



Bioimaging and pulmonary applications of self-assembled Flt1 peptide–hyaluronic acid conjugate nanoparticles

Hyemin Kim^a, Hyun Taek Park^b, You Me Tae^b, Won Ho Kong^a, Dong Kyung Sung^c,
Byung Woo Hwang^a, Ki Su Kim^a, Yoon Keun Kim^{b,**}, Sei Kwang Hahn^{a,*}

^a Department of Materials Science and Engineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea

^b Department of Life Science, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Republic of Korea

^c Samsung Biomedical Research Institute, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Republic of Korea

ARTICLE INFO

Article history:

Received 9 May 2013

Accepted 18 July 2013

Available online 6 August 2013

Keywords:

Hyaluronic acid

Flt1 peptide

Dexamethasone

Nanoparticle

Pulmonary delivery

Asthma

ABSTRACT

Despite wide exploitation of corticosteroid drugs for the treatment of asthma, the poor therapeutic effect on a neutrophilic subtype of asthma prohibits the full recovery of asthma patients. In this work, dexamethasone (Dexa) was loaded in Flt1 peptide–hyaluronic acid (HA) conjugate nanoparticles to overcome the limitation of corticosteroid resistance for the treatment of neutrophilic pulmonary inflammation. Flt1 peptide–HA conjugates are self-assembled to nanoparticles because of hydrophobic Flt1 peptide conjugated to HA by benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) chemistry. *In vitro* bioimaging showed efficient internalization of Flt1 peptide–HA conjugate nanoparticles into lung epithelial cells by HA-receptor mediated endocytosis. Also, *ex vivo* imaging for the biodistribution in ICR mice revealed long-term retention of Flt1 peptide–HA conjugate nanoparticles in deep lung tissues possibly due to mucoadhesive property of HA. On the basis of bioimaging results for pulmonary drug delivery applications, we prepared Dexa-loaded Flt1 peptide–HA conjugate nanoparticles. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) confirmed the formation of nanoparticles, which reduced cytokine levels of lipopolysaccharide (LPS)-stimulated cells more efficiently than free Dexa. Furthermore, according to the bronchoalveolar lavage (BAL) cellularity and histological analysis, Dexa loaded Flt1 peptide–HA conjugate nanoparticles showed remarkable therapeutic effects in both eosinophilic and neutrophilic asthma model mice.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Asthma is a chronic inflammatory immune disease exhibiting reversible airway obstruction and airway hyper-responsiveness (AHR) [1,2]. T helper 2 (Th2) cell response and eosinophilic inflammation have been considered as key factors for the progress of asthma, but recent clinical and experimental evidences have indicated that severe asthma can be characterized by neutrophilic inflammation [3,4]. Although symptoms of asthma can usually be well relieved with inhaled corticosteroids and beta-2-agonists, refractory or steroid resistant asthma, which is hard to manage using

conventional treatments, occurs in some severe asthmatic patients [5]. This kind of asthma accompanies immoderate neutrophilic infiltration, and high levels of cytokines including interferon gamma (IFN- γ) and interleukin 17 (IL-17) related with Th1 and Th17 cell responses [6,7]. Accordingly, neutrophilic airway inflammation induced by Th1 and Th17 cell responses has been considered as an important feature of steroid-resistant asthma. In our previous studies, we established asthma model mice which contain type 1 and type 2 immune responses to inhaled allergen. High-dose lipopolysaccharide (LPS) treatment induced type 1 (neutrophilic) asthma accompanying both Th1 and Th17 cell responses, whereas low dose LPS treatment did type 2 (eosinophilic) asthma accompanying Th2 cell response [8,9].

Vascular alterations may significantly contribute to airway remodeling in asthma and vascular endothelial growth factor (VEGF), the most specific growth factor for vascular endothelium, is strongly involved in the pathophysiology of airway vascular

* Corresponding author. Tel.: +82 54 279 2159; fax: +82 54 279 2399.

** Corresponding author. Tel.: +82 54 279 2125; fax: +82 54 279 8379.

E-mail addresses: juinea@postech.ac.kr (Y.K. Kim), skhanb@postech.ac.kr (S.K. Hahn).

remodeling in asthma [10,11]. In patients with asthma, the expression of VEGF in biopsy specimens of bronchial mucosa and sputum was up-regulated as compared to controls. In addition, administration of VEGF receptor inhibitors reduced the pathophysiological signs of asthma [12,13]. VEGF receptor 1 (VEGFR1, Flt1) is expressed on vascular endothelial cells and non-endothelial cells such as monocytes and macrophages. Our previous study showed that VEGFR1-mediated pathway was important in the development of Th1 and Th17 cell responses to inhaled allergens in neutrophilic asthma model mice. The treatment of Flt1 peptide in high-dose LPS model reduced IFN- γ and IL-17 levels in the lung and lymph-node (LN). Flt1 peptide of GNQWFI has been reported to specifically bind VEGFR1 and inhibit the binding of VEGF to VEGFR1 [14]. The peptide alleviated Th1 and Th17 immune responses, and the development of T cell priming via reducing maturation and migration of lung dendritic cells [15]. Hence, the inhibition of VEGF and its receptor may be effective for the treatment of the vascular alterations in the airway. We hypothesized that the combination treatment of Flt1 peptide and dexamethasone (Dexa) might be more effective to relieve neutrophilic pulmonary inflammation elicited by Th1 and Th17 cell responses.

In this work, Flt1 peptide was conjugated to hyaluronic acid (HA) and the self-assembled Flt1 peptide–HA conjugate nanoparticle was chosen as a delivery carrier of Dexa for the treatment of asthma. HA is a biodegradable, non-immunogenic, and nontoxic glycosaminoglycan with a unique mucoadhesive property [16,17]. HA is mostly present in soft connective tissues. It is also on the surface of alveolar epithelial cells to protect lung elastin from tissue damage and injury in respiratory diseases [18,19]. Due to the mucoadhesive property of HA [20], its nanoparticles would prolong the residence time and remain close to the main absorption sites in the deep lung. The efficient pulmonary delivery of Dexa loaded in Flt1 peptide–HA conjugate nanoparticles may reduce the amount of dose required for the same therapeutic effect with systemically administered drug. The minimized systemic exposure to Dexa would alleviate its adverse effect including adrenal suppression, bone loss, hypertension, and hyperglycemia [21,22]. Several kinds of HA receptors such as CD44, LYVE-1, and HARE have been known to bind specifically to HA [23,24]. The existence of CD44 positive epithelial cells was confirmed in asthmatic epithelium [25]. After confocal imaging for *in vitro* receptor-mediated endocytosis and *ex vivo* fluorescence analysis of Flt1 peptide–HA conjugate nanoparticles into alveolar epithelial cells in the lung, we investigated the effect of Dexa loaded Flt1 peptide–HA conjugate nanoparticles on the treatment of airway inflammation. The formation of self-assembled nanoparticles was assessed by transmission electron microscopy (TEM), dynamic light scattering (DLS), zeta potential analysis, and high performance liquid chromatography (HPLC). The efficacy of Dexa/Flt1 peptide–HA nanoparticles has been evaluated and discussed in model mice of eosinophilic asthma induced by allergens plus aluminum hydroxide (Alum) and neutrophilic asthma induced by LPS-containing allergens.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (HA) with a molecular weight of 100 kDa was kindly provided from Shiseido Co. (Tokyo, Japan). Flt1 peptide with a sequence of Gly–Gly–Asn–Gln–Trp–Phe–Ile (GNQWFI) was purchased from Pepttron Co. (Daejeon, Korea). Dowex® 50WX8–400 ion-exchange resin, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), N,N-diisopropylethylamine (DIPEA), dexamethasone, fluorescein isothiocyanate (FITC), 5-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), dexamethasone 21-phosphate disodium salt, aluminum hydroxide [Al(OH)₃], and ovalbumin (OVA) were obtained from Sigma–Aldrich (St. Louis, MO). Tetra-n-butyl ammonium hydroxide (TBA-OH) and dimethyl sulfoxide (DMSO) were obtained from Alfa Aesar (Ward Hill, MA). HiLyte

647 amine was purchased from AnaSpec Inc. (Fremont, CA) and 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Lipopolysaccharide (LPS) was purchased from Calbiochem (Darmstadt, Germany). TRITC conjugated anti-IgG mouse antibody was purchased from Dako Corporation (Carpinteria, CA) and ED1 antibody was obtained from Chemicon International (Temecula, CA). VECTOR shield mounting medium and DAPI were obtained from Vector Laboratories Inc. (Burlingame, CA). Diff-Quik was purchased from Dade Behring (Newark, DE). All chemicals were used without further purification. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in a pathogen-free facility at the Pohang University of Science and Technology (POSTECH). All live animal experiments were approved by the Ethics Committee of POSTECH.

2.2. Synthesis of Flt1 peptide–HA conjugate

HA-TBA was prepared as described in our previous report [26]. Ion exchange resin of Dowex® 50WX8–400 (2.5 g) was washed with 50 mL of distilled water three times. The washed Dowex resin was mixed with 1.5 M excess of TBA-OH (4.9 mL), which stirred for 30 min. The resulting Dowex–TBA resin was filtered and mixed with 100 mL of HA (M_w = 100 kDa, 1 g) aqueous solution for 3 h. The resulting solution was filtered through 0.45 μ m filter to remove the Dowex resin and get a clear HA-TBA solution, and lyophilized for 3 days. After dissolution of the prepared HA-TBA in DMSO, carboxyl groups of HA-TBA were activated with 2.5 M excess of BOP [27]. The HA-TBA solution was mixed with Flt1 peptide dissolved in DMSO at a molar ratio of 1/10. Then, the equimolar amount of DIPEA was added to the solution. The resulting solution stirred at 37 °C for a day and was mixed with 1 M NaCl aqueous solution at a volume ratio of 1/1. The reaction was terminated by dropping the pH of the solution to 3.0 with 1 M HCl and raising it to 7.0 with 1 M NaOH. The final reaction solution was poured into the pre-washed dialysis membrane tube (MWCO of 10 kDa), dialyzed against a large excess amount of 0.3 M NaCl solution, 25% ethanol, and water, and lyophilized for 3 days. The peptide content in Flt1 peptide–HA conjugate was determined by ¹H NMR analysis (DPX500, Bruker, Germany). Flt1 peptide–HA conjugate containing 25 peptide molecules per single HA chain was prepared and used for the following experiments.

