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Supplementary information

Synthesis and use of an amphiphilic dendrimer for siRNA delivery into primary immune cells

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Supplementary Information

Synthesis and use of an amphiphilic dendrimer for siRNA delivery into primary immune cells

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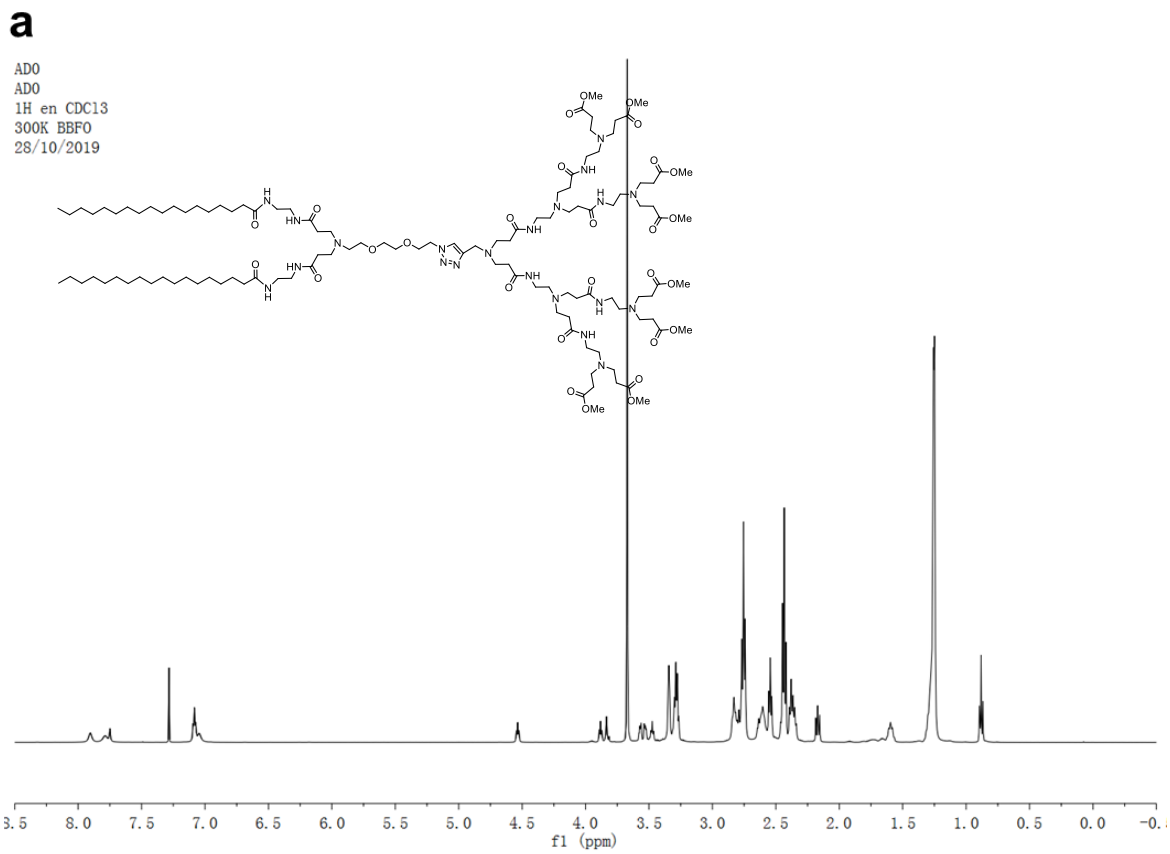
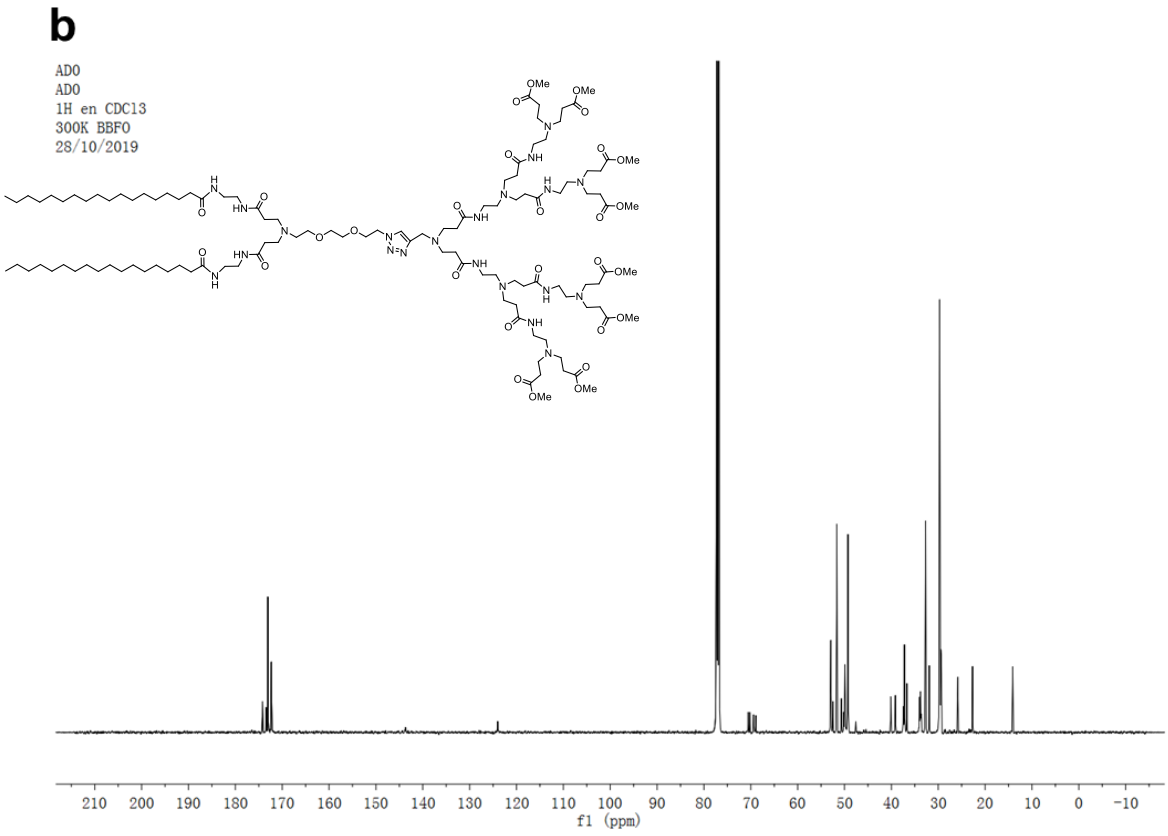
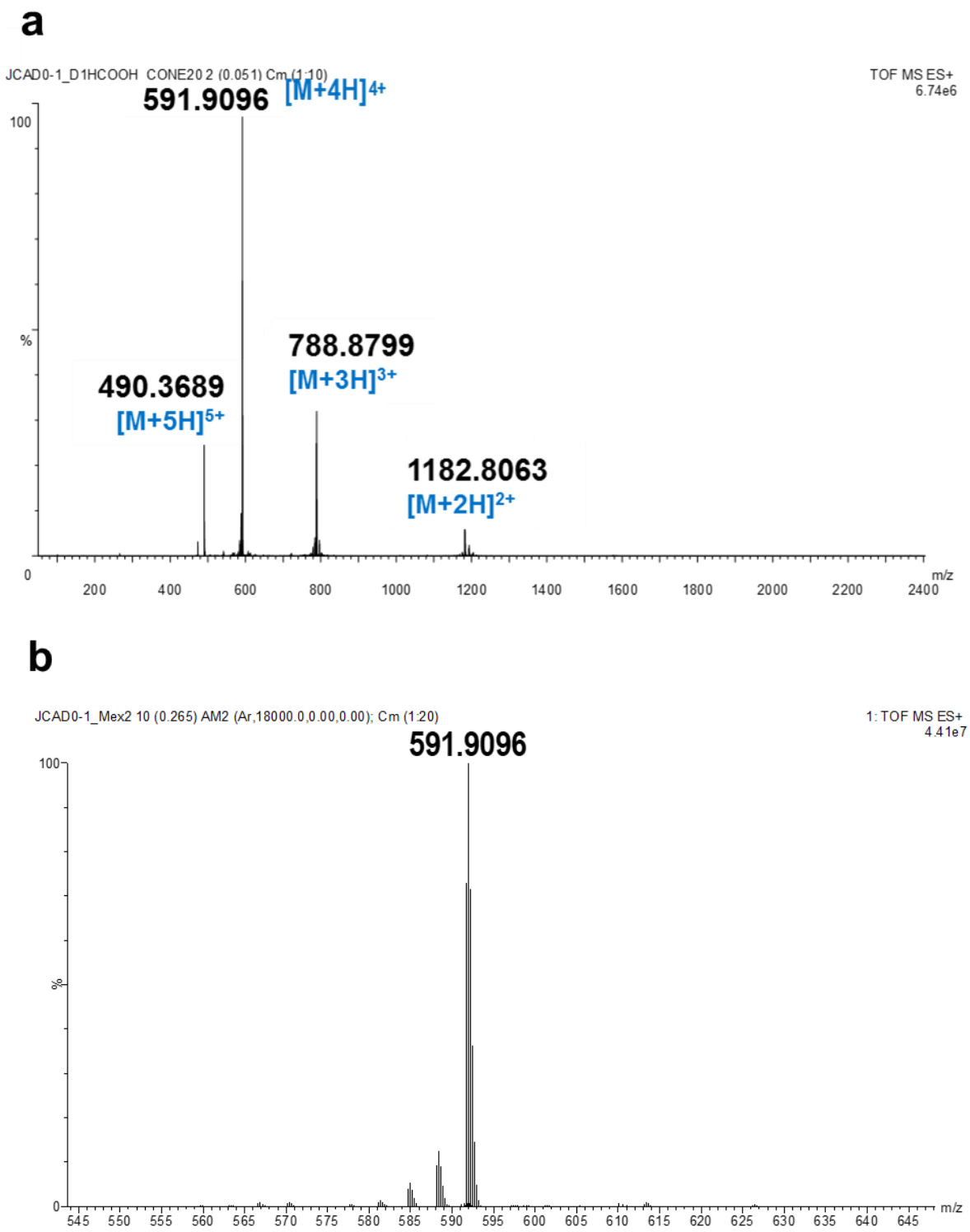
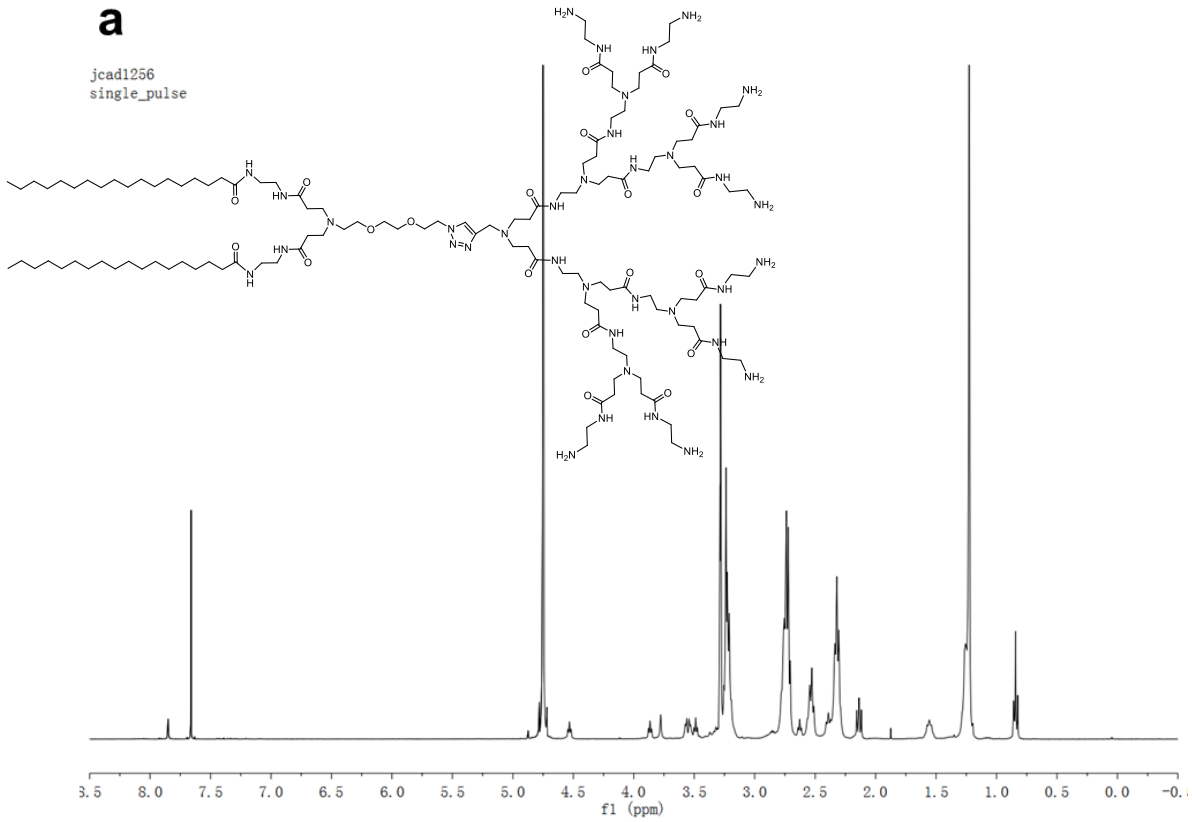


