultured with Ag-presenting cells (APC) and $hIRBP_{1-20}$ as described elsewhere (Kitamura et al. 2007). In brief, T cell-enriched fraction was prepared as Nylon wool non-adherent cells by passing dispersed cells of draining lymph nodes from $hIRBP_{1-20}$-primed mice over nylon wool column. Then the T-enriched fraction (5×10^5/well) were co-cultured with mitomycin-C (MMC)-treated splenocytes as APC (5×10^5/well) and $hIRBP_{1-20}$ peptide at the indicated concentration in serum-free medium (RPMI 1640 medium, 10 mM Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamicin sulfate, supplemented with 0.1 % bovine serum albumin, and ITS+1 liquid media supplement [2 μg/ml insulin from bovine pancreas, 1.1 μg/ml iron-free transferrin, 1 ng/ml sodium selenite, 100 μg/ml bovine serum albumin, and 1 μg/ml linoleic acid] (Sigma Chemical Co.). Cells in 96-well flat-bottomed plates were incubated for 64 hours at 37°C in 5 % CO_2 in air, pulsed with 18.5 kBq of [^{3}H]-thymidine (Perkin Elmer Japan, Tokyo) per well during the last 16 hours of incubation, and then were harvested. Incorporation of [^{3}H]-thymidine was quantified with a direct β-counter (Packard, Meriden, CT), and the data were presented as the mean counts per minute (CPM) minus the background (medium alone; ΔCPM) (Kitamura et al. 2007).

2.9 Quantification of Cytokine

Cytokine concentrations in each culture supernatant were quantified with either BD™ Cytometric Bead Array kit (BD Bioscience, San Diego, CA, USA) (Cook et al. 2001) or FlowCytomix™ multiplex kit (Bender MedSystems GmBH, Vienna, Austria). In brief, as for CBA set-up, 50 μL samples or known concentrations of standard samples (0–5000 pg/ml) were added to capture beads conjugated with Ab for each cytokine followed by an addition of anti-cytokine Ab-phycoerythrin (PE) reagent (detection reagent). The mixture was then incubated for 2 h at room temperature in the dark, and washed to remove unbound detection reagent. As for FlowCytomix, 25 μL samples or known concentrations of standard samples (0–20000 pg/ml) were added to capture beads conjugated with Ab for each cytokine followed by an addition of biotinylated anti-cytokine Abs and the mixture was incubated for 2hr at room temperature in the dark. After washing, the beads were incubated with streptavidin-PE for 2 h at room temperature in the dark, and washed to remove unbound detection reagent. Data acquisition was performed with FACS Calibur flow cytometer (BD Bioscience) and analyzed on a computer (CBA software 1.1; BD Bioscience or FlowCytomix Pro2.2 software). Amounts of IFN-γ, TNF-α and Interleukin (IL)-1α, -2, -4, -5, -6, -10, -17, and granulocyte macrophage colony-stimulating factor (GM-CSF) were quantified.

2.10 Statistical analysis

Data are presented as mean ± SD in clinical and histopathological scoring and as mean ± SEM in analyses of cell proliferation and cytokine production. Statistical analysis of EAU scoring was performed using the nonparametric Mann-Whitney *U*-test. Analyses of cell proliferation and

cytokine production were performed using two-tailed Student's *t*-test. P values <0.05 were considered statistically significant.

3. Results

3.1 OPN-siRNA treatment inhibited the increase of OPN plasma level during EAU

We demonstrated that the plasma concentration of OPN elevated from the basal level (4~5 ng/ml) over time after $IRBP_{1-20}$, peaked around 14 d, and then gradually waned (Kitamura et al. 2007), suggesting that plasma OPN concentration correlates well with disease development. First, we quantified the plasma OPN level to examine whether administration of OPN-siRNA could actually reduce the OPN level *in vivo*. To this end, we introduced siRNA twice at 24 h before and at the same time of $IRBP_{1-20}$ immunization. In the control-siRNA-treated group, OPN concentration in plasma again peaked around 2 wk, thus reproducing our previous results. On the other hand, in the OPN-siRNA-treated group, the OPN concentration in plasma was not elevated from the basal level during EAU. In comparison with that of the control group, plasma concentration of OPN upsurge was significantly suppressed following immunization in the OPN-siRNA-treated group at days 7 and 14 (Fig .1).

3.2 OPN-siRNA reduced EAU scores

To investigate the potential of OPN-siRNA to prevent EAU, B6 mice were immunized with $hIRBP_{1-20}$ and treated twice with either OPN-siRNA or control-siRNA 24hours before and simultaneously with immunization. From day 7 after immunization, clinical assessment was performed every 3 or 4 days. As compared to the control group, the EAU clinical score was low

in the OPN-siRNA-treated group during the entire period of observation (Fig.2). In the OPN-siRNA-treated group, EAU reached a peak at day 28 after immunization. In contrast, control group mice peaked at day 21 (Fig. 2). The maximum clinical scores were significantly lower in the OPN-siRNA-treated group (average scores: 0.89 ± 0.68) than those in the control-siRNA-treated group (2.44 ± 0.78).

To further examine the effect of OPN-siRNA on EAU, histopathological examinations were performed. Eyes were removed from either OPN-siRNA- or control-siRNA-treated EAU mice 21 days after $hIRBP_{1–20}$ immunization. Representative histopathology of the eyes from mice treated with control-siRNA or OPN-siRNA is shown in Fig. 3A and 3B, respectively. In control mice, inflammatory cells were found in the retina, vitreous and choroid along with retinal folds and granulomatous lesions (Fig. 3A). Retinas collected from mice treated with OPN-siRNA showed almost normal histology (Fig. 3B). The histological scores of retinal sections were significantly lower in OPN-siRNA-treated mice (average scores: 0.88 ± 0.69) than in control mice (1.5 ± 0.73; Fig. 3C).

3.3 OPN-siRNA treatment only partly reversed the disease

We next examined whether OPN-siRNA treatment could reverse an ongoing disease process. EAU was induced as usual by $hIRBP_{1–20}$ immunization at day 0, and the mice were treated with two injections of either OPN- or control-siRNA at day 7 and day 8 when ocular symptoms first appeared overt after the immunization (reversal regimen). The clinical severity of EAU appeared to be slightly lower around day 21 but was not significantly reduced during the course of observation with the reversal regimen (Fig. 4A). The histopathological scores of retinal sections

were not significantly lower in OPN-siRNA-treated mice (average scores: 0.95 ± 0.63) than in control mice (1.31 ± 1.11; Fig. 4B).

These results suggest that OPN-siRNA treatment more efficiently targets the priming rather than effector function of pathogenic T cells.

