Title: Targeting tumor associated macrophages with the immune-activating nanomedicine for achieving strong anti-tumor activity with rapid clearance from the body

Yosuke Ota[1#], Ryosaku Inagaki[1#], Yosuke Takanashi[2#], Hiro Uemachi[2], Kimiya Matsuda[1], Makoto Matsuoka[2], Risa Taoda[1], Seina Ohe[1], Yukari Ishitsubo[1], Megumi Nakamura[1], Masashi Goto[1], Hitoshi Ban[3], and Yasuhiro Nagai[1*]

[1]Cancer Research Unit, Sumitomo Pharma Co Ltd, Osaka 5540022 Japan
[2]Modality Research Unit, Sumitomo Pharma Co Ltd, Osaka 5540022 Japan
[3]Oncology, Sumitomo Pharma Co Ltd, Osaka 5540022 Japan

[#]Authors contributed equally.
[*]Correspondence to: Yasuhiro Nagai,
e-mail: yasuhiro1.nagai@sumitomo-pharma.co.jp

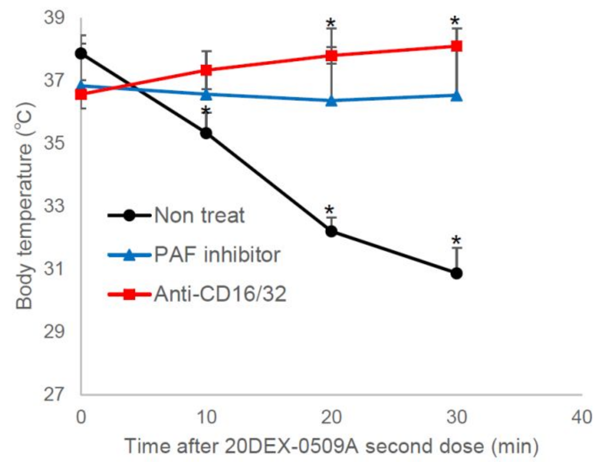


Figure S1. Body temperature changes of mice treated with 20DEX-0509A and PAF inhibitor WEB-2086 or anti-CD16/32. Mice were treated with each dextran (0.2 mpk as equivalent dose of DSP0509) at day 0 and day 7. PAF inhibitor was injected i.v. at 10 min and anti-CD16/32 at 1 day before the second injection. Statistical differences were evaluated by the parametric Dunnett test *$P<0.05$ vs 0 min.