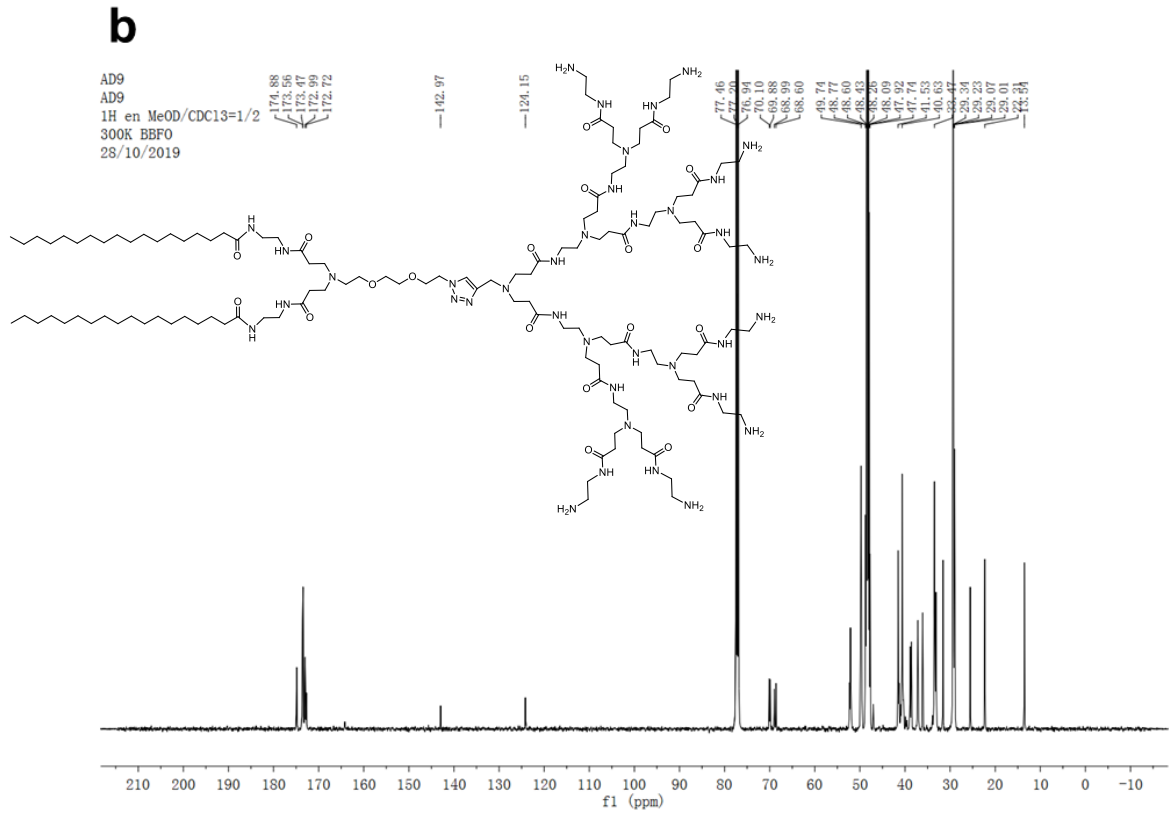


Supplementary Figure 2. High-resolution mass spectra of **3**. (a) ESI-HRMS full spectrum; (b) Enlarged spectrum of the expected ionic molecular weight peak with the highest intensity and the corresponding isotopic pattern.