2.3. Fluorescence imaging for the cellular uptake of Flt1 peptide–HA conjugate nanoparticles

To visualize the cellular uptake of Flt1 peptide–HA conjugate nanoparticles, the nanoparticles were modified with hexamethylene diamine (HMDA, 5 mol%) and reacted with excess FITC in 0.1 M sodium carbonate buffer (pH 9, 5 mg/mL) at a dark place. The mixed solution stirred at room temperature overnight. The unreacted FITC was removed by centrifugal filtration (MWCO: 10 kDa). Meanwhile, A549 cells were seeded on an eight-chamber glass slide at a density of 2×10^4 cells/well and cultured in DMEM supplemented with 10 vol% of FBS and 1 wt% of antibiotics in a humidified 5% CO₂ incubator at 37 °C. The culture medium was replaced with FBS-free DMEM. FITC-labeled Flt1 peptide–HA conjugate in 200 μ L DMEM (1 mg/mL) was added to the wells of culture slides. For HA preincubation tests, 100-fold molar excess of HA was added to the well before the treatment with the sample. The cells were incubated for 2 h, washed with PBS three times, and fixed with 4% paraformaldehyde in PBS. After DAPI staining for 10 min, the cells were observed with a confocal microscope (FV1000, Olympus America Inc.).

2.4. Ex vivo imaging for the biodistribution of Flt1 peptide–HA conjugate nanoparticles

Ex vivo imaging for the biodistribution of Flt1 peptide–HA conjugate nanoparticles and their cargoes was carried out using CFSE/Flt1 peptide–HA–HiLyte 647 nanoparticles. The residual carboxyl group of Flt1 peptide–HA conjugate was activated with 2 M excess of EDC and labeled with 4 mol% of HiLyte 647 amine. The reaction was performed for 2 h, and the product was dialyzed against excess amount of 0.3 N NaCl aqueous solution and distilled water to remove EDC and unreacted HiLyte 647 amine. Then, CFSE was loaded into HiLyte 647 labeled Flt1 peptide–HA conjugate nanoparticles. Briefly, CFSE (5 mg) was dissolved in DMSO, which was mixed with Flt1 peptide–HA conjugate (10 mg) aqueous solution. The mixture was sonicated with an ultrasonicator (Ultrasonic 410, Hwashin Tech., Seoul, Korea) for 10 min. The resulting product was transferred to the pre-washed dialysis tube (MWCO of 3.5 kDa) and dialyzed against a large excess amount of distilled water for a day to remove unloaded CFSE and DMSO. The amount of fluorescent dyes conjugated and loaded were quantified by measuring the absorbance of the labeled samples at 650 nm for HiLyte 647 and 490 nm for CFSE. CFSE standard solutions and CFSE-labeled samples were treated with 10 μ L esterase solution (0.25 mg/mL) overnight before the measurement. For the *ex vivo* bioimaging, ICR mice were treated with free CFSE, free HiLyte 647 amine, and CFSE/Flt1 peptide–HA–HiLyte 647 nanoparticles via orotracheal route. At pre-determined time intervals, the mice were anesthetized with sodium pentobarbital and the organs were extracted after cardiac perfusion with PBS. Fluorescence images of the organs were obtained with an optical imaging system (IVIS: Xenogen, Alameda, CA). Photonic emissions were assessed with Living image® 2.2.0.1 software (Xenogen). The data are presented by

pseudocolor representation for light intensity and mean photon in the region of interest (ROI). Three replicates were performed for the *ex vivo* imaging.

2.5. *In vitro* cytotoxicity test of Flt1 peptide–HA conjugate nanoparticles

A549 cells were seeded in a 24-well plate at a density of 2×10^5 cells/well and cultivated at 37 °C for 24 h. The cells were treated with Flt1 peptide–HA conjugate nanoparticles at concentrations up to 600 µg/mL in the FBS-supplemented medium. For comparison, the cells were also treated with polyethyleneimine (PEI, M_w 25 kDa) at concentrations up to 75 µg/mL in the same condition. At 12 h post-treatment, the cytotoxicity was examined with an EZ-Cytox cell viability assay kit. Typically, 10 µL of EZ-Cytox reagent was added to 100 µL of serum-free medium in each well of the plate. After incubating the plate at 37 °C for 2 h, the absorbance at 450 nm was measured with a Bio-Rad microplate reader. The relative cell viability was expressed as the percentage of viable cells with respect to the untreated control group.

2.6. *In vivo* cytotoxicity test of Flt1 peptide–HA conjugate nanoparticles

The cytotoxicity of Flt1 peptide–HA conjugate nanoparticles was evaluated by ED1 staining and histological analysis. Twenty-week-old male ICR mice with a mean body weight of 29 ± 1.2 g were used for the cytotoxicity analysis. The mice were anesthetized by injecting the mixture of ketamine (45 mg/kg) and xylazine (8 mg/kg) intraperitoneally. After that, light guided oral endotracheal intubation was performed using a 20 gage central vein catheter for orotracheal instillation of samples. Aliquots of each sample containing 3 mg of Flt1 peptide–HA conjugate nanoparticles were orotracheally instilled and the cytotoxicity of the samples was evaluated 24 h after the treatment. For comparison, *Escherichia coli*

strain E69 was harvested as reported elsewhere [28] and used for the treatment of ICR mice (50 µL, 10^7 colony forming units in 0.5 mL PBS). The animals were anesthetized with sodium pentobarbital (100 mg/kg) 24 h post-treatment and the lungs were exposed via thoracotomy, followed by transcardial perfusion with ice cold PBS (pH 7.4). The lungs were fixed by tracheal instillation of 4% formaldehyde at a constant inflation pressure. After that, the trachea was ligated, and the lung was removed and immersed in 4% formaldehyde. Lung was embedded in paraffin and transverse serial sections (4 µm) were stained with hematoxylin and eosin (H&E) for histological analysis. The lung sections were also analyzed by ED1 antibody staining. The lung sections were incubated for 60 min with ED1 antibody. After rinsing with PBS, the sections were incubated with TRITC conjugated anti-IgG mouse antibody (1:1000) for 60 min. After washing with PBS, the sections were mounted in VECTOR shield mounting medium with DAPI and examined with a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA).

2.7. Preparation and characterization of Dexa/Flt1 peptide–HA nanoparticles

Dexa (5 mg) was dissolved in ethanol, which was mixed with Flt1 peptide–HA conjugate (10 mg) aqueous solution. The mixture was sonicated with an ultrasonicator (Ultrasonic 410, Hwashin Tech., Seoul, Korea) for 10 min. The resulting product was transferred to the pre-washed dialysis tube (MWCO of 3.5 kDa) and dialyzed against a large excess amount of distilled water for a day to remove unloaded Dexa and ethanol. The formation of Dexa/Flt1 peptide–HA nanoparticle was analyzed by TEM (Hitachi, Tokyo, Japan). For the TEM analysis, 20 µL of the nanoparticle solution was dropped on 300 mesh copper TEM grids with a carbon film and then dried in a desiccator. The hydrodynamic size and the zeta potential of the nanoparticles in aqueous solution were measured by DLS analysis (Zetasizer

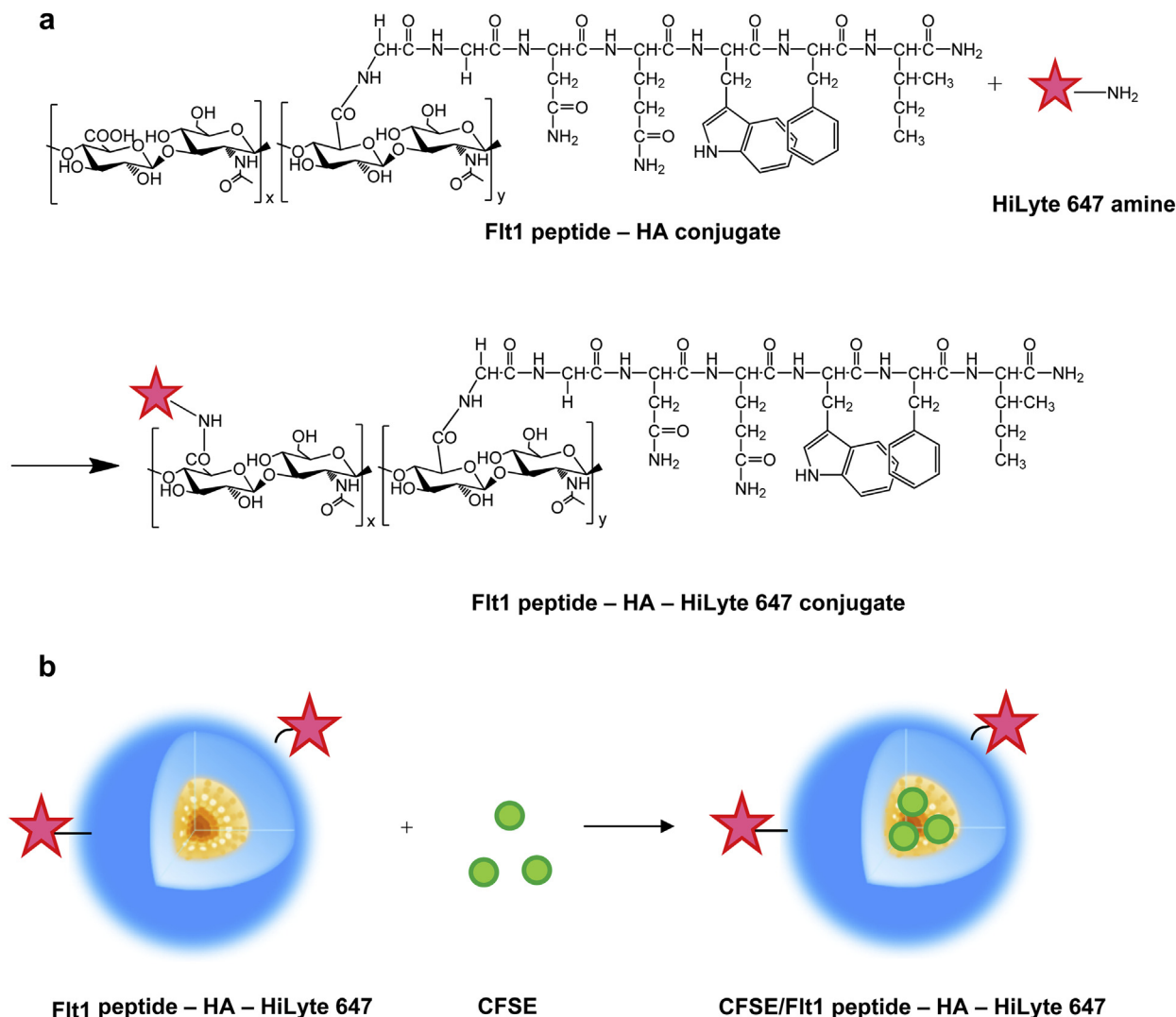


Fig. 1. Schematic representations for (a) the conjugation of Flt1 peptide–hyaluronic acid (HA) with HiLyte 647 amine and (b) the CFSE loaded Flt1 peptide–HA–HiLyte 647 nanoparticles.