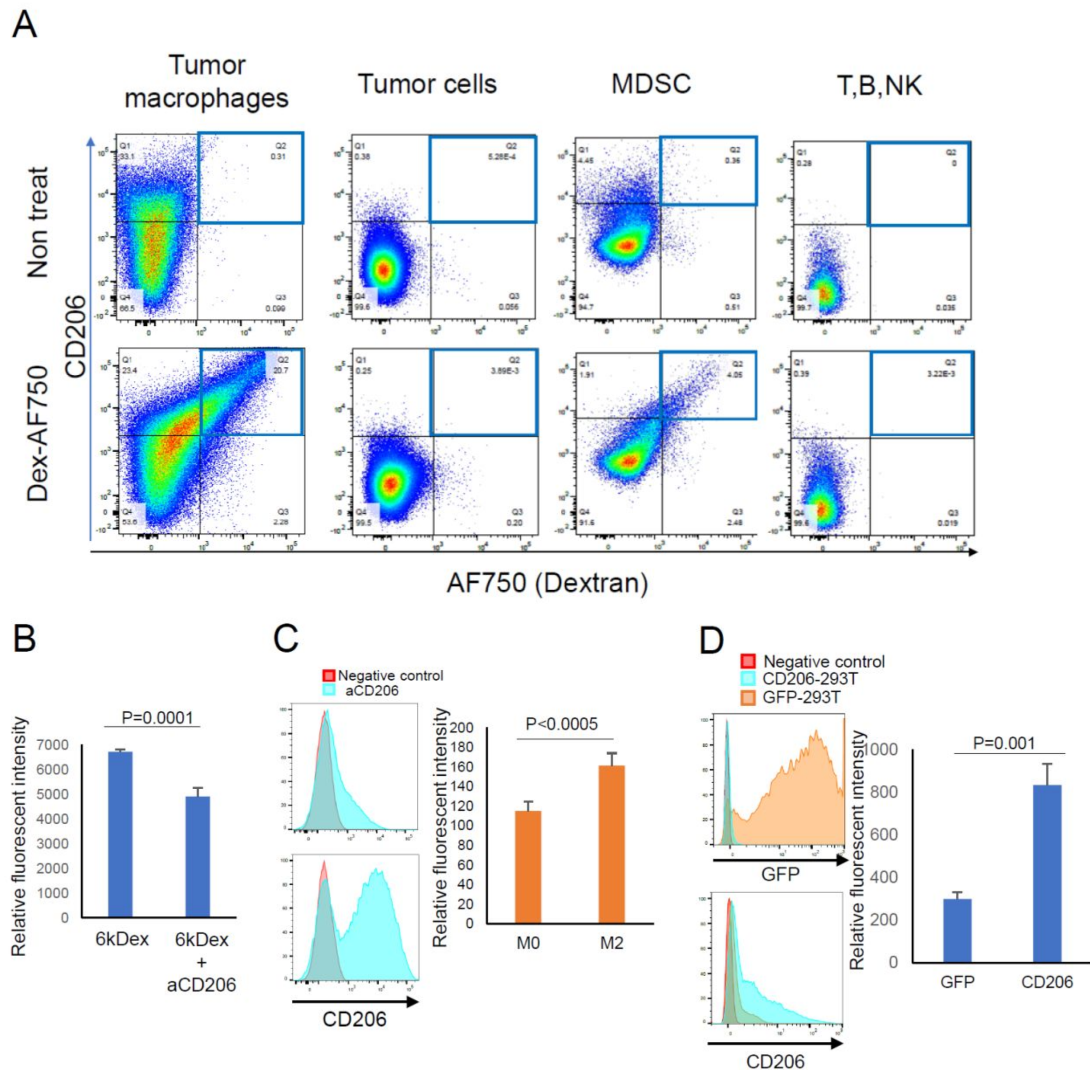


Figure S2. Cellular uptakes of 5 kDa dextran conjugated with AF750 at the reduced end. A. Mice were inoculated with EMT6 tumor, and the dextrans were injected i.v. at 1 mg/kg of AF750 (n=3). After 24 hr, tumors and spleens were dissected and dissociated into single cell suspensions, then analyzed with flow cytometry. Macrophages were categorized as CD45+ CD11b+ F4/80+ cells; MDSC as CD45+ CD11b+ Gr1high cells; other myeloid cells as CD45+ CD11b+ F4/80- cells, and T, B, NK, etc. cells as CD45+ CD11b-F4/80- cells. Data are representative of n=3 mice. **B**. Human monocyte-derived M2 macrophages were incubated with dextran-AF750 for 1hr, and their uptakes were analyzed with or without blocking anti-CD206 antibody (n=3). **C**. M2 type macrophages were differentiated from the M0 type macrophage cell line DH82, and their dextran uptakes were measured using dextran-AF750 (n=5). **D**. Human CD206 or GFP plasmid vectors were transfected into 293T cells, then their dextran uptakes were measured using dextran-AF750 (n=3). Data are shown as mean ± S.D. Statistical differences were evaluated by *t*-tests.

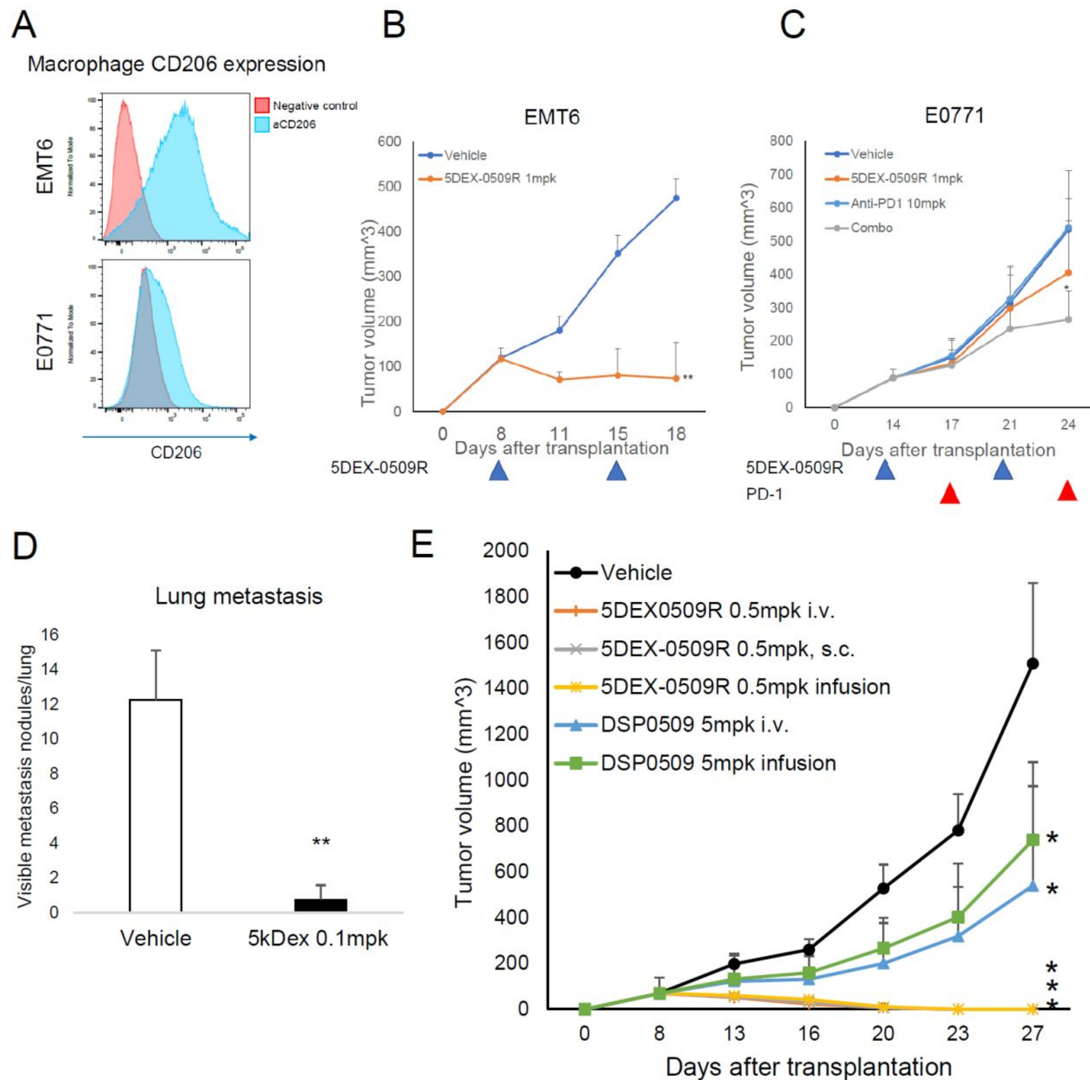


Figure S3. *In vivo* anti-tumor profiles of 5DEX-0509R. A, B. *In vivo* efficacy of EMT6 and E0771 triple-negative breast tumor models. **(A)** Tumor infiltrating macrophages were analyzed to show CD206 expression. **(B)** EMT6-bearing mice (n=6 per group) were injected intravenously once a week with 5DEX-0509R (0.1 mpk) for 2 weeks. Data show average tumor sizes in each of the groups. Data are shown as mean ± S.D. Statistical differences were evaluated by the parametric Dunnett test vs vehicle (PBS treatment, **P<0.01). **C.** E0771-bearing mice (n=6 per group) were treated with vehicle (PBS, i.v., once a week), anti-PD1 (10 mpk, i.p., twice a week), 5DEX-0509R (1 mpk as equivalent dose of DSP-0509, i.v., once a week) and combination of anti-PD-1 and 5DEX-0509R for 2 weeks. Data are shown as mean ± S.D. Statistical differences were evaluated by the parametric Dunnett test vs vehicle (PBS treatment) (*P<0.05). **D.** Lung metastasis of EMT6. Tumor-bearing mice were treated with vehicle (PBS) or 5DEX-0509R (0.1 mpk, i.v., once a week for 3 weeks). One week after final treatment, the lungs were dissected and metastatic nodules were counted using a stereo microscope (Leica). Data are shown as mean ± S.D. Statistical differences were evaluated by *t*-test (**P<0.01). **E.**

Anti-tumor effect of 5DEX-0509R injected via different routes. Colon26-bearing mice (n=5 per group) were injected once a week with DSP-0509 by the i.v. bolus and i.v. infusion (5 mpk) routes, or 5DEX-0509R (0.5 mpk as equivalent dose of DSP-0509) by the i.v. bolus, s.c. bolus. and i.v. infusion routes. Data are shown as mean ± S.D. Statistical differences were evaluated by the parametric Dunnett test vs vehicle (PBS treatment) (*$P<0.01$).