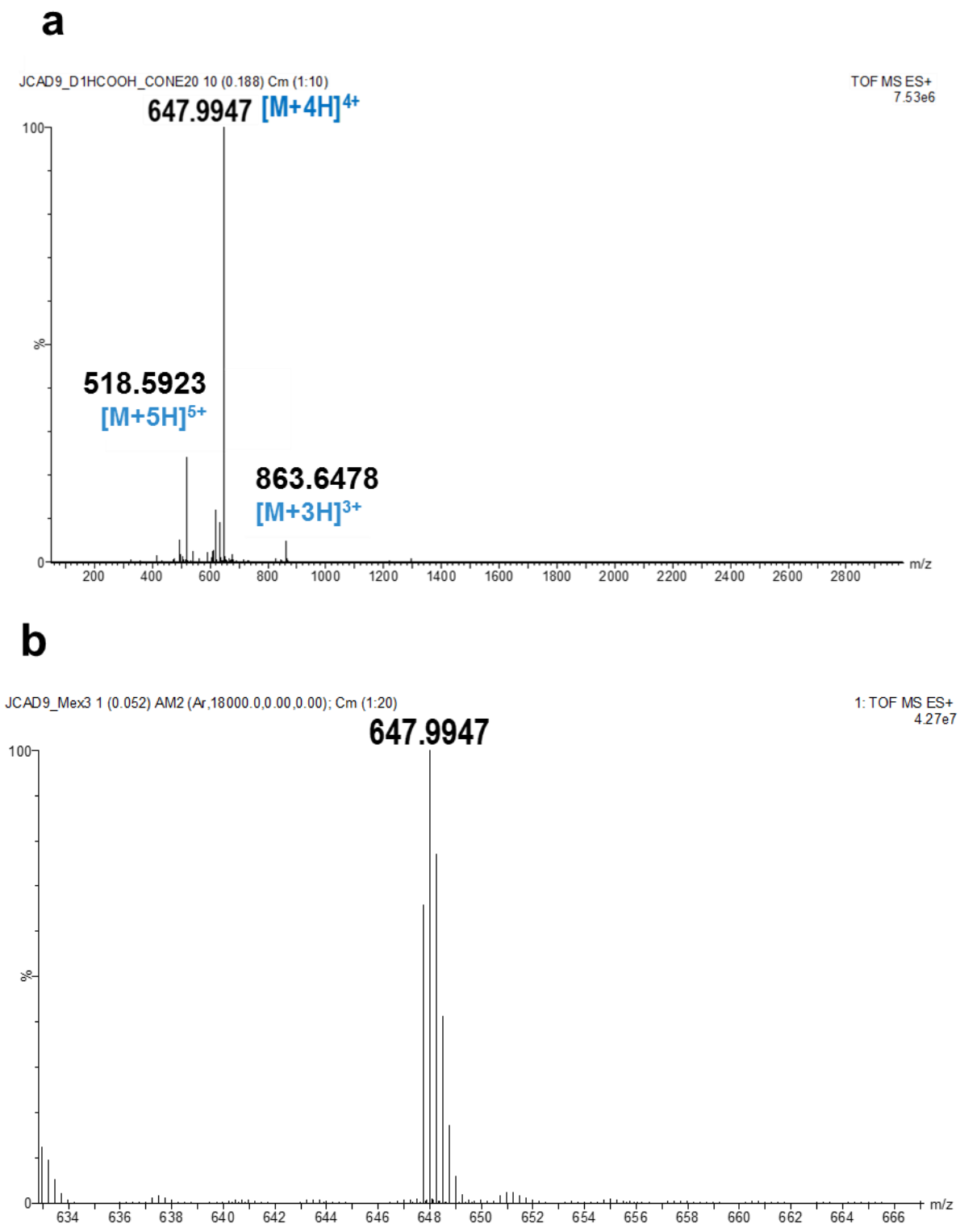


<u>Supplementary Figure 3.</u> (a) ^{1}H- and (b) ^{13}C-NMR spectra of **AD**.