Nano ZS, Malvern Instruments, Worcestershire, UK). The loading content of Dexa was quantified by HPLC analysis. DMSO was added to the aqueous nanoparticle solution at a volume ratio of 4/1 to dissolve Dexa completely in the nanoparticles. HPLC analysis was performed using the following systems: Waters 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector, Waters 717 plus autosampler, Symmetry™ 300C18 5 μ m column (Waters, MA). The mobile phase was a mixture of acetonitrile and 0.05 M ammonium formate buffer at a volume ratio of 27/73, and the flow rate was 1 mL/min. The detection wavelength was 240 nm for Dexa.

2.8. *In vitro* release test of Dexa from Dexa/Flt1 peptide–HA nanoparticles

In vitro release tests of Dexa from Dexa/Flt1 peptide–HA nanoparticle were performed over 24 h using a pre-washed dialysis tube (MWCO 10 kDa). The dialysis tube contained 2 mL of the Dexa/Flt1 peptide–HA nanoparticle aqueous solution with 22 μ g Dexa or the equivalent amount of water soluble Dexa (Dexa 21-phosphate disodium salt) aqueous solution. Then, the dialysis tubes were incubated in 10 mL of PBS at pH 7.4 and 37 °C for a day. At pre-determined time intervals, samples of the incubation medium were collected and the whole medium was replaced with 10 mL of fresh PBS. The amount of Dexa in the collected samples was quantified by HPLC as described above. Three replicates were performed for the *in vitro* release tests.

2.9. *In vitro* VEGFR binding test of Dexa/Flt1 peptide–HA nanoparticles

VEGF₁₆₅ was dissolved in PBS at a concentration of 0.5 μ g/mL and the solution was coated on each well of a 96-black well-plate at 4 °C overnight. After washing the plate with PBS, the wells were blocked with 3 wt% bovine serum albumin (BSA) blocking buffer at room temperature for 2 h and washed with PBS again. Three kinds of samples, Flt1 peptide, Flt1 peptide–HA conjugate nanoparticles, Dexa/Flt1 peptide–HA nanoparticles containing 20–20,000 nm of Flt1 peptide were added to the wells with Flt1-Fc in 1 wt% BSA solution and incubated at room temperature for 1 h. After washing thrice with PBS containing 0.05% Tween 20, anti-human IgG–HRP–Fc in 0.3 wt% BSA solution was added and incubated at room temperature for 1 h. After washing thrice with PBS

containing 0.05% Tween 20 and once with PBS, the amount of bound Flt1-Fc was quantified with a BM chemiluminescence ELISA substrate system (Victor 3 luminometer, Perkin Elmer, MA). ELISA was performed twice with three replicates.

2.10. *In vitro* biological activity test of Dexa/Flt1 peptide–HA nanoparticles

The effect of Dexa/Flt1 peptide–HA nanoparticles was evaluated on the mediator release from LPS-stimulated epithelial cells and macrophages. A human lung epithelial cell line (A549) was treated with Dexa/Flt1 peptide–HA nanoparticles for 1 h. After removal of the supernatant, human interleukin-6 (hIL-6) level was measured by ELISA 3 h and 6 h after 10 μ g/mL LPS stimulation. A mouse alveolar macrophage cell line (MH-S) was treated with Dexa/Flt1 peptide–HA nanoparticles for 24 h in the presence of 10 μ g/mL LPS. Then, IL-6 and tumor necrosis factor-alpha (TNF- α) levels were measured by ELISA.

2.11. *In vivo* evaluation of Dexa/Flt1 peptide–HA nanoparticles in asthma model mice

2.11.1. Preparation of neutrophilic and eosinophilic asthma model mice for pharmacologic intervention

Neutrophilic asthma model (high-dose LPS model) mice characterized by Th1 and Th17 cell responses were prepared as described elsewhere [8]. Briefly, 6-week-old mice were sensitized intranasally with 75 μ g LPS-depleted OVA plus 10 μ g LPS at days 0, 1, 2, and 7, and then challenged with 50 μ g of OVA alone at days 14, 15, 21, and 22. The control group was sensitized with OVA and then challenged with PBS. Eosinophilic asthma model (Alum model) characterized by Th2 cell responses was also prepared as described previously [29]. Most part of the protocol was same with that for high-dose LPS induced neutrophilic asthma model, but eosinophilic asthma model was prepared by using Alum instead of LPS. The mice were intraperitoneally sensitized with 75 μ g of LPS-depleted OVA plus 2 mg Alum at days 0 and 7, and then intranasally challenged with 50 μ g OVA alone at days 14, 15, 21, and 22.

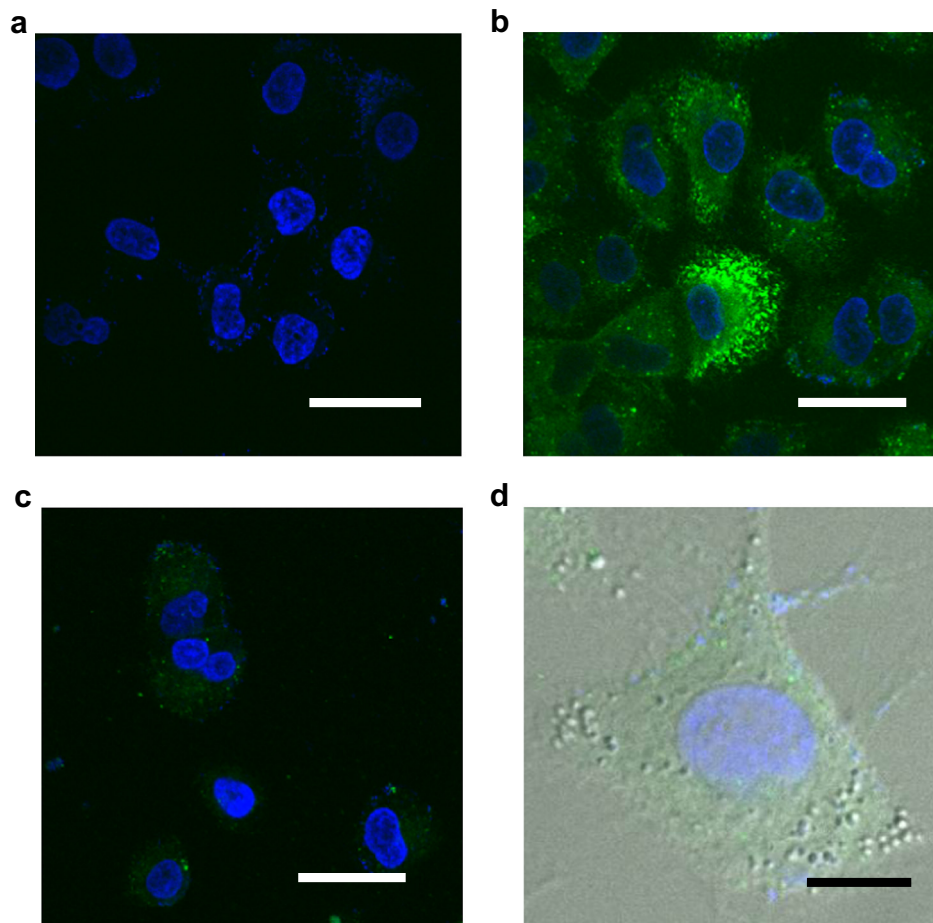


Fig. 2. Confocal microscopic images of A549 cells treated with (a) free medium, (b) FITC- labeled Flt1 peptide – HA nanoparticles, (c) FITC- labeled Flt1 peptide – HA nanoparticles after pre-incubation with HA, and (d) a magnified image of (b). White bars correspond to 30 μ m and black bar does 10 μ m.

2.11.2. Cellularity in bronchoalveolar lavage fluids

Bronchoalveolar lavage (BAL) cellularity for the evaluation of lung inflammation was analyzed as described elsewhere [8]. Briefly, BAL cells were stained with Diff-Quik and 300 inflammatory cells were counted for the analysis. Inflammatory cells were classified as macrophages, lymphocytes, neutrophils, and eosinophils.

2.11.3. Lung tissue histology

We entrusted lung tissues to Asan Medical Center (Seoul, Republic of Korea) and received histologic sample slides for the analysis. All histologic sample slides were compared at the same magnification ($\times 100$). Lung inflammation was measured by assessing the degree of peribronchiolar and perivascular inflammation [8].