3.4 OPN-siRNA showed a slight influence on priming of hIRBP-specific T cells, but significantly inhibited Th1 and Th17 cytokine responses

To examine the mechanism underlying the suppressive effect of siRNA, we analyzed proliferative responses of lymphocytes from regional lymph nodes of hIRBP-immunized mice treated with OPN-siRNA or control-siRNA upon stimulation with hIRBP *in vitro*. As shown in Fig. 5A, lymphocytes from both groups mounted a considerable response. No significant differences were observed in the cell proliferation between OPN-siRNA-treated and control-siRNA-treated EAU mice, although the response in the OPN-siRNA group was slightly lower than that in the control group.

Next, we examined cytokine levels in the cultures of hIRBP peptide and lymphocytes collected from hIRBP-immunized mice treated with either OPN-siRNA or control-siRNA. We quantified IFN-γ and TNF-α concentrations in the culture supernatants. The levels of both IFN-γ and TNF-α were significantly reduced in the supernatants from cells of siRNA-treated mice compared to those of control siRNA-treated mice at any concentrations of hIRBP analyzed (Fig 5. B). Furthermore, the production levels of IL-2, GM-CSF, and IL-17 were also significantly reduced in the supernatants from cells of OPN siRNA-treated mice compared to those of control siRNA-treated mice, whereas there no difference in the production of IL-1α, -4, 5, 6, and 10

between the two groups (Fig 5. C, and data not shown).

4. Discussion

In our previous study (Kitamura et al. 2007), we demonstrated that the plasma OPN levels were significantly elevated in EAU B6 mice by day 3 after immunization and peaked at day 14, which was concordant with the clinical course. Notably, OPN knockout (KO) mice displayed a considerably milder EAU and delayed disease onset compared with those of $OPN^{+/+}$ littermates (Hikita et al. 2006; Kitamura et al. 2007). In addition, EAU induced in B6 mice was ameliorated by administration of M5, an anti-OPN antibody. These findings demonstrated that OPN played a role in EAU development and might be an appropriate target for controlling ocular inflammation.

In the present study focusing on the blockade of OPN production, we used siRNA targeting the OPN coding sequence (OPN-siRNA). The OPN-siRNA was introduced into the animal with a hydrostatic pressure-mediated technique, hydrodynamic delivery (Liu et al. 1999). OPN is thought to function not only in soluble form (OPN-s) as a cytokine but also in intracellular form, OPN-i (Shinohara et al. 2006; Cantor and Shinohara 2009). Although anti-OPN Ab can only be accessible to and block OPN-s, OPN-siRNA may block both forms by reducing the expression in both compartments.

First, we quantified the plasma level to evaluate the duration for the inhibition of OPN following *in vivo* siRNA treatment with the prevention regimen (day 1 and 0 of immunization) in EAU mice. OPN-siRNA treatment inhibited the increase of the plasma OPN level during the entire period of EAU to the basal level (Fig 1). It was reported that OPN protein expression

was significantly suppressed 5 days after *in vitro* siRNA treatment (Saito et al. 2007). In our study, OPN level remained significantly reduced at day 7 and day 14 in the OPN-siRNA-treated group (Fig. 1). This result suggested that OPN-siRNA treatment could have a longer period of efficacy than anticipated and thus may be applicable to chronic inflammatory diseases. When RNAi for OPN was induced with a prevention regimen, significant prevention of EAU was indeed manifested as had been shown with anti-OPN Ab (M5) treatment (Fg 2, 3). As to the clinical score, OPN-siRNA appeared to be more efficient than M5 (Kitamura et al. 2007).

We also examined whether OPN-siRNA treatment could reverse ongoing EAU. Mice were immunized by $IRBP_{1-20}$ peptide at day 0 and treated with two injections of OPN-siRNA at day 7 and day 8 after EAU induction. At the 7-day time point, uveitogenic effector cells had already been primed and could induce EAU (Agarwal et al. 2000). As anticipated with this report, the reversal regimen was not effective for amelioration of the ongoing disease (Fig. 4A, B). These results suggested that the effects of OPN-siRNA were induced by the blockade of upsurge of OPN following immunization and thus preventing generation of primed T cells more than by inhibiting the effector function of induced T cells. However, the ineffectiveness of OPN blockade with siRNA after disease onset may not ruin its application for ongoing diseases. This is because the consecutive priming and generation of autoreactive T cells may take place even in chronic diseases. [Moreover, co-administration of anti-OPN Ab may also compensate the effect of siRNA administration after onset, which should be pursued in further investigation.]

We then examined the antigen-specific proliferative responses of lymphocytes upon *ex vivo* hIRBP peptide restimulation in the preventive regimen. Proliferation of hIRBP peptide-primed cells was slightly reduced by administration of OPN-siRNA *in vivo*. This finding is partially

compatible with the result with the anti-OPN antibody (Kitamura et al. 2007). Notably, the production of IFN-γ and TNF-α was significantly reduced in the culture supernatants of the OPN-siRNA-treated group compared to that of the control group. The suppressed production of IFN-γ and TNF-α appeared to be interpreted by the blockade of Th1 cells, which led to the amelioration of EAU, a Th1-mediated autoimmune model (Caspi et al. 1988; Caspi 2002; Schiffelers et al. 2005). IFN-γ induces macrophage activation and nitric oxide production, which leads to destruction of retina in EAU (Hoey et al. 1997). TNF-α provokes inflammatory responses (Green and Flavell 1999) and TNF p55 receptor deficient mice are resistant to EAU (Calder et al. 2005).

OPN has been recognized as a key player in the Th1-responses for several reasons. First, the expression of OPN is mediated by T-bet, which is indispensable for the polarization of Th1 immune response (Shinohara et al. 2005). The secreted OPN further affects the expression of IL-12 (enhancement) and IL-10 (inhibition) that favors Th1-deviation (Ashkar et al. 2000). Second, the specific form of OPN, intracellular OPN, could induce IFN-α secretion from plasmacytoid dendritic cells (pDC) in the presence of CpG in TLR9-MyD88- and IRF7-dependent manner (Shinohara et al. 2006). IFN-α also favors Th1-deviation. Third, OPN, especially the NH_2-terminal fragment of OPN cleaved by thrombin, promotes adhesion and migration of leukocytes and neutrophils and directly binds to $\alpha_9\beta_1$, which interacts with vascular cell adhesion molecule-1 (VCAM-1) in extravasation of neutrophils at sites of acute inflammation (Taooka et al. 1999). Thus, the migration of inflammatory cells might be blocked by the reduction of OPN content in the tissue.

It has been reported that OPN enhances survival of activated T cells by inhibiting transcription

factor Foxo3a, activating NF-κB, and altering pro-apoptotic proteins (Hur et al. 2007). Thus, OPN function seems to be superfluous and not only supports Th1 deviation but also plays complex roles in immunological responses. This finding may explain the various influences on the manifestation of autoimmune diseases observed in different disease models.

On the other hand, there were conflicting results that EAU development was aggravated in IFN-γ KO and IFN-γ receptor KO mice (Fukushima et al. 2005; Hikita et al. 2006), which implied that IFN-γ might inhibit generation of pathogenic Th17 cells in EAU.