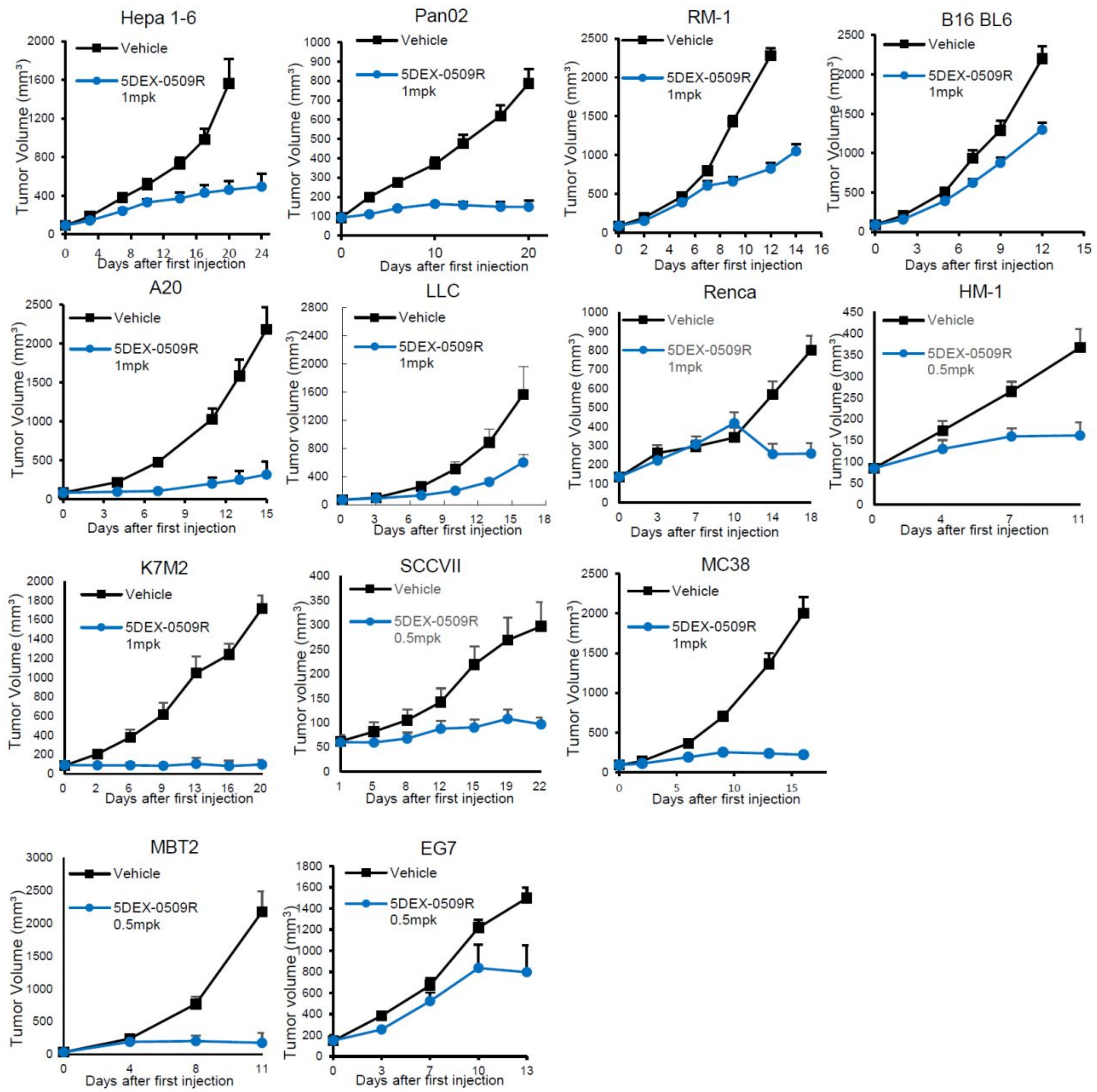


Figure S4. *In vivo* efficacy data of 5DEX-0509R summarized in Table 1. Data are shown as mean ± S.D. Statistical differences were evaluated by *t*-tests