2.12. Statistical analysis

The data are expressed as means $\pm$ standard deviation from several separate experiments. Statistical analysis was carried out via the two-way analysis of variance

(ANOVA) test using the software of SigmaPlot10.0. A value for $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation and fluorescent labeling of Flt1 peptide–HA conjugate nanoparticles

Although Flt1 hexa-peptide of GNQWFI showed an excellent biological activity as an antagonist for VEGFR1 [14], the use of Flt1 peptide has been limited due to its poor water solubility. To overcome the inherent limitation for further application, Flt1 peptide was conjugated to HA-TBA in DMSO by the amide bond formation between carboxyl groups of HA and N-terminal α -amine groups

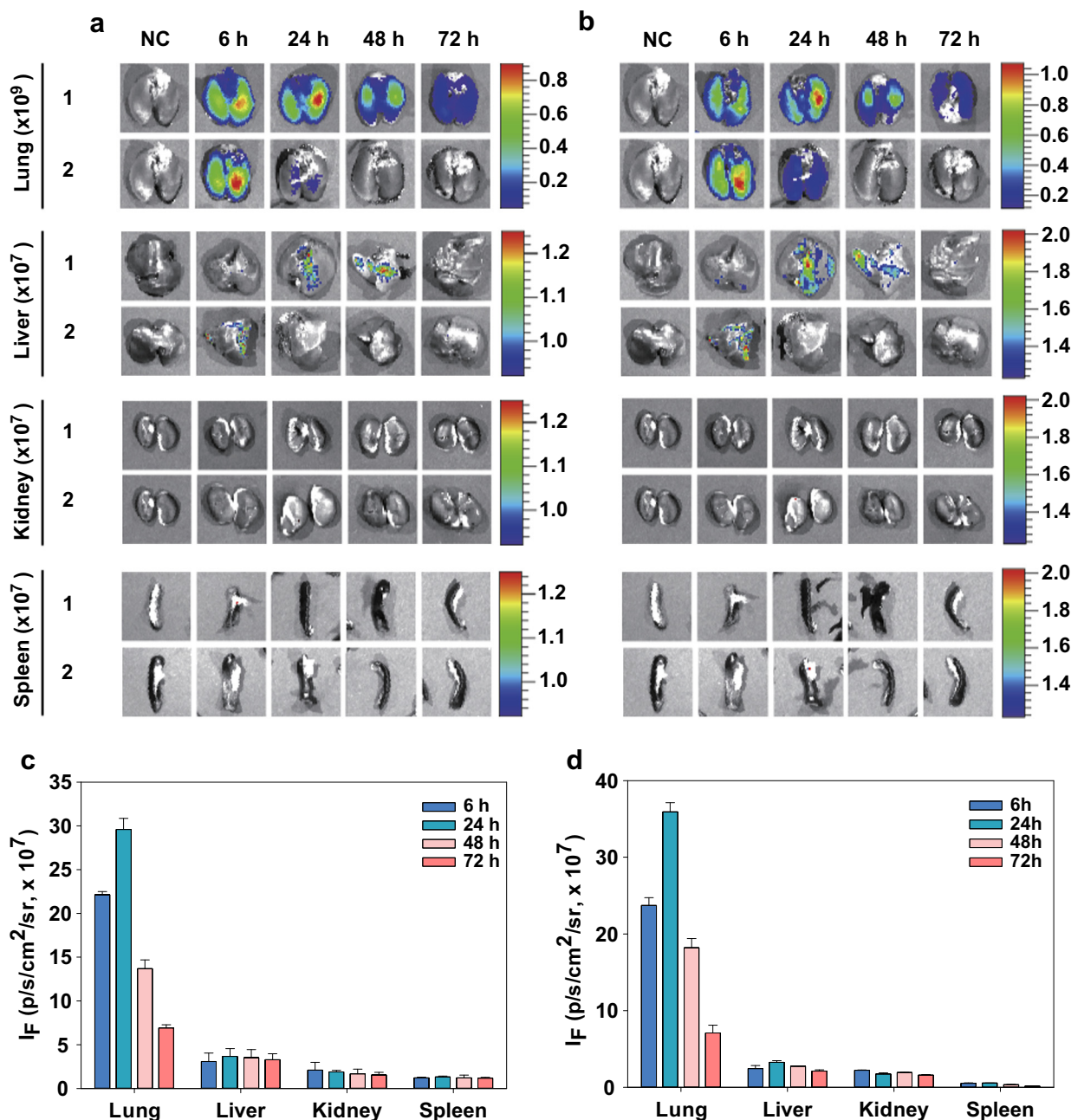


Fig. 3. Ex vivo imaging for the biodistribution of fluorescent dyes after orotracheal instillation of CFSE/Flt1 peptide – HA – HiLyte 647 nanoparticles (row 1) and dye mixtures (HiLyte 647 + CFSE) (row 2) under (a) a red filter for HiLyte 647 and (b) a green filter for CFSE. The fluorescence signal intensity presented with pseudo-colors was quantified in the region of interest (ROI) for (c) red and (d) green fluorescences.

of Flt1 peptide. The successful synthesis and self-assembly of Flt1 peptide–HA conjugates were confirmed in our previous work [30]. The formation of nanoparticles in aqueous solution could be attributed to the hydrophobic interaction between Flt1 peptide molecules conjugated to hydrophilic HA chain. The Flt1 peptide–HA conjugate nanoparticles have shown improved therapeutic effect on various ocular neovascularization diseases with the loading of hydrophobic drugs [31]. For *ex vivo* tracking of inhaled Flt1 peptide–HA conjugate nanoparticles, the nanoparticles were decorated with two kinds of fluorescent dyes. To examine the biodistribution of Flt1 peptide–HA conjugate nanoparticles, HiLyte 647 amine was conjugated to Flt1 peptide–HA conjugate. Ten percent of carboxyl groups in HA were biochemically conjugated with Flt1 peptide and some of residual carboxyl groups were labeled with HiLyte 647 amine by the amide bond formation using EDC chemistry as shown in Fig. 1a. The aqueous solution of Flt1 peptide–HA–HiLyte 647 nanoparticles was mixed with CFSE

dissolved in DMSO with ultrasonication. CFSE was loaded into Flt1 peptide–HA–HiLyte 647 nanoparticles by the hydrophobic interaction between CFSE molecules and Flt1 peptides (Fig. 1b). The concentrations of fluorescent dyes decorated to Flt1 peptide–HA conjugate nanoparticles were determined by measuring the absorbance at 650 nm for HiLyte 647 and at 490 nm for CFSE. CFSE is colorless and non-fluorescent until the acetate groups are removed by intracellular esterase to yield highly fluorescent carboxyfluorescein succinimidyl ester [32]. After esterase treatment overnight, we could observe the fluorescence of CFSE in the nanoparticles.

3.2. Bioimaging of Flt1 peptide–HA conjugate nanoparticles

Before *ex vivo* fluorescence imaging for the biodistribution of Flt1 peptide–HA conjugate nanoparticles, *in vitro* cellular uptake of the nanoparticles was bioimaged in human lung epithelial cells. As

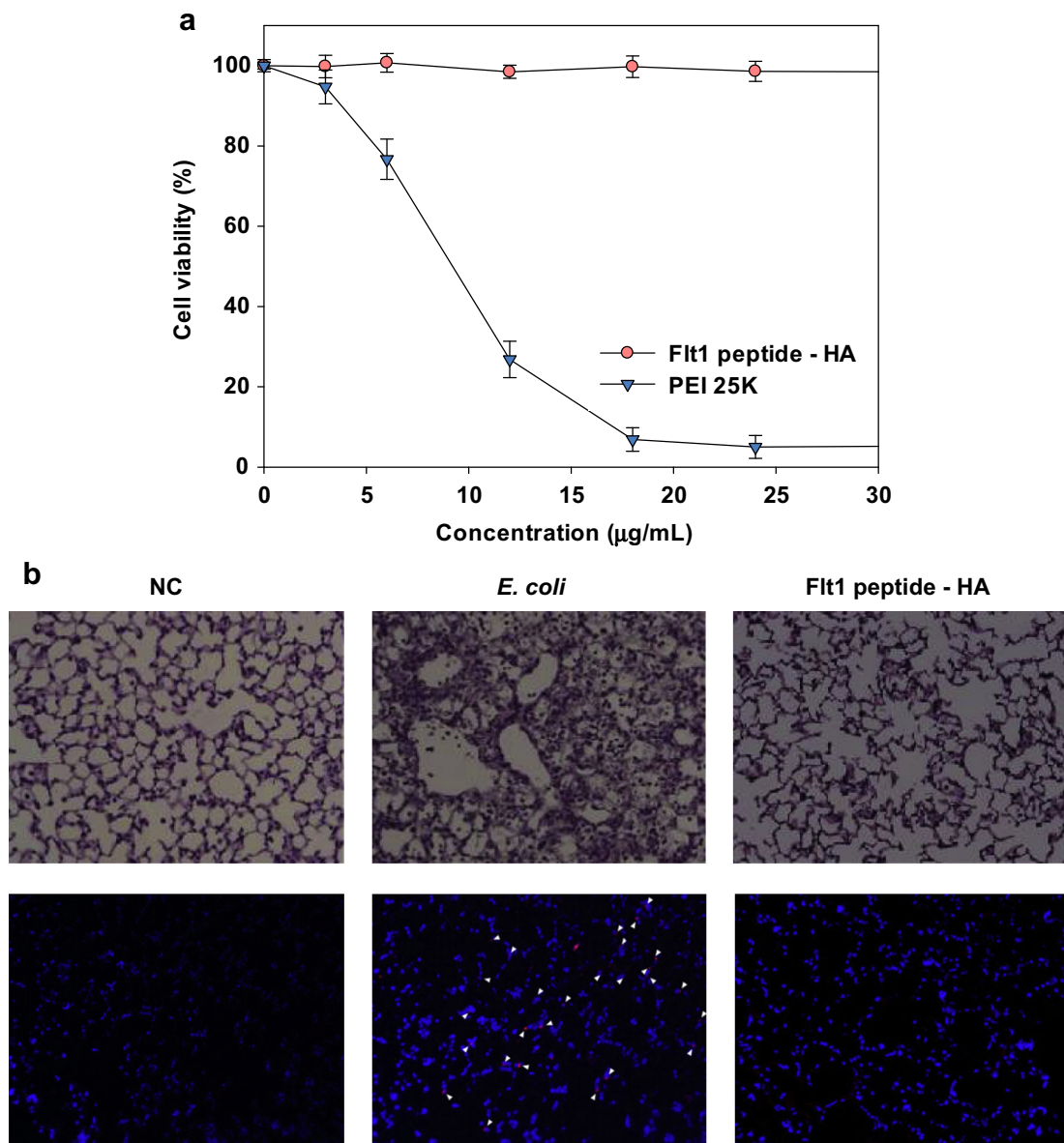


Fig. 4. (a) *In vitro* cytotoxicity of Flt1 peptide–HA conjugate nanoparticles assessed by the viability of A549 cells 24 h after treatment with Flt1 peptide–HA conjugate nanoparticles and PEI 25K as a control. (b) *In vivo* cytotoxicity of Flt1 peptide–HA conjugate nanoparticles by histological analysis after H&E staining (top) and ED1 staining (bottom) of the lung sections in comparison with *E. coli*-treatment. White arrows indicate red dots stained by ED1 antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shown in Fig. 2, FITC-labeled Flt1 peptide–HA nanoparticles were evenly distributed in the cytosol of A549 cells revealing the effective cellular uptake of the nanoparticles. A549 cells are well known to have HA receptors like CD44 on their surfaces [33]. When A549 cells were preincubated with the excess amount of free HA to block HA receptors on the cell surface, cellular uptake of the nanoparticles was significantly reduced due to the competitive binding of HA to the receptors, reflecting the HA receptor-mediated endocytosis (Fig. 2c). The results confirmed the feasibility of Flt1 peptide–HA conjugate nanoparticles for the efficient intracellular drug delivery to HA receptor positive cells by the receptor-mediated endocytosis.