Recently, a new insight with Th17 cells has emerged for the pathogenesis of EAU (Amadi-Obi et al. 2007). In the present study, OPN-siRNA treatment suppressed not only Th1 cytokines but also IL-17 production, which could also account for the amelioration of EAU. These results suggest that OPN represents a good therapeutic target to ameliorate uveoretinitis as shown in our previous (Kitamura et al. 2007) and present studies. From a clinical viewpoint, OPN blockade seems to be not only potent but also beneficial for the treatment of human uveoretinitis without serious side effect, an obstacle of anti-TNF antibody therapy (Ohno et al. 2004). Thus far, no reports have demonstrated that OPN deficiency deteriorates host defense in mice (Rittling et al. 1998; Sato et al. 2005).

To date, OPN blockade has been shown to ameliorate various disease models in mice (Chabas et al. 2001; Jansson et al. 2002; Yumoto et al. 2002; Yamamoto et al. 2003; Hikita et al. 2006; Kitamura et al. 2007). Concordantly, OPN was elevated in human counterparts, including pulmonary sarcoidosis (Maeda et al. 2001), rheumatoid arthritis (Ohshima et al. 2002), and multiple sclerosis (Comabella et al. 2005). We thus presume that OPN blockade is also effective in these diseases. It is important to develop a safe and feasible technique for siRNA delivery to

render the RNAi treatment applicable to human patients, as the hydrodynamic method is rather intense. Several novel techniques are being developed for the efficient introduction and interference for siRNA especially for *in vivo*-use (Liu et al. 1999; Howard and Kjems 2007). The mechanistic elucidation and technical excellence will drive the RNAi for the treatment of immunological diseases with equal or better chance of use than monoclonal antibodies targeted to various molecules involved in disease development.

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Figure Legends

Figure 1. Persistence of inhibition of plasma OPN level following *in vivo* siRNA treatment during EAU. EAU was induced in B6 mice. These mice were treated with OPN-siRNA (○) or control-siRNA (●) with hydrodynamic methods 24 hours before and simultaneously with immunization. Blood was collected transcardially before immunization and on days 3, 7, 10, 14, 17 and 21 after immunization from each group of mice. All blood samples were collected under EDTA, centrifuged to remove cells and debris, and stored at -80°C until used. Plasma levels of OPN were measured by sandwich ELISA. The results are presented as mean ± standard deviation. Statistical significance was determined using two-tailed Student's *t*-test (**, $P<0.01$, *, $P<0.05$). Data are representative of two separate experiments with the same result.

Figure 2. Clinical score of EAU in mice treated with OPN-siRNA, 24 hours before and simultaneously with the immunization. EAU was induced in B6 mice. These mice were treated with OPN-siRNA (○) or control-siRNA (●). Funduscopic examination was carried out every 3 or 4 days from day 7 after immunization. The results are presented as mean clinical score for all eyes of each group of mice (9 mice per group) ± standard deviation. Significance was determined using Mann-Whitney *U*-test (**, $P<0.01$, *, $P<0.05$).

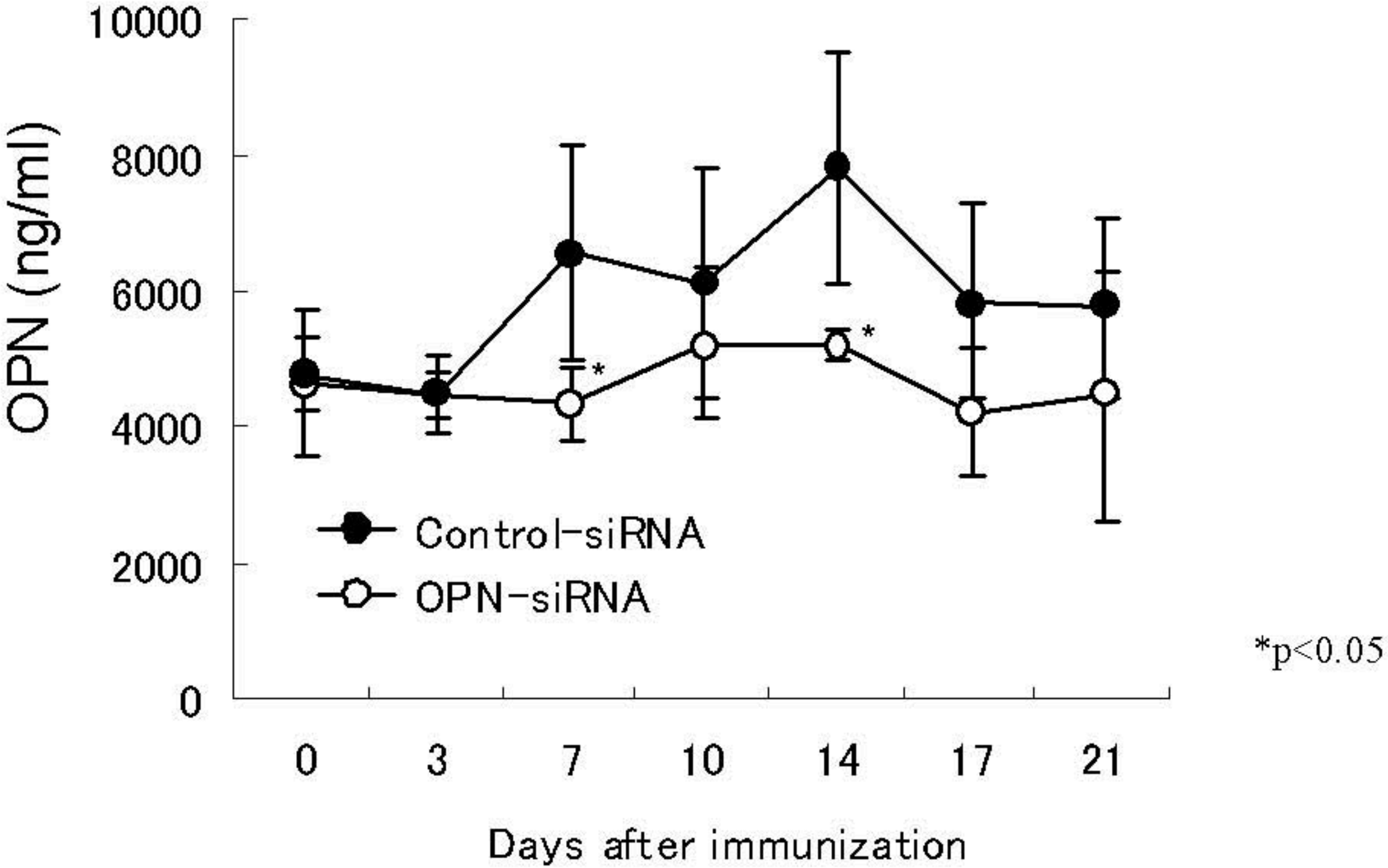
Figure 3. Histopathology and histopathological score of EAU mice treated with OPN-siRNA. EAU was induced in B6 mice. Histopathology of mice treated with either control-siRNA (A) or OPN-siRNA (B). Note that inflammatory cells are present in the retina, vitreous, and choroid with retinal folds and granulomatous lesions in mice treated with control-siRNA (A) and the almost normal architecture of the retina in OPN-siRNA-treated mice (B). C. Mice were treated with OPN-siRNA (○) or control-siRNA (●). On day 21, the eyes were enucleated and scored by examining the histopathological sections of these eyes as shown in A and B. The results are presented as the histopathological score of each eye, and the mean EAU score of each group is indicated by a bar. Significance was determined by Mann-Whitney *U*-test ($P<0.05$).