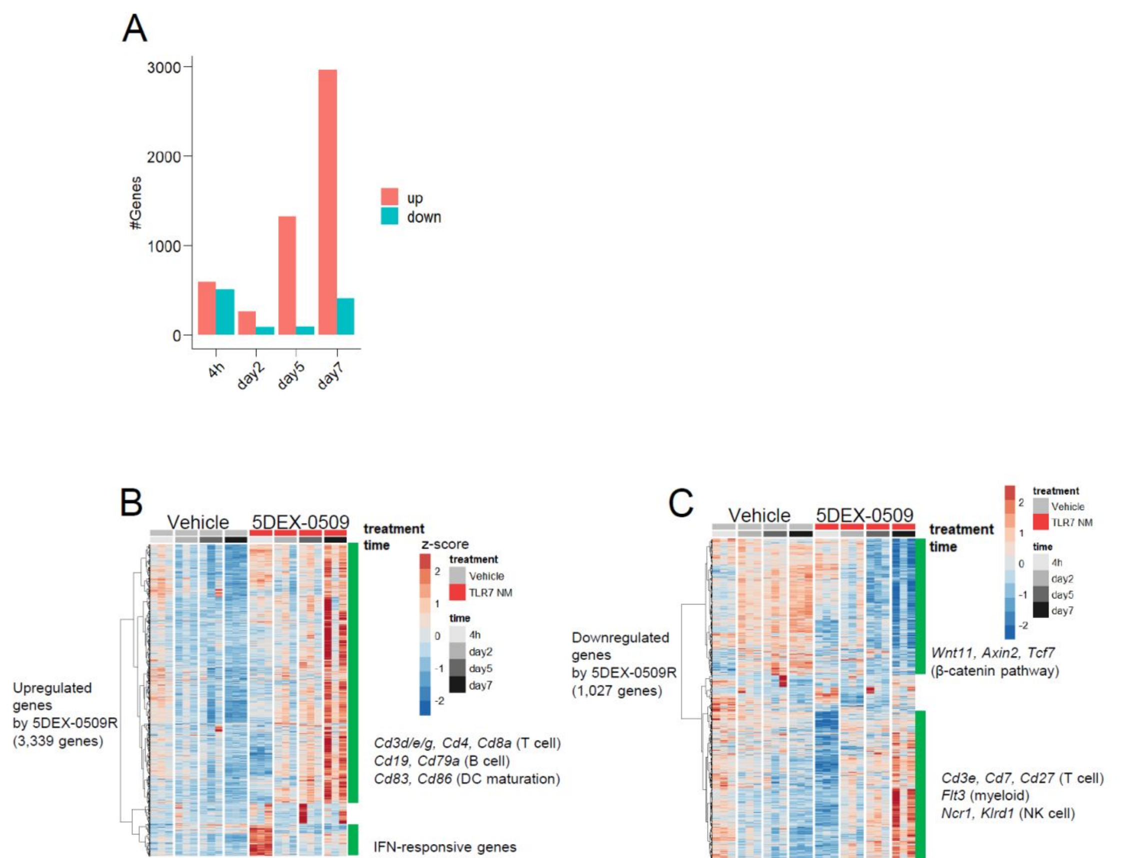


Figure S5. Bulk RNA-seq of tumor tissues A. Number of significantly upregulated or downregulated genes ($q < 0.25$) in the 5DEX-0509R group compared to the vehicle group at the indicated time points. **B, C.** Heatmap of gene expression for total upregulated (**B**) or downregulated (**C**) genes by 5DEX-0509R at one or more time points. Gene expression levels in tumors from individual mice across all time points were converted into z-scores. Representative gene names in clusters are shown on the right

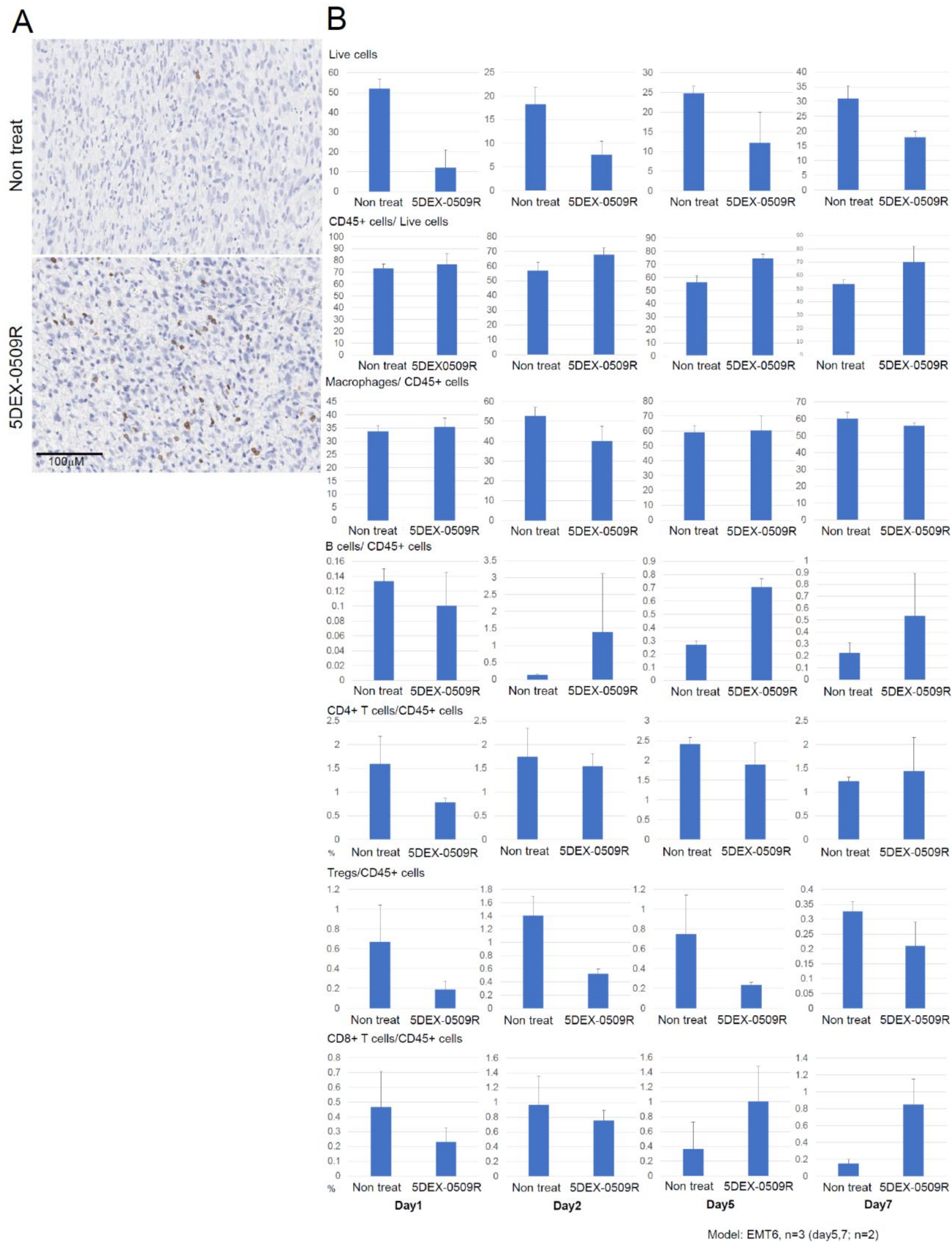


Figure S6. Changes in the tumor-infiltrating lymphocyte (TIL) population by 5DEX-0509R treatment. A. Immunohistochemical analysis of CD19+ cells. The figure shows data representative of n=3 mice. **B.** Flow cytometry analysis of TILs. EMT6-inoculated mice were treated with 5DEX-0509R, and TILs were analyzed at day 1, 2, 5, and 7 after the injection (day 1 and 2: n=3, day 5 and 7: pooled TILs from 2 mice, n=2). Macrophages were categorized as CD45+ CD11b+ F4/80+ cells;

B cells as CD45+ CD11b- B220+ CD19+ cells; CD4+ T cells as CD45+ CD3+ CD4+ cells; Tregs as CD45+ CD3+ CD4+ Foxp3+ cells, CD8+ T cells as CD45+ CD3+ CD8+ cells. Data are shown as mean ± S.D.