Ex vivo fluorescence imaging was carried out for the bio-distribution of CFSE/Flt1 peptide–HA–HiLyte 647 nanoparticles after their pulmonary delivery. Fig. 3 shows the fluorescence images of dissected organs from ICR mice treated with HiLyte 647, CFSE, and CFSE/Flt1 peptide–HA–HiLyte 647 nanoparticles under red and green filters. Both Flt1 peptide–HA conjugate nanoparticles (Fig. 3a) and encapsulated hydrophobic molecules (Fig. 3b) were more significantly delivered to the lung than free fluorescent dyes, and detected for up to 72 h post-treatment. The fluorescence intensities of dissected organs were quantified by the ROI method (Fig. 3c–d). The maximum accumulation was observed in 24 h for CFSE/Flt1 peptide–HA–HiLyte 647 nanoparticles and only tiny amount of the nanoparticles was delivered to the liver

systemically. In the case of free fluorescent dyes, the maximum accumulation was observed in 6 h and the dyes completely disappeared within 24 h. As delineated above, Flt1 peptide–HA conjugate nanoparticles appeared to be internalized into lung epithelial cells by HA receptor-mediated endocytosis. Once the nanoparticles and loaded hydrophobic drugs were delivered to the lung, the amount of drugs systemically delivered to the liver was reduced significantly. In addition, HA might prolong the residence time of the nanoparticles in the lung due to its mucoadhesivity [20]. The efficient local delivery and prolonged residence of Dexa/Flt1 peptide–HA conjugate nanoparticles can reduce the necessary dose of Dexa and alleviate the adverse effect of systemic delivery of Dexa such as adrenal suppression, bone loss, hypertension and hyperglycemia.

3.3. Cytotoxicity of Flt1 peptide–HA conjugate nanoparticles

In vitro cytotoxicity of Flt1 peptide–HA conjugate nanoparticles was assessed by cell proliferation assay in A549 cells with HA receptors for further therapeutic applications. Fig. 4a shows the cell viability 24 h after incubation with the nanoparticles and 25 kDa PEI. PEI was used as a control exhibiting a dose-dependent cytotoxicity. Flt1 peptide–HA conjugate nanoparticles showed a cell viability over 98% up to the concentration of 20 $\mu\text{g/mL}$, whereas PEI exhibited a cell viability lower than 10% at the

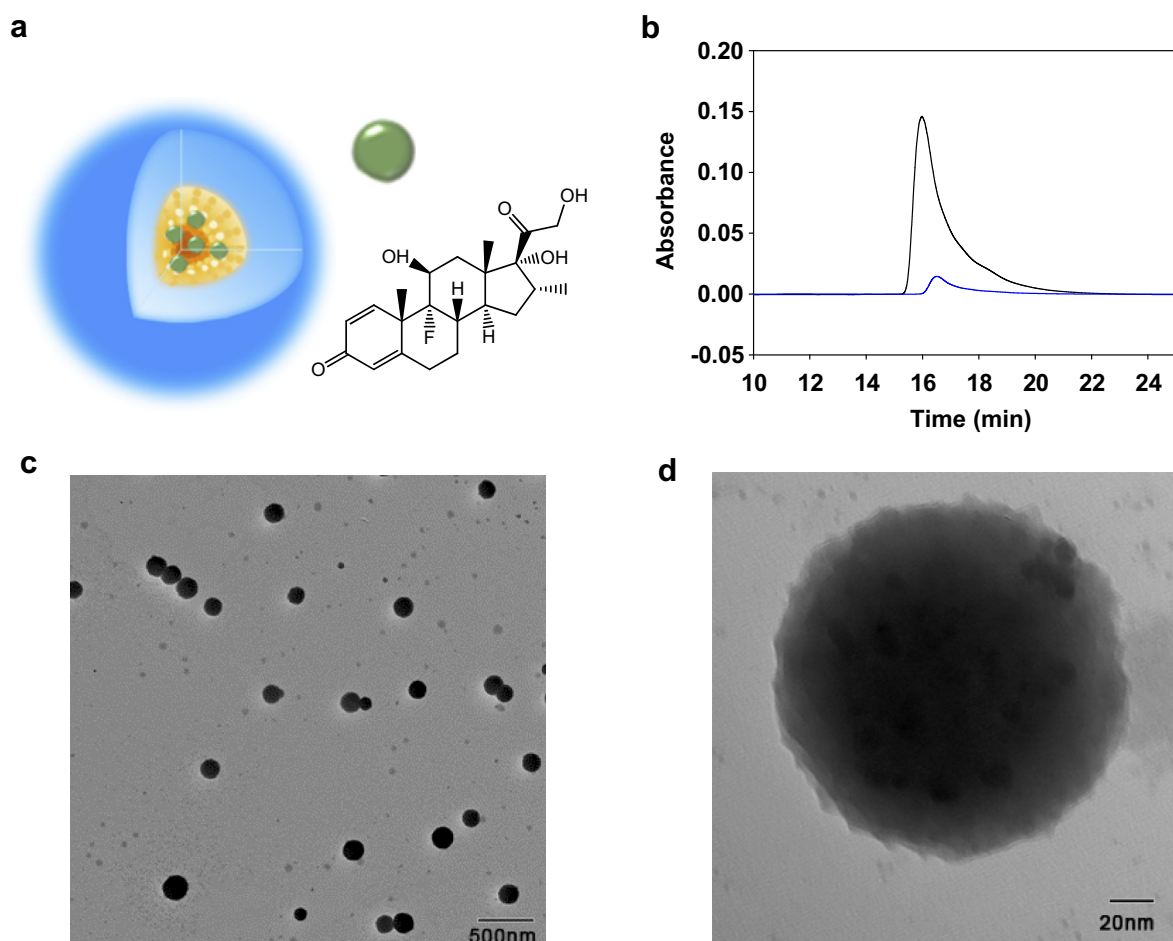


Fig. 5. (a) Schematic representation for dexamethasone (Dexa) loaded Flt1 peptide–HA nanoparticles. (b) High performance liquid chromatograms (HPLCs) showing the absorbance of Dexa at 240 nm before (black line) and after (blue line) drug loading to Flt1 peptide–HA conjugate nanoparticles. (c) Transmission electron microscopic (TEM) image and (d) magnified TEM image of Dexa/Flt1 peptide–HA nanoparticles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration of PEI higher than 20 $\mu\text{g/mL}$ (Fig. 4a). According to the further analysis, Flt1 peptide–HA conjugate nanoparticles showed a cell viability over 95% up to the concentration of 600 $\mu\text{g/mL}$. *In vivo* cytotoxicity of the Flt1 peptide–HA conjugate nanoparticles was also evaluated as shown in Fig. 4b. The upper row of Fig. 4b shows representative photomicrographs of pulmonary parenchyma 24 h after orotracheal instillation of *E. coli* strain E69 and 3 mg/head nanoparticles. The nanoparticle dose was ten times of the therapeutic dose of 300 $\mu\text{g/head}$. In a positive control group treated with *E. coli* strain E69, severe congestion and infiltration of inflammatory cells such as macrophages and neutrophils were observed. In contrast, there was no significantly higher inflammatory cell infiltration in the lung parenchyma treated with Flt1 peptide–HA conjugate nanoparticles. To visualize the macrophages, anti-CD68 (ED1) antibody staining was performed as shown in the lower row of Fig. 4b. ED1 is a general marker of macrophages. While lung parenchyma treated with *E. coli* revealed a significant increase in ED1 levels, ED1 staining of lung parenchyma treated with the nanoparticles didn't show any visible red fluorescent dots, showing the similar ED1 level with the normal

control group. The results clearly confirmed the remarkable safety of Flt1 peptide–HA conjugate nanoparticles as a pulmonary drug delivery system.

3.4. Preparation of Dexa/Flt1 peptide–HA nanoparticles

On the basis of the results in the previous sections, Flt1 peptide–HA conjugate nanoparticles were exploited for pulmonary therapeutic applications. Herein, Dexa, one of the glucocorticoid drugs, was encapsulated in the Flt1 peptide–HA conjugate nanoparticles for the treatment of asthma. Glucocorticoid drugs like Dexa exhibit excellent therapeutic effects on eosinophilic asthma characterized by Th2 immune responses. In contrast, it has poor therapeutic effect on neutrophilic asthma due to the steroid resistance. Meanwhile, Flt1 peptide was reported as a key mediator regulating neutrophilic inflammation and showed significant therapeutic effect on neutrophilic asthma characterized by Th1 immune responses. Accordingly, it was expected that Dexa/Flt1 peptide–HA nanoparticles might show formidable therapeutic effects both in eosinophilic and