Figure 4. Clinical and histopathological score of EAU in mice treated with OPN-siRNA with reversal regimen. A. EAU was induced by $hIRBP_{1-20}$ immunization at day 0. These mice were treated with two injections of either OPN-siRNA (○) or control-siRNA (●) on day 7 and day 8 after the immunization. Funduscopic examination was carried out every 3 or 4 days from day 7 after immunization. The results are presented as mean clinical score for all eyes of each group of mice (10 mice per group) ± standard deviation. Representative data of two separate experiments with similar results are presented. B. Histopathological score of EAU in mice treated with OPN-siRNA with reversal regimen. On day 21, the eyes from EAU mice were enucleated and scored of each eye. The mean EAU score of each group is indicated by a transverse bar.

Figure 5. Cell proliferative response and cytokine production of lymphocytes from regional

lymph nodes of hIRBP-immunized and OPN-siRNA-treated mice. A. [^{3}H]-thymidine incorporation by primed lymphocytes. Lymphocytes were obtained from B6 mice immunized with hIRBP and treated with OPN-siRNA (●) or control-siRNA (○). Lymphocytes were incubated with indicated dose of hIRBP peptide and with [^{3}H]-thymidine for the last 16 hours. B. IFN-γ and TNF-α produced in the culture supernatant. C, IL-17, IL-2 and GM-CSF produced in the culture supernatant. The results are presented as mean ± standard deviation. Statistical significance is determined using two-tailed Student's *t*-test (**, $P<0.01$, *, $P<0.05$). Data are representative of two separate experiments with the similar result.

Fig. 1


10000
8000
6000
4000
2000
0
OPN (ng/ml)
0
3
7
10
14
17
21
Days after immunization
Control-siRNA
OPN-siRNA
*
*
*

*p<0.05

Fig. 2

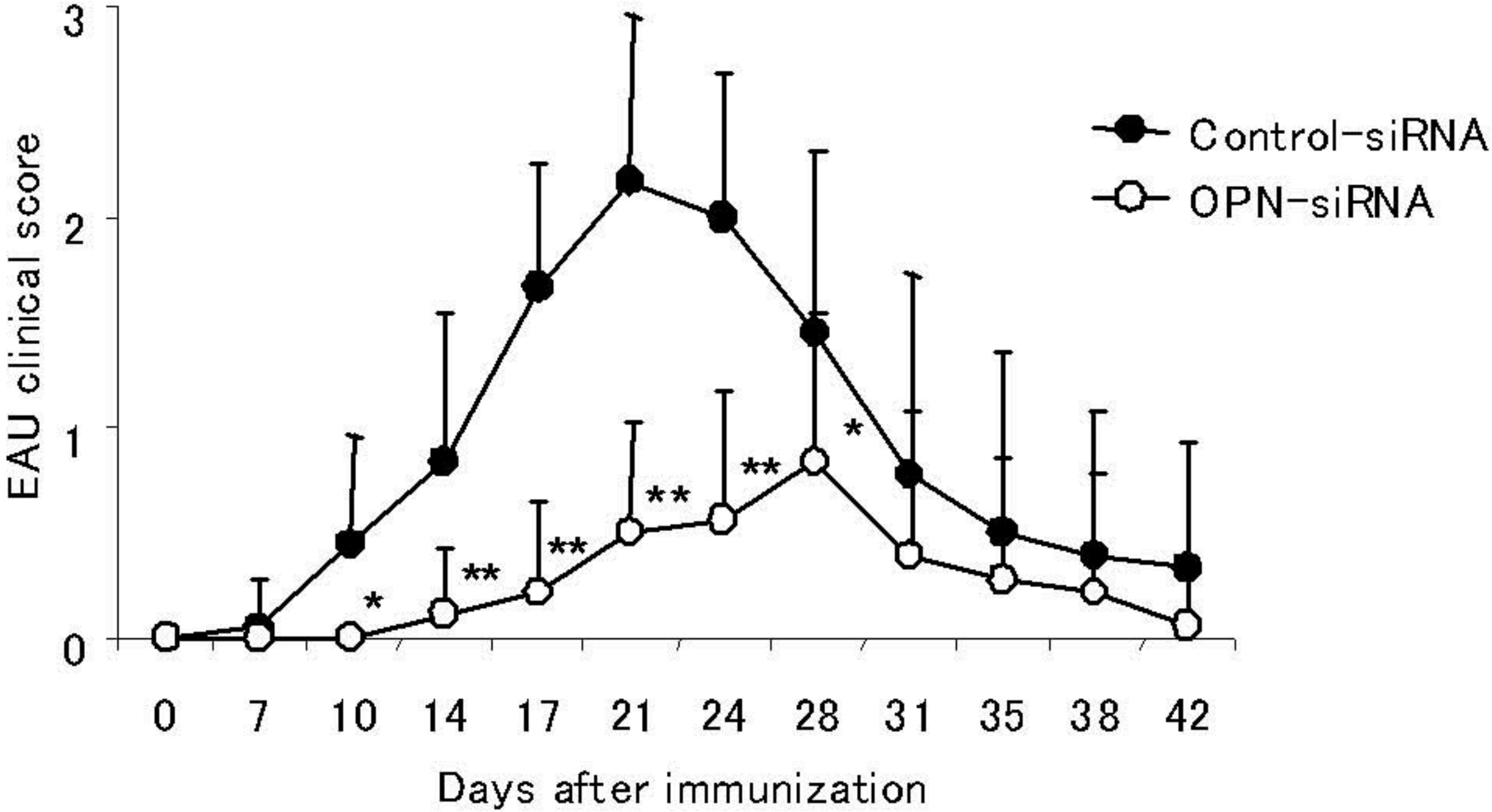

EAU clinical score
3
2
1
0
0
7
10
14
17
21
24
28
31
35
38
42
Days after immunization
Control-siRNA
OPN-siRNA
*
**
**
**
**
*