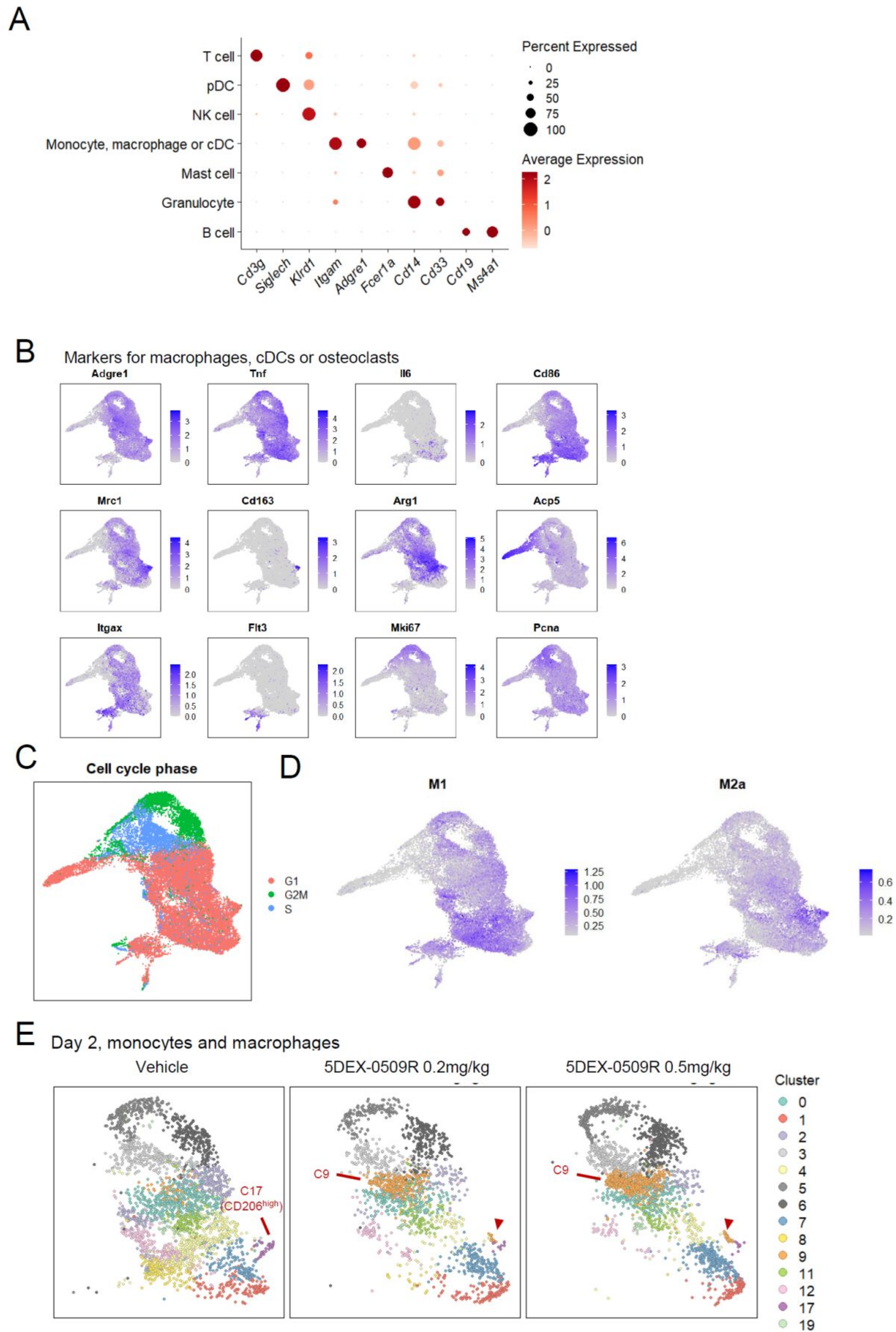


Figure S7. Single-cell RNA-seq clustering. A. Expression levels of representative marker genes (horizontal axis) in cell type-annotated cell clusters (vertical axis). **B.** Expression levels of marker

genes for macrophages, classical dendritic cells (cDCs), or osteoclasts in the monocyte, macrophage, and cDC populations. **C.** Estimated cell cycle phases. **D.** Expression scores summarizing expression of marker genes for macrophage M1 or M2a. **E.** Separate Uniform Manifold Approximation and Projection representations for monocytes and macrophages at day 2 after vehicle or 0.2 or 0.5 mpk 5DEX-0509R administration.