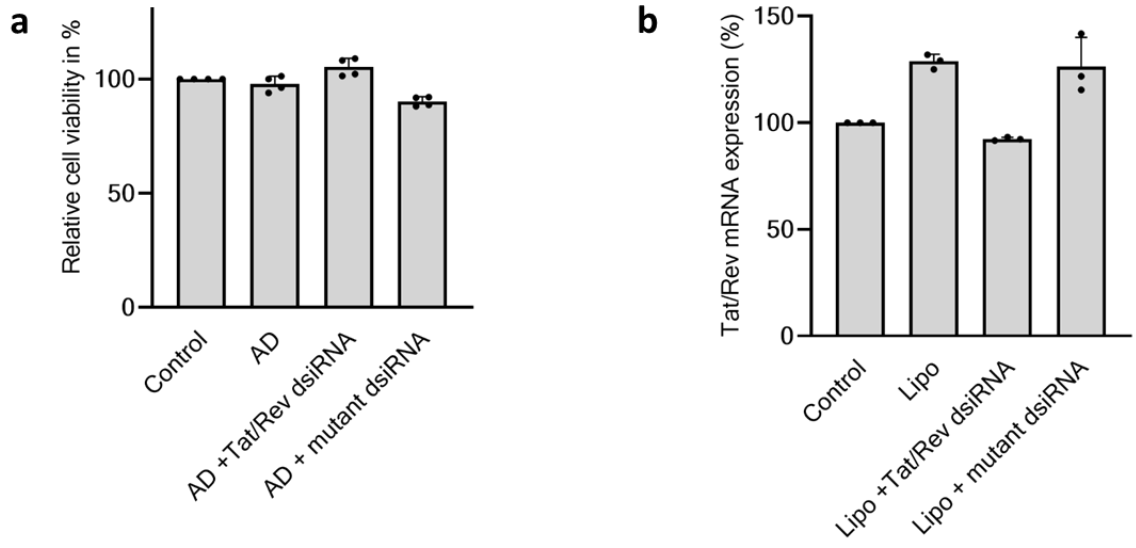




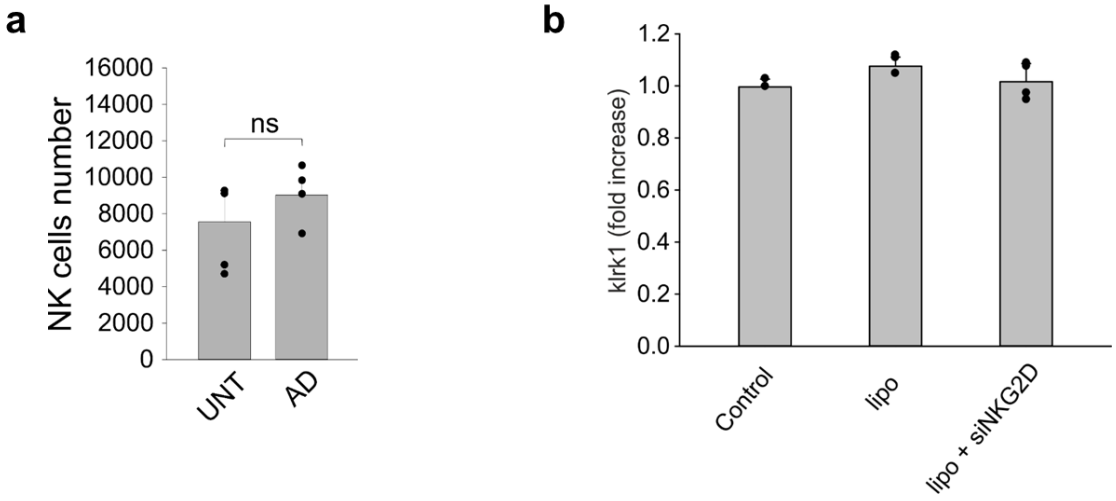
Supplementary Figure 4. High-resolution mass spectra of **AD**. (a) ESI-HRMS full spectrum; (b) Enlarged spectrum of the expected ionic molecular weight peak with the highest intensity and the corresponding isotopic pattern.