Fig 3A, B

A. Control-siRNA

B. OPN-siRNA

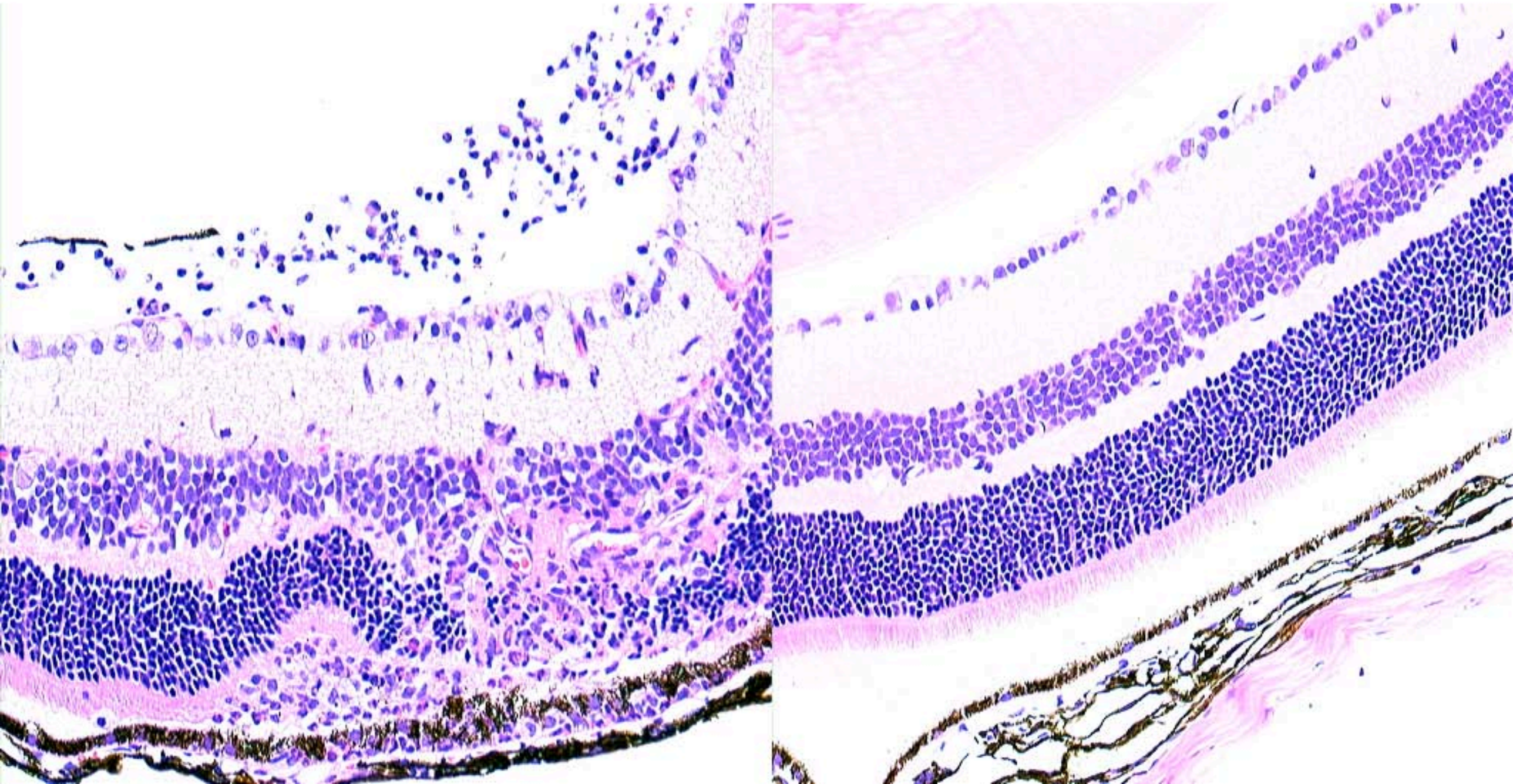


Fig 3C

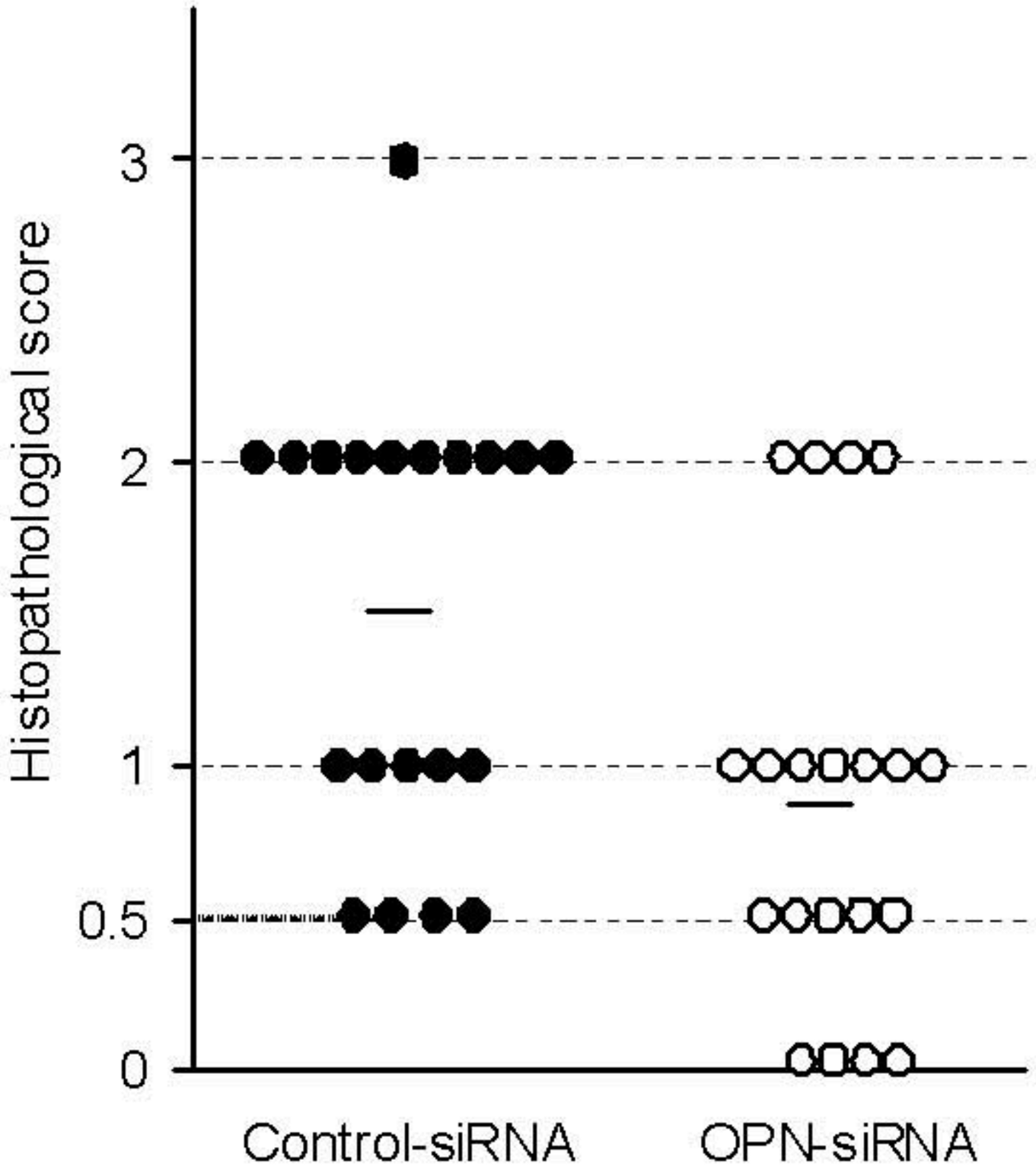

P<0.05
Histopathological score
3
2
1