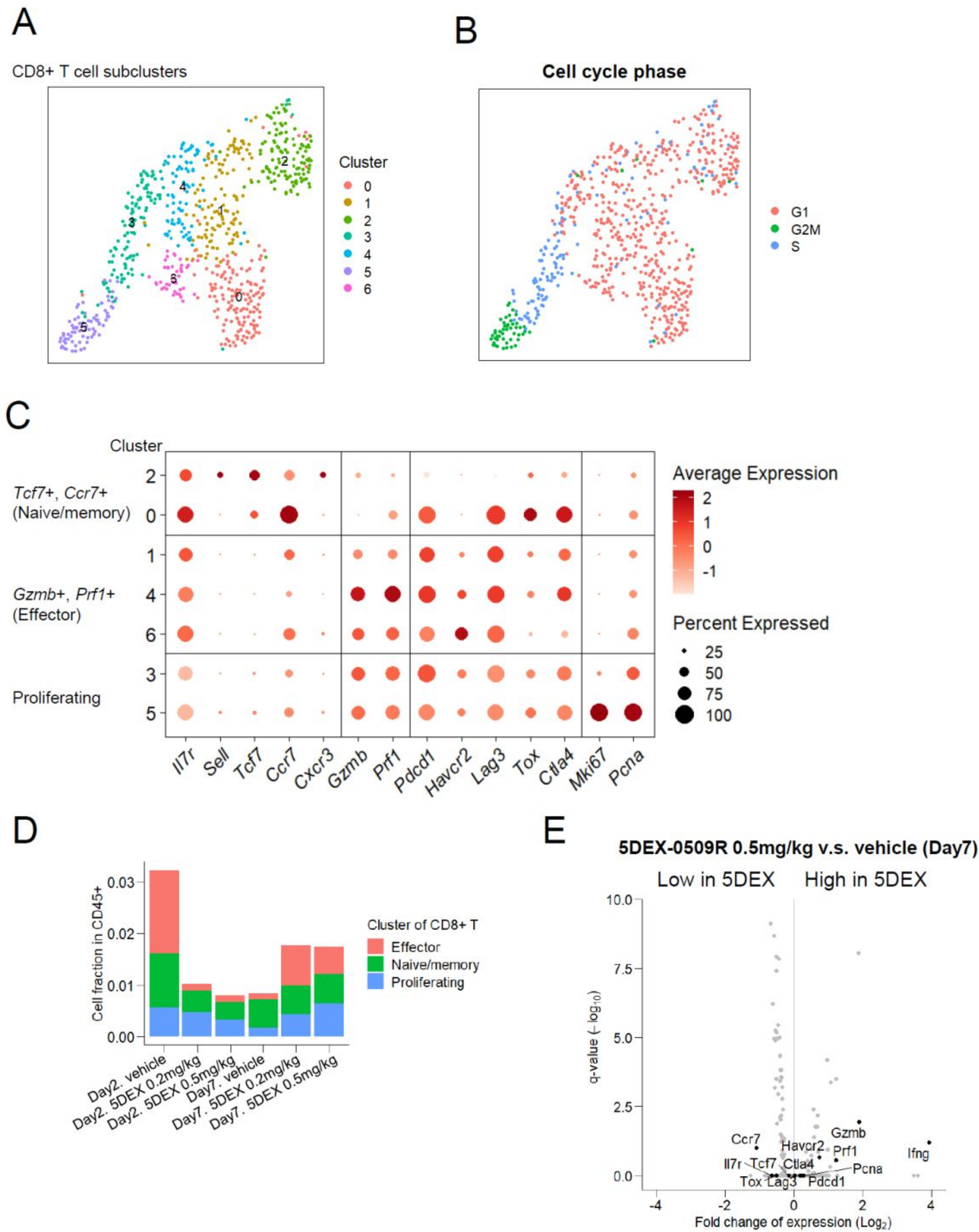


Figure S8. Single-cell RNA-seq of tumor infiltrating CD8+ T cells. A. Uniform Manifold Approximation and Projection representation for CD8+ T cells. Distinct cell clusters are shown in colors. **B.** Estimated cell cycle phases. **C.** Expression levels of marker genes for CD8+ T cells subsets in each cluster. Sizes of dots indicate percent of cells expressing the gene. **D.** Cell frequency of each CD8+ T cell cluster in the entire CD45+ cell population. **E.** Differentially expressed genes in the 5DEX-0509R 0.5 mpk group versus the vehicle group at day 7

Supplementary methods

Synthesis of dextran conjugates

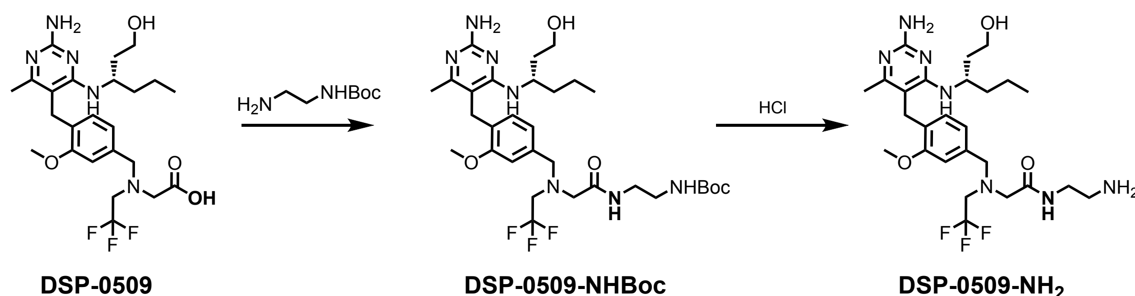
List of abbreviations

CPME = cyclopentyl methyl ether; DIPEA = N,N-diisopropylethylamine; DMF = dimethylformamide; DMSO = dimethyl sulfoxide; TFA = trifluoroacetic acid; THF = tetrahydrofuran; Boc = tert-butoxycarbonyl; Fmoc = fluorenylmethoxycarbonyl; EtOAc = ethyl acetate; HATU = 1-[(bis(dimethylamino)- methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3- oxide hexafluorophosphate; LC-MS = liquid chromatography-mass spectrometry; HPLC = high-performance liquid chromatography; MeOH = methanol; PBS = phosphate buffered saline; TSTU = O-(N-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

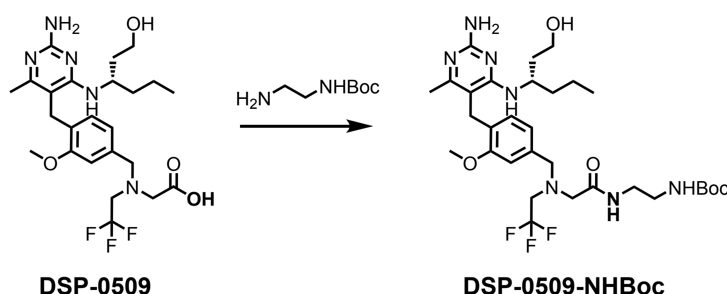
Experimental conditions and reagents

All reactions were performed in single-neck, round-bottom flasks or in centrifuge tubes (10, 25, or 50 mL). Liquid reagents were transferred via micropipettes. Crude materials were purified with Biotage® Sfär Amino D columns (11, 28, 55, 110, or 220 g) (Biotage, Charlotte, NC), and by elution with linear gradients of Chloroform/MeOH on a Yamazen automated flash purification system (Yamazen, Osaka, Japan). Reverse-phase purification was conducted using a GILSON PrepHPLC System (GILSON, Middleton WI, USA) equipped with YMC CombiPrep ODS-A columns (5 μm, 50 x 30 mm, YMC Co. Ltd., Kyoto, Japan) irrigated with linear gradients of 0.05% TFA-H_2O and 0.035% TFA-MeCN. The fraction was concentrated and lyophilized using a freeze drier (EYELA, TOKYO RIKAKIKI, Tokyo, Japan). The anion-exchange resin of MP-Carbonate was purchased from Biotage and used without any pretreatment. MS data were obtained for purified compounds using a Waters ACQUITY UPLC BEH C18 (1.7 μm, 2.1 x 30 mm) column (Waters) on a ACQUITY SQdetecter/ACQUITY system. The eluting solvent was a 2% to 96% linear gradient consisting of 0.06% formic acid in water and 0.06% formic acid in MeCN (flow rate: 3.5 mL/min).