Supplementary Figure 5. Cell viability of primary T-cells after treatment with dendrimer **AD** and siRNA delivery using commercial transfection vector Lipofectamine RNAiMAX (lipo). The MTS assay was performed for cell viability test. (a) No significant toxicity was observed in PBMC CD4+ cells following treatment with **AD** and 50 nM dsiRNA at a N/P ratio of 5 (n = 4, data are expressed as mean ± SD, one-way ANOVA). (b) No effective gene silencing was achieved using commercial transfection reagent Lipofectamine RNAiMAX (lipo) and dsiRNA (50 nM) in primary PBMC CD4+ cells (n = 3, data are expressed as mean ± SD, one-way ANOVA). The control is cell alone without any treatment. All measurements were taken from distinct samples. Statistical significance was assessed by one-way ANOVA for parametrical data as indicated by using GraphPad Prism8.0 version. (Reproduced and adapted with permission from ref. 1, John Wiley and Sons.)

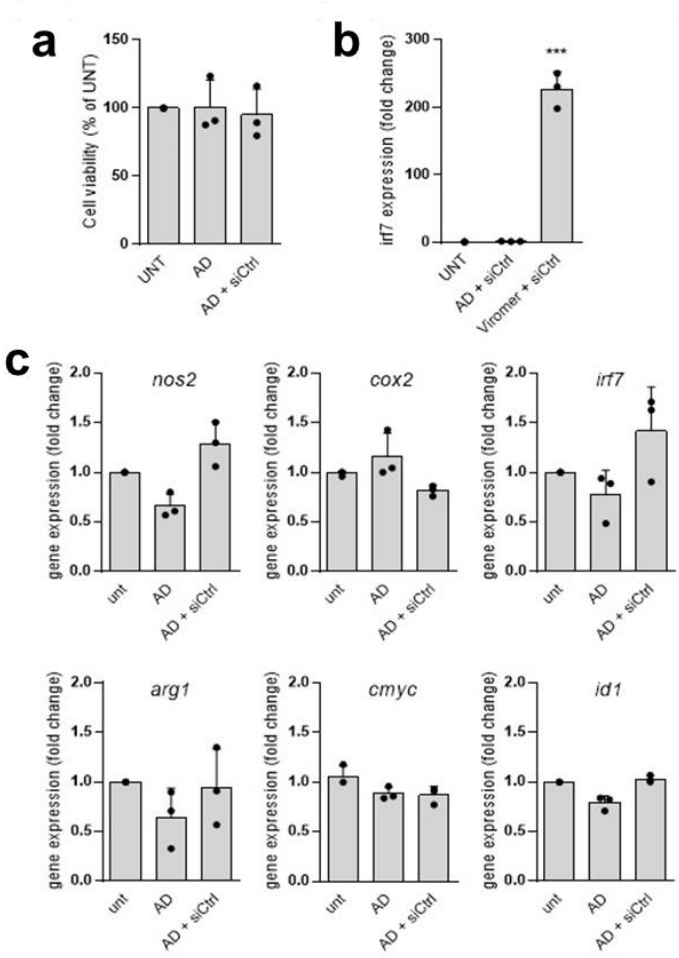


Supplementary Figure 6. Cell viability of primary NK-cells after treatment with dendrimer **AD** and *klrk1* gene expression following transfection with commercial transfection vector Lipofectamine RNAiMAX (lipo). (a) Dendrimer **AD** alone does not affect the number of NK cells. Murine NK cells were cultured alone or incubated with **AD** as indicated. The number of NK (NK1.1-/CD3+) cells after 24 h of stimulation was assessed by FACS. (n = 3; data are expressed as mean ± S.E.M. ns, not significant, one-way ANOVA). "UNT" represents cells without any treatment. (b) Transfection with commercial vector Lipofectamine RNAiMAX (lipo) did not reduce the expression of *klrk1* in murine NK cells (n = 4; data are expressed as mean ± SEM, one-way ANOVA).