0.5
0
Control-siRNA
OPN-siRNA

Fig 4A

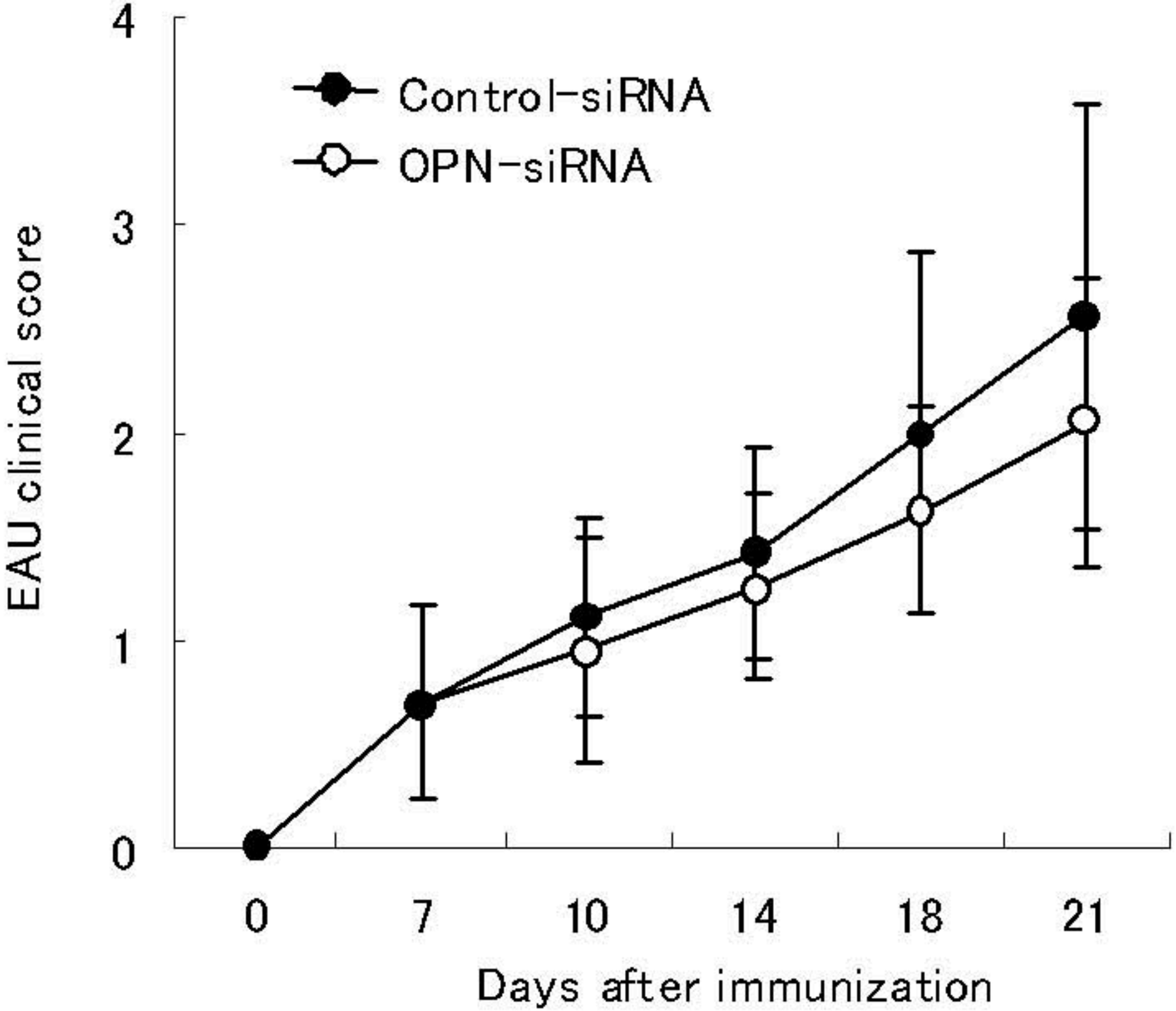

Control-siRNA
OPN-siRNA
EAU clinical score
0
1
2
3
4
0
7
10
14
18
21
Days after immunization