Synthesis of DSP-0509-NH_2

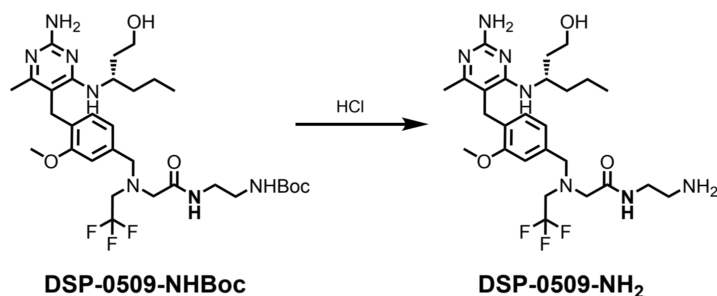


tert-butyl (S)-(2-(2-((4-((2-amino-4-((1-hydroxyhexan-3-yl)amino)-6-methylpyrimidin-5-yl)methyl)-3-methoxybenzyl)(2,2,2-trifluoroethyl)amino)acetamido)ethyl)carbamate (DSP-0509-NHBoc)



The DSP-0509 (1.53 g, 2.98 mmol) was dissolved in DMF (5 mL). To this solution was added TSTU (1.1 g, 3.65 mmol), DIPEA (1.54 g, 11.9 mmol) and tert-butyl *N*-(2-aminoethyl)carbamate (0.716 g, 4.47 mmol). The reaction mixture was allowed to stir at room temperature for 1 h. The reaction mixture was diluted with ethyl acetate and then washed with saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried with Na_2SO_4, filtered, and concentrated. The crude product was added to an amino silica gel column and eluted with chloroform/methanol to give tert-butyl (S)-(2-(2-((4-((2-amino-4-((1-hydroxyhexan-3-yl)amino)-6-methylpyrimidin-5-yl)methyl)-3-methoxybenzyl)(2,2,2-trifluoroethyl)amino)acetamido)ethyl)carbamate (1.67 g, 85% yield) as a white amorphous powder. LC-MS: Calc'd m/z = 655.8 for C31H48F3N7O5, found $[M+H]^+$ = 656.

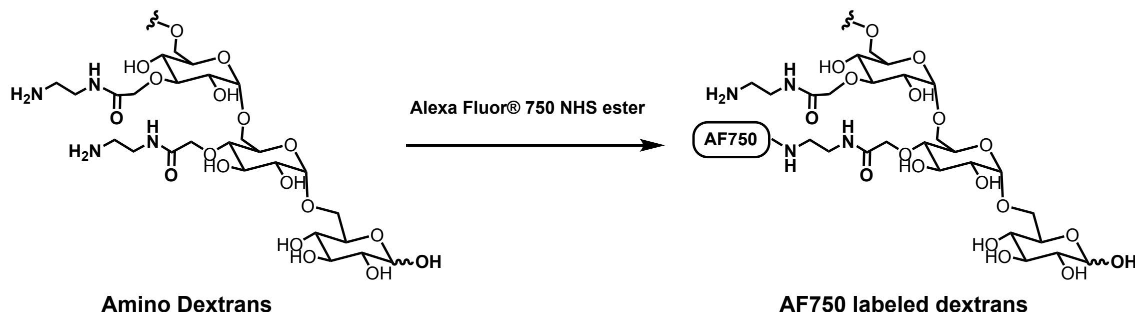
(S)-2-((4-((2-amino-4-((1-hydroxyhexan-3-yl)amino)-6-methylpyrimidin-5-yl)methyl)-3-methoxybenzyl)(2,2,2-trifluoroethyl)amino)-N-(2-aminoethyl)acetamide (DSP-0509-NH_2)



The DSP-0509-NHBoc (1.67 g, 2.55 mmol) was dissolved in CPME (25 ml) to give a white suspension. A 4N-HCl/CPME (25 mL) solution was added to the reaction mixture in one portion at room temperature. The reaction mixture was allowed to stir at room temperature for 3 h. The reaction mixture was concentrated *in vacuo* and dissolved in MeOH. MeOH was removed *in vacuo* to give (S)-2-((4-((2-amino-4-((1-hydroxyhexan-3-yl)amino)-6-methylpyrimidin-5-yl)methyl)-3-methoxybenzyl)(2,2,2-trifluoroethyl)amino)-N-(2-aminoethyl)acetamide, 3HCl (1.50 g, 89 % yield) as white solid.

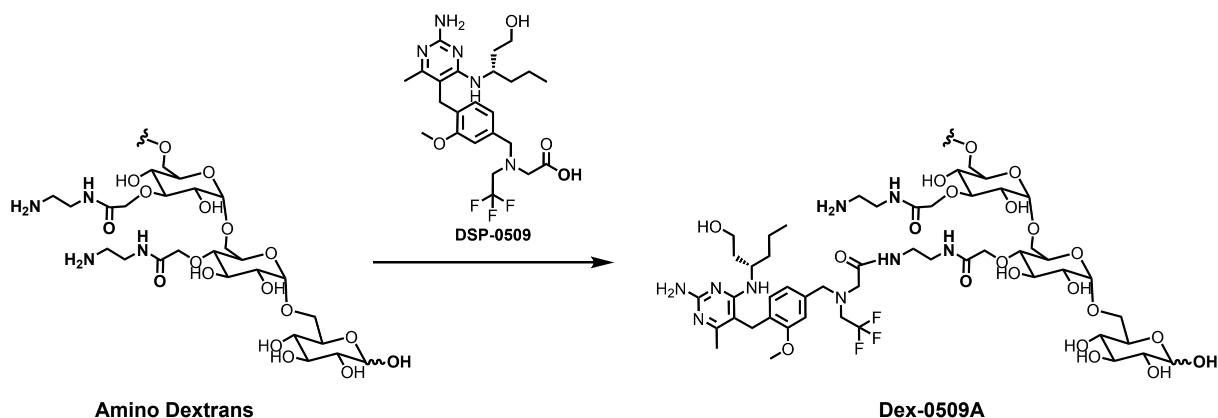
LC-MS: Calc'd m/z = 555.6 for C26H40F3N7O3, found $[M+2H]^{2+}$ = 278.