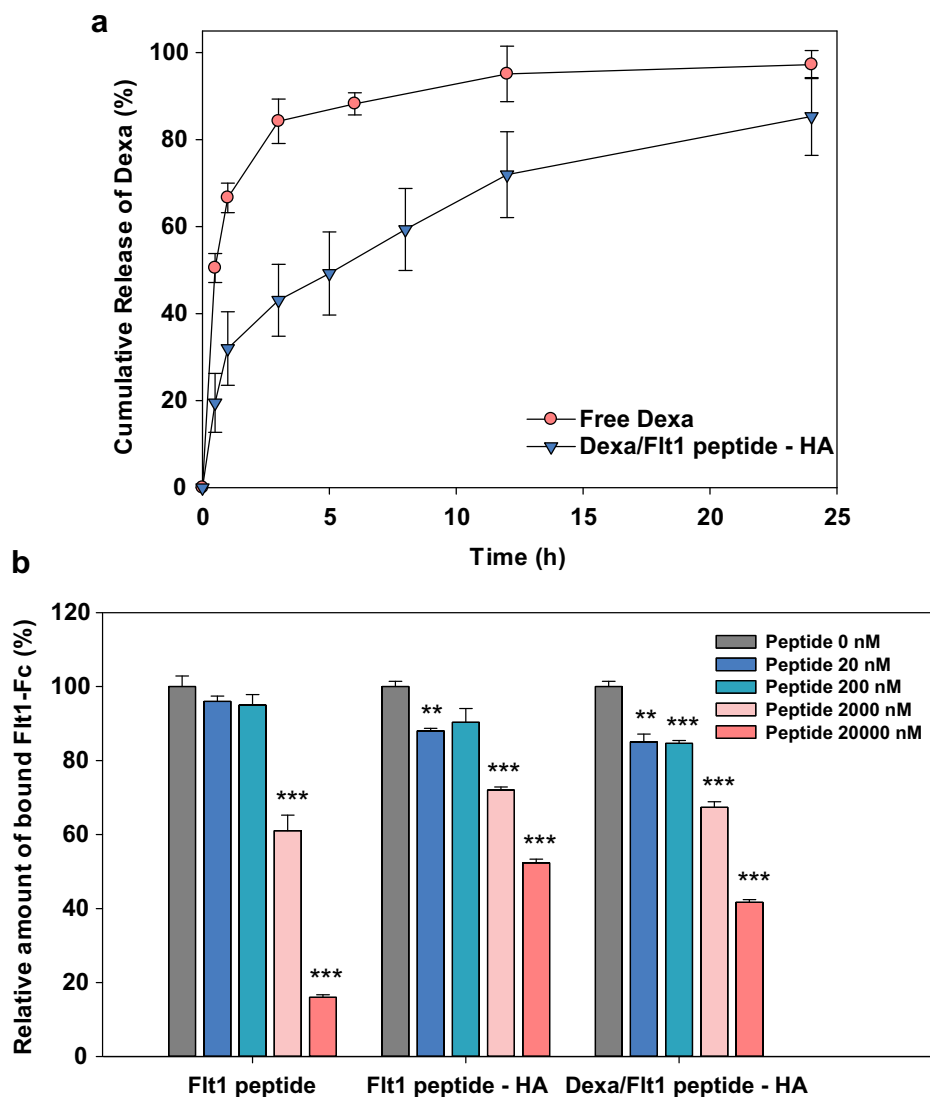


Fig. 6. (a) *In vitro* release profiles of dexamethasone (Dexa) from dialysis bags containing the aqueous solution of free Dexa and Dexa/Flt1 peptide–HA nanoparticles in PBS at pH 7.4 and 37 °C. Data are presented as means $\pm$ SD ($n = 3$). (b) *In vitro* biological activity of Flt1 peptide, Flt1 peptide–HA conjugate nanoparticles, and Dexa/Flt1 peptide–HA nanoparticles inhibiting the binding of Flt1-Fc to VEGF₁₆₅ coated on the well with increasing peptide concentrations. Data are presented as means $\pm$ S.D. ** $P < 0.01$ and *** $P < 0.005$ versus the non-treated control group ($n = 4$).

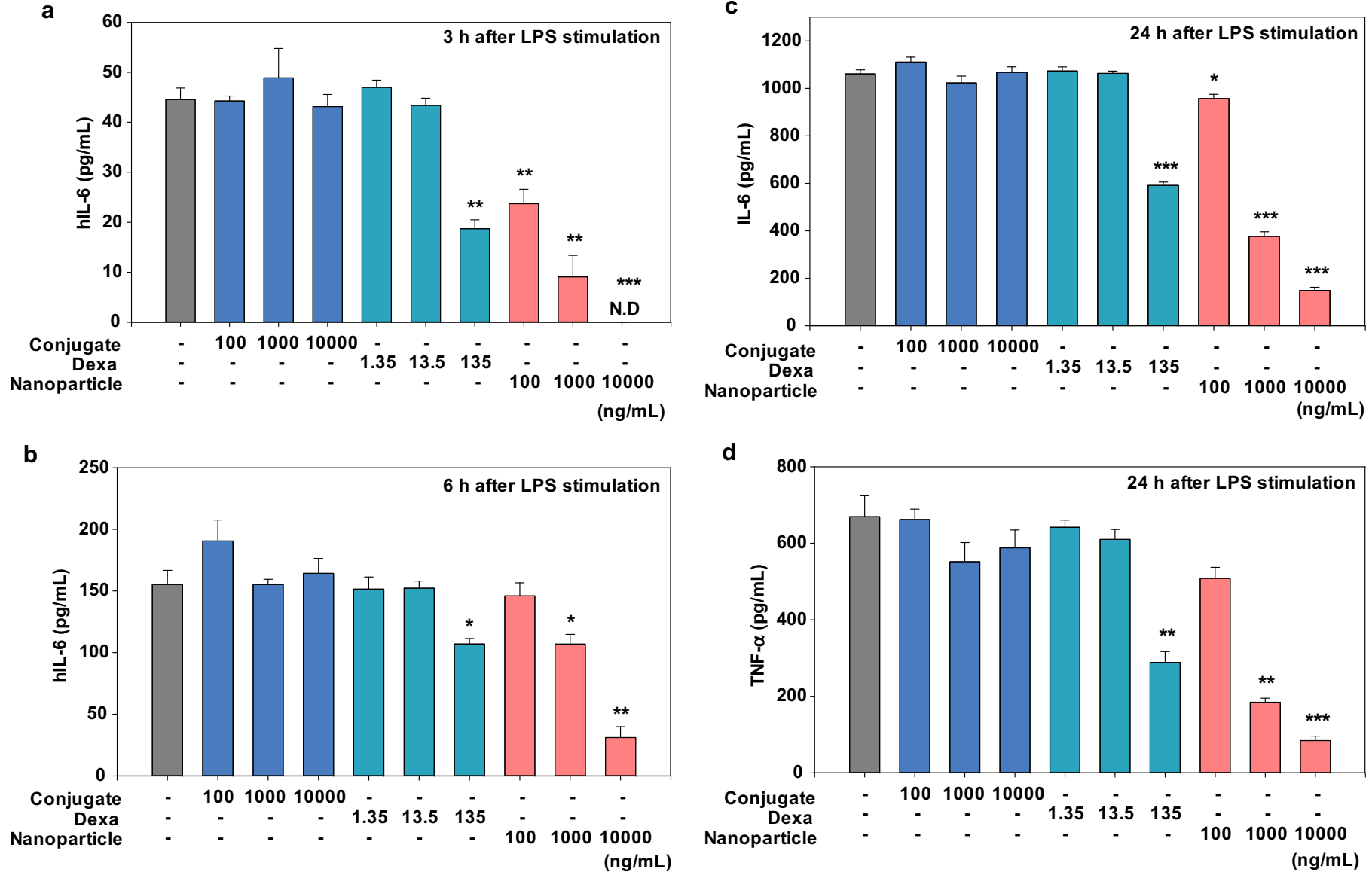


Fig. 7. Effect of Flt1 peptide–HA conjugate (conjugate), DEXA, and DEXA/Flt1 peptide–HA nanoparticles (nanoparticles) on the release of pro-inflammatory cytokines from alveolar macrophages and lung epithelial cells. Cytokine levels of human interleukin-6 (hIL-6) (a) 3 h and (b) 6 h after treatment of LPS-stimulated human lung epithelial cells with the samples. Cytokine levels of (c) IL-6 and (d) tumor necrosis factor alpha (TNF- α) 24 h after treatment of mouse alveolar macrophage (MH-S) cells with the samples and LPS. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ versus PBS plus LPS group.

neutrophilic asthma models with their synergistic pharmacodynamic interactions. The Dexa/Flt1 peptide–HA nanoparticles were prepared by mixing of aqueous solution of Flt1 peptide–HA conjugate nanoparticles and Dexa solution in ethanol with ultrasonication. After solvent exchange and removal of unloaded Dexa by dialysis, the nanoparticles were constructed via hydrophobic interactions between Flt1 peptides and Dexa molecules as schematically shown in Fig. 5a. The loaded amount of Dexa within the nanoparticles was quantified by HPLC analysis with C18 column (Fig. 5b). Approximately 10% of the added Dexa was loaded in the nanoparticles (exactly 12.0% in Fig. 5b). The formation of the polymeric nanoparticles was confirmed by TEM (Fig. 5c). In a magnified TEM image of Fig. 5d, small drug particles

embedded in polymeric nanoparticles were observed confirming the encapsulation of Dexa molecules. The average diameter of Dexa/Flt1 peptide–HA nanoparticles was 110.2 ± 17.4 nm by DLS, which was in accordance with that in TEM images. The nanoparticles had a negative charge with a zeta potential around -20 mV due to the negatively charged HA on the surface.

3.5. *In vitro* VEGFR binding of Dexa/Flt1 peptide–HA nanoparticles

Before evaluating *in vitro* biological activity of the nanoparticles, *in vitro* release tests of Dexa were performed in PBS (pH 7.4) at 37°C with dialysis bags containing the aqueous solutions of water soluble Dexa and the Dexa/Flt1 peptide–HA