Fig 4B

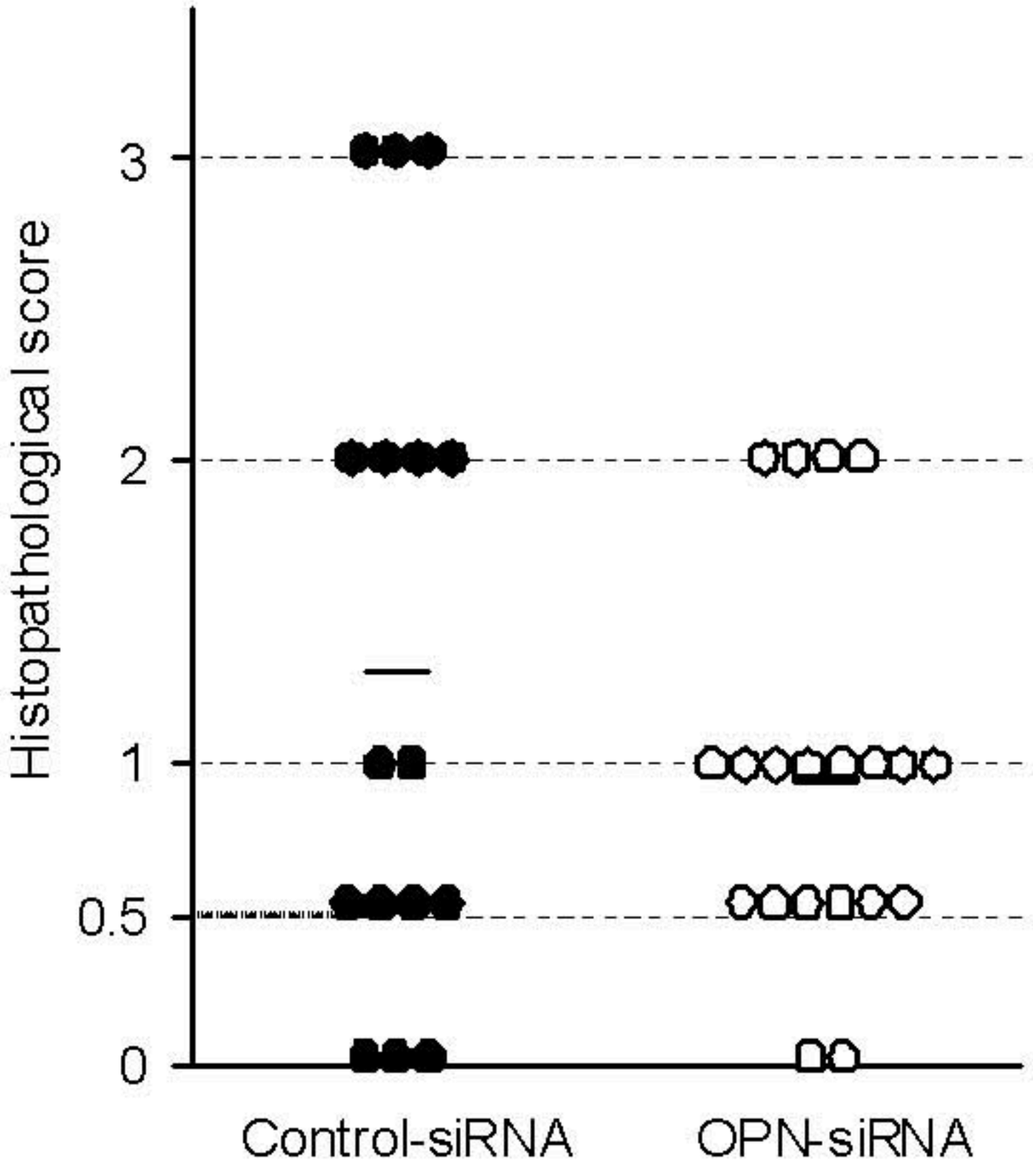


Fig. 5A

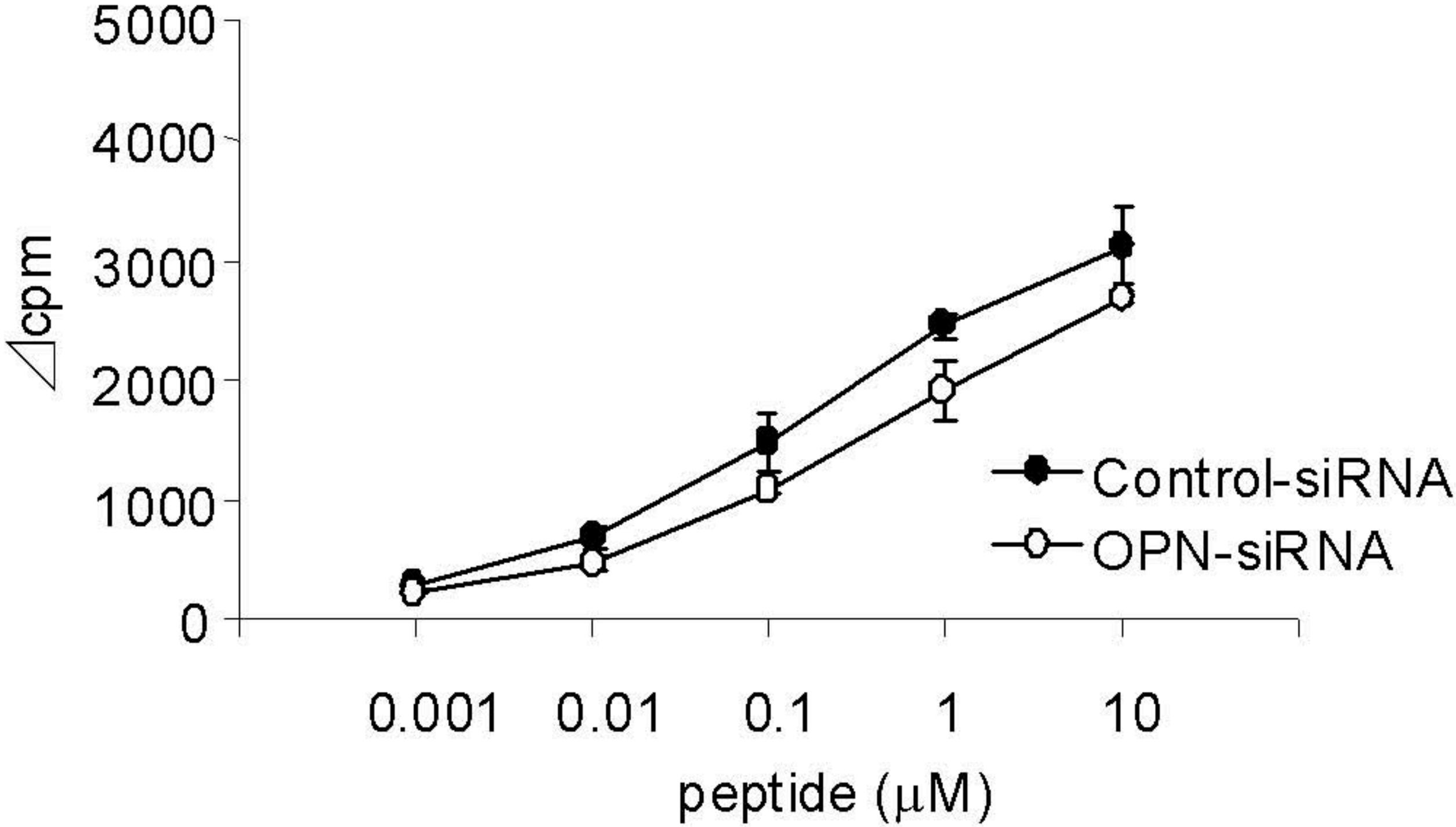

5000
4000
3000
2000
1000
0
Δcpm
0.001
0.01
0.1
1
10
peptide (μM)
Control-siRNA
OPN-siRNA