General synthesis procedure of AF750-labeled dextrans



The amino dextran (6 kDa or 20 kDa) was conjugated to Alexa Fluor® 750 NHS (Cat# A20111, Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The reaction mixture was purified with Amicon Ultra Centrifugal Filters (3K, Miliipore Sigma, Darmstadt, Germany) by using H_2O as the solvent. The purified AF750-labeled dextran was lyophilized and reconstituted with PBS as a 10 mg/mL solution. The concentration was calculated by using the extinction coefficient (λmax = 240,000) measured at 749 nm.

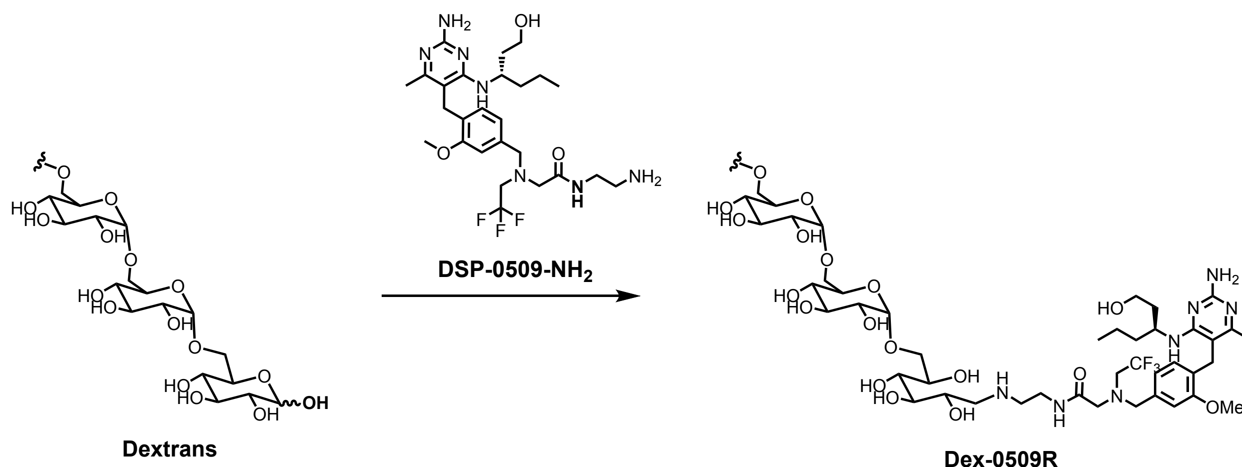
General synthesis procedure of Dex-0509A



The DSP-0509 (1 eq.) was dissolved in DMF to make an around 50 mM solution. To this solution was added TSTU (0.99 eq.) and DIPEA (3 eq.). The reaction mixture was allowed to stir at room temperature for 30 minutes before addition of the solution of amino dextran in H_2O (100 mg/mL). After stirring at room temperature overnight, the reaction mixture was purified by passage through PD-10 columns packed with Sephadex G25 resin (Cytiva, Marlborough, MA, USA) by using PBS as the eluent. The fractions were lyophilized to give white powder. The DSP-0509-equivalent

concentration of the conjugate was calculated from the peak area in the HPLC profile and based on a calibration curve prepared using a solution of DSP-0509-NH_2 of known concentration.

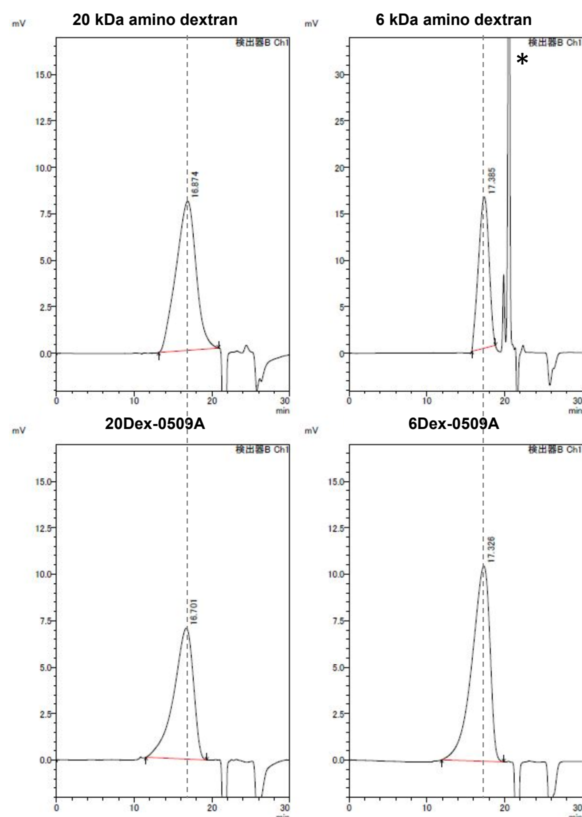
General synthesis procedure of Dex-0509R



To a solution of dextran (1 eq., Pharmacosmos A/S, Holbaek, Denmark) in H_2O (50 mM) was added acetic acid (1 eq.), DSP-0509-NH2 (1.1 eq.), and sodium cyanoborohydride (100 eq.). After heating at 60ºC for 24 hr, the reaction mixture was purified by reverse phase HPLC. The fractions were lyophilized to give a white powder. The DSP-0509-equivalent concentration of the conjugate was calculated from the peak area in the HPLC profile and based on a calibration curve prepared using a solution of DSP-0509-NH_2 of known concentration.

SEC-HPLC analysis of 20Dex-0509A and 6Dex-0509A

SEC-HPLC data were obtained by using a Shimazu HPLC system (Shimazu, Kyoto, Japan). Shodex OHpak SB-804 HQ (8.0 mm I.D. x 300 mm) x 2 colmuns were used with 0.1 M$NaNO_3$ aq. as the solvent with flow rate 1 mL/min. The column temperature was set at 40 °C. Refractive index detector was used to detect the analyte. *: artificial peaks derived from the solvent of sample.



SEC-HPLC analysis of 5Dex-0509R

SEC-HPLC data were obtained by using a Shimazu HPLC system (Shimazu, Kyoto, Japan). Shodex OHpak SB-803 HQ (8.0 mm I.D. x 300 mm) x 2 colmuns were used with 30% MeCN in 0.1 M$NaNO_3$ aq. as the solvent with flow rate 1 mL/min. The column temperature was set at 40 °C. Refractive index detector was used to detect the analyte. Analytical standard, for GPC, 5,000 (Aldrich, catalog No. 00269) was used as the 5 kDa dextran GPC standard.