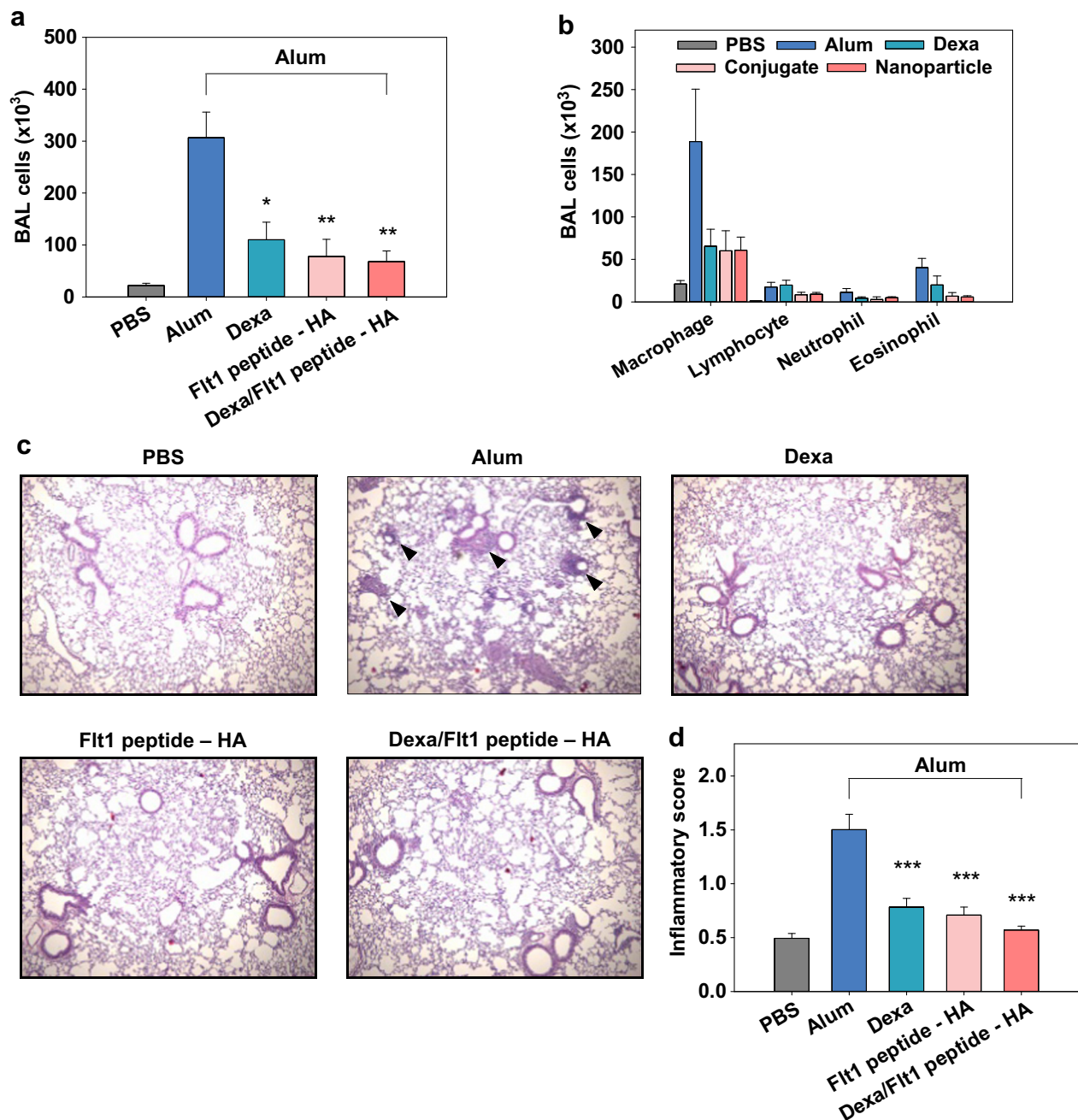


Fig. 8. Anti-inflammatory effect of Dexa, Flt1 peptide–HA conjugate (conjugate), and Dexa/Flt1 peptide–HA nanoparticles (nanoparticles) in eosinophilic asthma model mice. (a, b) BAL cellularity 48 h after the last OVA challenge. (c) Histological images of the lung with H&E staining (original magnification $\times 100$) and (d) the inflammatory scores. Black arrows indicate the regions of inflammatory cell infiltration. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ versus PBS plus Alum group.

nanoparticles in PBS. Fig. 6a shows the cumulative amount of Dexa released from Dexa/Flt1 peptide–HA nanoparticles compared with free Dexa. The half of the loaded Dexa was released from the nanoparticles over the first 6 h by an initial burst release and the residual Dexa was released continuously over the following 18 h. Then, *in vitro* dose-dependent biological activity of Flt1 peptide–HA conjugate nanoparticles was assessed by monitoring the inhibition effect on the binding of Flt1-Fc to VEGF₁₆₅ coated on the well. Flt1 peptide is an antagonistic peptide for VEGFR1 (Flt1), interrupting the interaction of VEGFR1 with other VEGFR1 ligands like VEGF₁₆₅ [14]. After treatment of the mixed solution of Flt1 peptide and Flt1-Fc, unbound Flt1-Fc was removed and the remaining Flt1-Fc bound to VEGF₁₆₅ was

quantified by ELISA. The luminescence value without any treatment was set to be 100% and the other data were normalized in comparison to the value. Flt1 peptide reduced the binding of Flt1-Fc to VEGF₁₆₅ in a dose-dependent manner at the concentrations above 200 nM. Flt1 peptide–HA conjugate nanoparticles with or without loading of Dexa showed less efficient inhibitory effect than pristine Flt1 peptide due to the steric hindrance. However, they still interacted with VEGFR1 and reduced the binding of Flt1-Fc to VEGF₁₆₅ even after formation of the nanoparticles. The results clearly confirmed that Flt1 peptides maintained their biological binding affinity to VEGFR1 and regulated the VEGFR1-mediated interactions even after formation of the Flt1 peptide–HA conjugate nanoparticles.

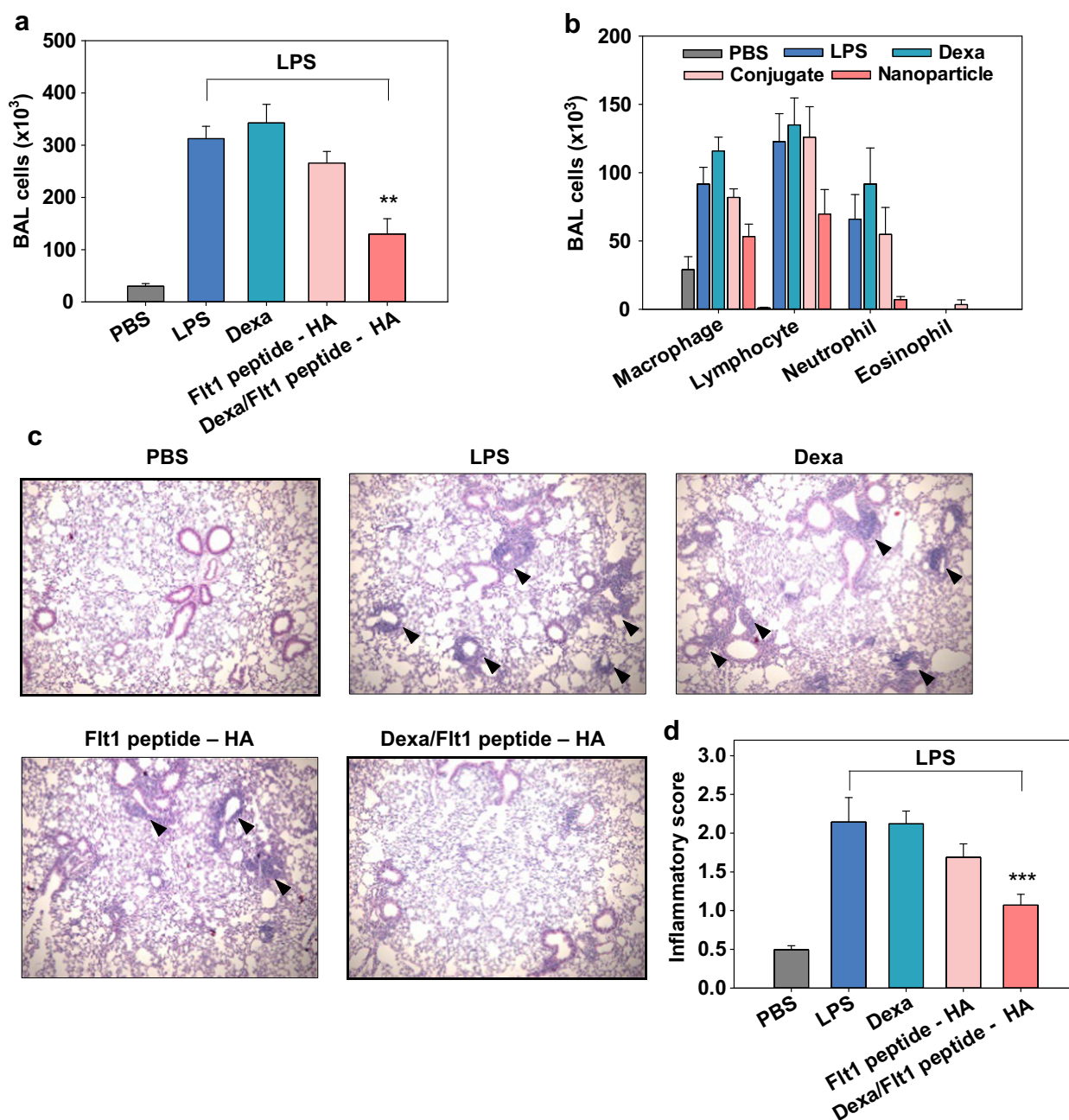


Fig. 9. Anti-inflammatory effect of Dexa, Flt1 peptide–HA conjugate (conjugate), and Dexa/Flt1 peptide–HA nanoparticles (nanoparticles) in neutrophilic asthma model mice. (a, b) BAL cellularity 48 h after the last OVA challenge. (c) Histological images of the lung with H&E staining (original magnification $\times 100$) and (d) the inflammatory scores. Black arrows indicate the regions of inflammatory cell infiltration. ** $P < 0.01$ and *** $P < 0.005$ versus PBS plus LPS group.

3.6. *In vitro* biological activity of Dexa/Flt1 peptide–HA nanoparticles

The first defense line for pathogenic particles and molecules is airway epithelial cells and alveolar macrophages. We used a human lung epithelial cell line of A549 cells and a mouse alveolar macrophage cell line of MH-S cells to evaluate the efficacy of Dexa/Flt1 peptide–HA nanoparticles in pro-inflammatory condition induced by LPS. As reported elsewhere [33,34], HA receptors such as CD44 are highly expressed in both lung epithelial cells and alveolar macrophages. To investigate the release and action of Dexa loaded in the nanoparticles, A549 cells were treated with Dexa, Flt1 peptide–HA conjugate nanoparticles, and Dexa/Flt1 peptide–HA nanoparticles at the same dose for 1 h. After stimulation with 10 ng/mL LPS for 3 and 6 h, the secretion of hIL-6 increased in the A549 cells (Fig. 7a and b). Interestingly, the pro-inflammatory cytokine release after 3 h and 6 h was reduced in both groups treated with Dexa and Dexa/Flt1 peptide–HA nanoparticles in a dose-dependent manner. The efficacy of Dexa/Flt1 peptide–HA nanoparticles was much higher and longer than free Dexa. The results revealed that Dexa/Flt1 peptide–HA nanoparticles could be internalized to the cells, leading to the slow release of Dexa and the reduction of pro-inflammatory mediator like IL-6. Then, the anti-inflammatory efficacy of Dexa/Flt1 peptide–HA nanoparticles was evaluated in MH-S cells after treatment with the samples and LPS together for 24 h. The treatment of MH-S cells with Dexa and Dexa/Flt1 peptide–HA nanoparticles resulted in the dose-dependently decreased levels of pro-inflammatory cytokines such as IL-6 and TNF- α (Fig. 7c and d). From the results, Dexa/Flt1 peptide–HA nanoparticles appeared to be more effective than free Dexa to alleviate airway inflammation in both the human lung epithelial cells and the mouse alveolar macrophage cells.