Fig. 5B

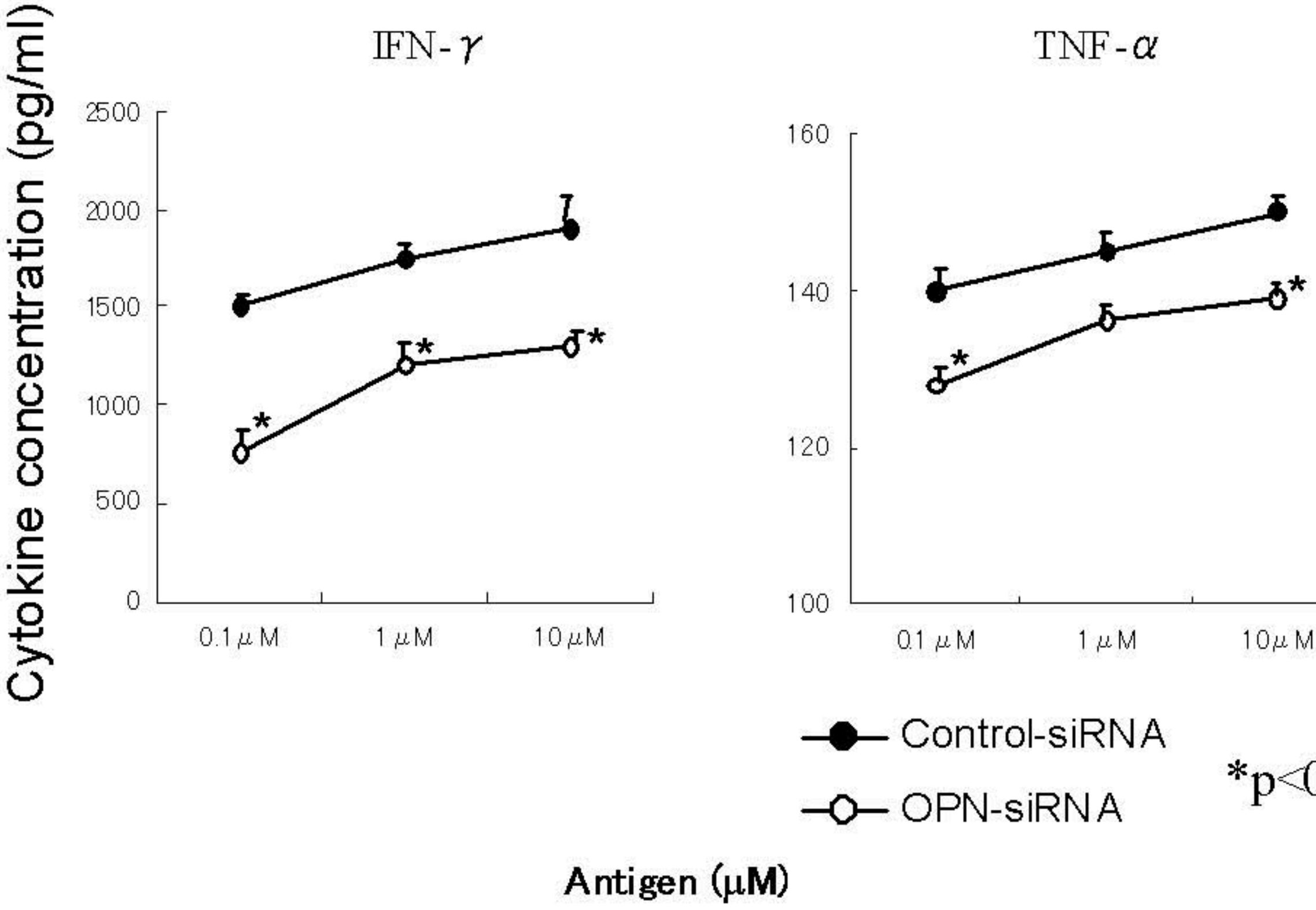
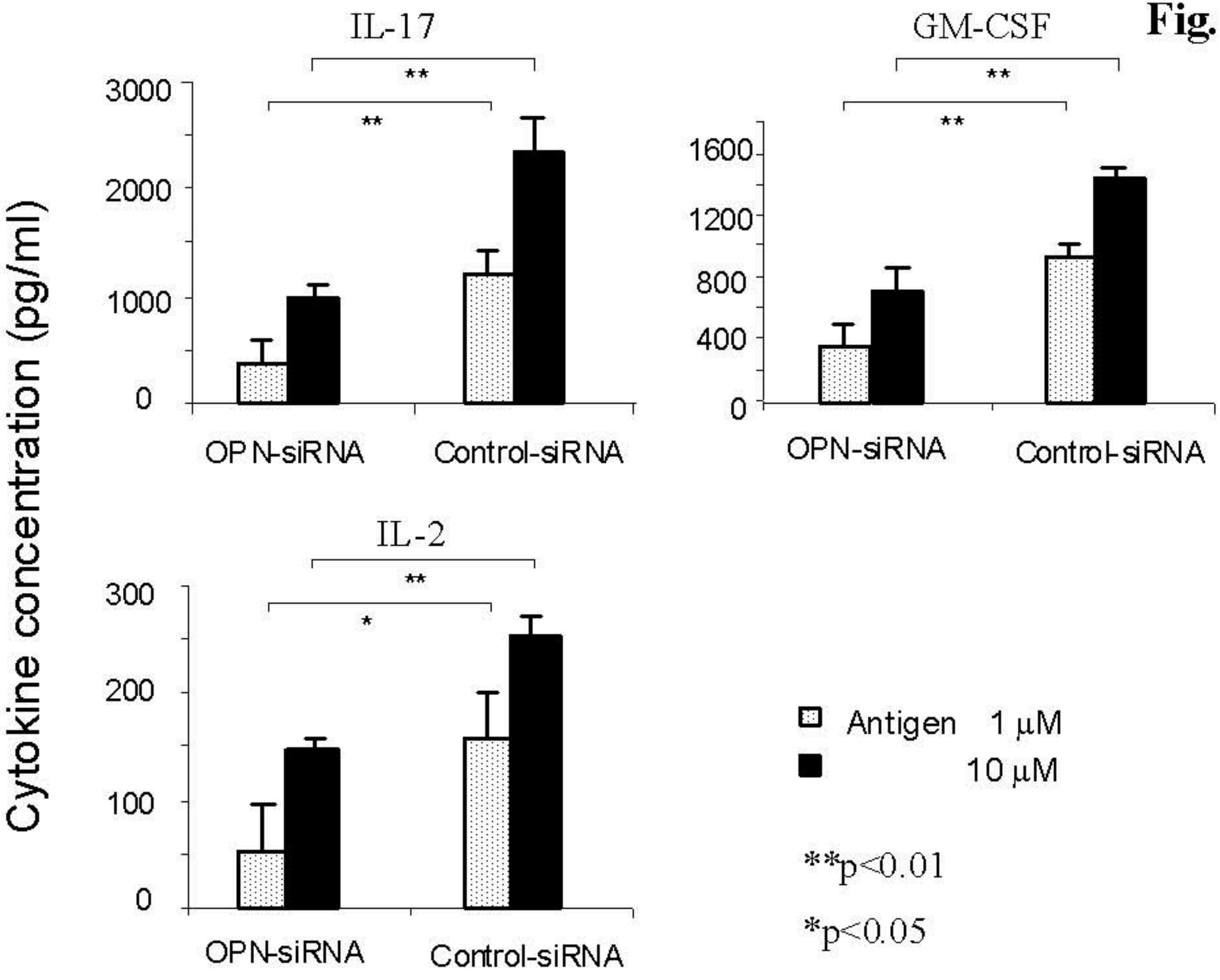

IFN-γ
TNF-α
Cytokine concentration (pg/ml)
2500
2000
1500
1000
500
0
160
140
120
100
0.1μM
1μM
10μM
*
Control-siRNA
OPN-siRNA
*p<0.05
Antigen (μM)

Fig. 5C


IL-17
**
**
3000
2000
1000
0
OPN-siRNA
Control-siRNA
GM-CSF
**
**
1600
1200
800
400
0
OPN-siRNA
Control-siRNA
IL-2
**
*
300
200
100
0
OPN-siRNA
Control-siRNA
Cytokine concentration (pg/ml)
Antigen 1 μM
10 μM
**p<0.01
*p<0.05