3.7. *In vivo* efficacy of Dexa/Flt1 peptide–HA nanoparticles in asthma model mice

Previously, we prepared a mouse model of eosinophilic asthma gathering Th2 response by the intraperitoneal sensitization with OVA together with Alum [29]. After intraperitoneal sensitization with OVA in Alum and intranasal challenges with OVA, the mice showed eosinophilic inflammation with increasing BAL eosinophils and macrophages. Remarkably, the treatment groups with both Flt1 peptide–HA conjugate nanoparticles and Dexa/Flt1 peptide–HA nanoparticles showed significantly decreased BAL cellularity including macrophages and eosinophils in OVA/Alum induced eosinophilic inflammation model mice (Fig. 8a and b). Histological analysis and inflammatory scores also confirmed the therapeutic efficacy of Dexa, Flt1 peptide–HA conjugate nanoparticles, and Dexa/Flt1 peptide–HA nanoparticles (Fig. 8c and d). The results indicate that Dexa conventionally used for the treatment of asthma is effective to alleviate eosinophilic asthma [5]. However, Dexa was not effective to BAL cellularity in the neutrophilic asthma model mice induced by repeated inhalation of LPS-containing allergens (Fig. 9a and b). In contrast, Dexa/Flt1 peptide–HA nanoparticles resulted in the significantly reduced cellularity including macrophages and neutrophils. Although neutrophilic asthma model mice showed the steroid resistance and neutrophilic airway inflammation, Dexa/Flt1 peptide–HA nanoparticles alleviated neutrophilic pulmonary inflammation. Histological analysis also showed the relief of lung inflammation by both Flt1 peptide–HA conjugate nanoparticles and Dexa/Flt1 peptide–HA nanoparticles (Fig. 9c and d). Taken together, Dexa/Flt1 peptide–HA nanoparticles appeared to have a significant therapeutic effect on both eosinophilic and neutrophilic subtypes of asthma, showing the feasibility for the

treatment of corticosteroid-sensitive and corticosteroid-resistant asthma.

4. Conclusions

We successfully developed Flt1 peptide–HA conjugate nanoparticles for pulmonary drug delivery applications. *In vitro* and *ex vivo* bioimaging revealed that Flt1 peptide–HA conjugate nanoparticles were effectively delivered and up-taken to the lung tissues by HA receptor mediated endocytosis with a prolonged residence time. *In vitro* and *in vivo* cytotoxicity were negligible confirming the feasibility of Flt1 peptide–HA conjugate nanoparticles for further therapeutic applications. Dexa could be loaded into the Flt1 peptide–HA conjugate nanoparticles for the treatment of asthma. The formation of self-assembled nanoparticles was verified by HPLC, DLS, and TEM analyses. The Dexa loaded nanoparticles reduced the cytokine levels in LPS-stimulated cells more significantly than free Dexa. Furthermore, Dexa/Flt1 peptide–HA nanoparticle showed remarkable therapeutic effects on both eosinophilic and neutrophilic subtypes of asthma.

Acknowledgment

This work was financially supported by the Converging Research Center Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0081871 and 2011-0016474) and a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A080711). This study was also supported by Mid-career Researcher Program through NRF grant funded by the MEST (No. 2012R1A2A2A06045773).

References

- [1] Busse WW, Lemanske Jr RF. Asthma. *N Engl J Med* 2001;344:350–62.
- [2] Bochner BS, Undem BJ, Lichtenstein LM. Immunological aspects of allergic asthma. *Annu Rev Immunol* 1994;12:295–335.
- [3] Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* 2004;22:789–815.
- [4] Cho SH, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *Am J Respir Crit Care Med* 2005;171:224–30.
- [5] National Heart, Lung, and Blood Institute. Guidelines for the diagnosis and management of asthma (EPR-3). p. 214. Available from: URL: <http://www.nhlbi.nih.gov/guidelines/asthma/>; 2007.
- [6] Dominique MB, Els T, Liesbeth C, Ellen D, Peter WH, Lieven JD, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006;7:135–9.
- [7] Bryan CP, Alison LB, Alan PB, Matthew AS, Nicholas WL. Interleukin-25 induces type 2 cytokine production in a steroid-resistant interleukin-17RB+ myeloid population that exacerbates asthmatic pathology. *Nat Med* 2012;18:751–8.
- [8] Kim YK, Oh SY, Jeon SG, Park HW, Lee SY, Chun EY, et al. Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *J Immunol* 2007;178:5375–82.
- [9] Kim YS, Hong SW, Choi JP, Shin TS, Moon HG, Choi EJ, et al. Vascular endothelial growth factor is a key mediator in the development of T cell priming and its polarization to type 1 and type 17 T helper cells in the airways. *J Immunol* 2009;183:5113–20.
- [10] Chetta A, Zanini A, Foresi A, Donno MD, Castagnaro A, D'Ipollito R, et al. Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. *Am J Respir Crit Care Med* 2003;167:751–7.
- [11] Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. *Chest* 2005;127:965–72.
- [12] Chetta A, Zanini A, Foresi A, D'Ipollito R, Tipa A, Castagnaro A, et al. Vascular endothelial growth factor up-regulation and bronchial wall remodeling in asthma. *Clin Exp Allergy* 2005;35:1437–42.
- [13] Lee KS, Min KH, Kim SR, Park SJ, Park HS, Jin GJ, et al. Vascular endothelial growth factor modulates metalloproteinase-9 expression in asthma. *Am J Respir Crit Care Med* 2006;174:161–70.
- [14] Bae DG, Kim TD, Li G, Yoon WH, Chae CB. Anti-Flt1 peptide, a vascular endothelial growth factor receptor 1-specific hexapeptide, inhibits tumor growth and metastasis. *Clin Cancer Res* 2005;11:2651–61.

- [15] Kim YS, Choi SJ, Tae YM, Lee BJ, Jeon SG, Oh SY, et al. Distinct roles of vascular endothelial growth factor receptor-1- and receptor-2-mediated signaling in T cell priming and Th17 polarization to lipopolysaccharide-containing allergens in the lung. *J Immunol* 2010;185:5648–55.
- [16] Varghese OP, Sun W, Hilborn J, Ossipov DA. In situ cross-linkable high molecular weight hyaluronan-bisphosphonate conjugate for localized delivery and cell-specific targeting: a hydrogel linked prodrug approach. *J Am Chem Soc* 2009;131:8781–3.
- [17] Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, et al. Regulation of lung injury and repair by toll-like receptors and hyaluronan. *Nat Med* 2005;11:1173–9.
- [18] Al-Qadi S, Grenha A, Remunan-Lopez C. Microspheres loaded with polysaccharide nanoparticles for pulmonary delivery: preparation, structure and surface analysis. *Carbohydr Polym* 2011;86:25–34.
- [19] Surendrakumar K, Martyn GP, Hodgson ECM, Jansen M, Blair JA. Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs. *J Control Release* 2003;91:385–94.
- [20] Alpar HO, Somavarapu S, Atuah KN, Bramwell VW. Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. *Adv Drug Deliv Rev* 2005;57:411–30.
- [21] Hanania NA, Chapman KR, Kesten S. Adverse effects of inhaled corticosteroids. *Am J Med* 1995;98:196–208.
- [22] Ng PC. The effectiveness and side effects of dexamethasone in preterm infants with bronchopulmonary dysplasia. *Arch Dis Child* 1993;68:330–6.
- [23] Zhou B, Weigel JA, Fauss LA, Weigel PH. Identification of the hyaluronan receptor for endocytosis. *J Biol Chem* 2000;275:37733–41.
- [24] Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 1999;144:789–801.
- [25] Holgate ST, Lackie PM, Davies DE, Roche WR, Walls AF. The bronchial epithelium as a key regulator of airway inflammation and remodeling in asthma. *Clin Exp Allergy* 1999;29:90–5.
- [26] Oh EJ, Kim JW, Kong JH, Ryu SH, Hahn SK. Signal transduction of hyaluronic acid-peptide conjugate for formyl peptide receptor like 1 receptor. *Bioconjug Chem* 2008;19:2401–8.
- [27] Oh EJ, Park K, Choi JS, Joo CK, Hahn SK. Synthesis, characterization, and preliminary assessment of anti-Flt1 peptide – hyaluronate conjugate for the treatment of corneal neovascularization. *Biomaterials* 2009;30:6026–34.
- [28] Kim ES, Chang YS, Choi SJ, Kim JK, Yoo HS, Ahn SY, et al. Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells attenuates *Escherichia coli*-induced acute lung injury in mice. *Respir Res* 2011;12:108–18.
- [29] Jeon SG, Moon HG, Kim YS, Choi JP, Shin TS, Hong SW, et al. 15-Lipoxygenase metabolites play an important role in the development of a T-helper type 1 allergic inflammation induced by double-stranded RNA. *Clin Exp Allergy* 2009;39:908–17.
- [30] Oh EJ, Choi JS, Kim H, Joo CK, Hahn SK. Anti-Flt1 peptide-hyaluronate conjugate for the treatment of retinal neovascularization and diabetic retinopathy. *Biomaterials* 2011;32:3115–23.
- [31] Kim H, Choi JS, Kim KS, Yang JA, Joo CK, Hahn SK. Flt1 peptide – hyaluronate conjugate micelle-like nanoparticles encapsulating genistein for the treatment of ocular neovascularization. *Acta Biomater* 2012;8:3932–40.
- [32] Breeuwer P, Drocourt J, Rombouts FM, Abée T. A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-)carboxyfluorescein succinimidyl ester. *Appl Environ Microbiol* 1996;62:178–83.
- [33] Qhattal HSS, Liu X. Characterization of CD44-mediated cancer cell uptake and intracellular distribution of hyaluronan-grafted liposomes. *Mol Pharm* 2011;8:1233–46.
- [34] McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, et al. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest* 1996;98:2403–13.