Supplementary Figure 7. Assay of cell viability and macrophage polarization upon treatment with siRNA/**AD** complexes as well as siRNA delivery using commercial transfection vector Lipofectamine RNAiMAX (lipo). (a) Cell viability of the primary macrophages was not affected upon treatment with **AD**-mediated delivery of siRNA *JAK1* (siJAK1) and siRNA control (siCtrl). Cell viability was measured using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (n = 3, data are expressed as mean ± SD); (b) The polarization of macrophages was assessed by analyzing the expression of iNOS and Arginase1 (ARG1) using western blotting. M0 stands for unpolarized primary macrophages, M1 for LPS polarized macrophages expressing high level of iNOS and M2 for IL-4 polarized macrophages expressing high level of Arginase1. In the protocol, M0 unpolarized macrophages, which were transfected with siRNA control (siCtrl) complexed with **AD** at N/P ratio of 5, remained unpolarized. The presented blot is representative of three independent experiment. (c) The transfection of siRNA *JAK1* (siJAK1) and siRNA control (siCtrl) in primary macrophages using lipofectamine RNAiMAX (lipo) was not effective. The protein expression was assessed using western blotting (n = 1)

Supplementary Figure 8. The effect of **AD** on viability and polarization of primary microglia and siRNA delivery using a commercial transfection reagent Viromer. (a) Viability of rat microglial cells was not affected by **AD** alone or **AD** complexed with non-targeting siRNA (siCtrl) at N/P=10, 12.5 nM siRNA. Number of proliferating cells was evaluated using BrdU incorporation assay at 72 h post-treatment (n = 3, mean ± SD). "UNT" represents cells without any treatment. (b) **AD**-mediated siRNA delivery did not upregulate the inflammatory response in rat microglia in contrast to the commercial vector Viromer. Primary rat microglia cultures were transfected with 25 nM of the control non-targeting siRNA (siCtrl) using **AD** at N/P 10 or commercial vector Viromer. The mRNA level of the inflammatory gene *irf7* was measured using qPCR 24 h post-transfection and presented as a fold change relative to untreated cells (n = 3, mean ± SD). (c) There was no significant change in the expression of genes related to pro-inflammatory (*nos2*, *cox2* and *irf7*) or pro-invasive (*arg1*, *cmyc* and *id1*) phenotype in microglia upon treatment with **AD** alone or **AD** complexed with non-targeting siRNA (siCtrl) at N/P=10, 12.5 nM siRNA. The mRNA levels were measured using qPCR 48 h post-transfection and presented as a fold change relative to untreated cells (n = 3, mean ± SD). All measurements were taken from distinct samples. Groups were compared by one-way ANOVA with a post-hoc Tukey test for multiple comparisons using GraphPad Prism version 6.04. *** $p<0.001$. (Reproduced and adapted with permission from ref. 3, Nanomedicine (as agreed by Future Medicine Ltd.))



Supplementary Results

Analytical data:

Dendrimer 3. Typical isolated yields are 60-70%, from a white to a faint-colored solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (br s, 2H, NH), 7.78 (br s, 2H, NH), 7.73 (s, 1H,CH), 7.08 (br s, 4H, NH), 7.01 (br s, 2H, NH), 4.53 (t, 2H, *J* = 4.8 Hz, CH_2), 3.87 (t, 2H, *J* = 4.8 Hz, CH_2), 3.82 (s, 2H, CH_2), 3.66 (s, 24H, CH_3), 3.53-3.56 (br, 4H, CH_2), 3.46 (t, 2H, *J* = 4.5 Hz, CH_2), 3.34 (br, 8H, CH_2), 3.26-3.29 (m, 12H, CH_2), 2.73-2.81 (m, 32H, CH_2), 2.52-2.62 (m, 14H, CH_2), 2.33-2.44 (m, 32H, CH_2), 2.16 (t, 4H, *J* = 7.8 Hz, CH_2), 1.58 (br, 4H, CH_2), 1.25 (br, 56H, CH_2), 0.87 (t, 6H, *J* = 7.2 Hz, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.21, 173.44, 173.07, 172.31, 124.03, 70.56, 70.21, 69.41, 68.93, 52.94, 52.48, 51.65, 50.66, 50.15, 49.90, 49.26, 40.12, 39.14, 37.44, 37.21, 36.70, 33.99, 33.79, 33.63, 32.71, 31.92, 29.71, 29.59, 29.46, 29.43, 29.35, 25.82, 22.68, 14.11. HRMS *m/z* calcd for $C_{117}H_{215}N_{21}O_{28}$, $[M+4H]^{4+}$ 591.9092, found 591.9096.

Dendrimer AD. Typical isolated yields are 90%, from a white to a faint-colored foam-like solid after lyophilization; ^{1}H NMR (400 MHz, $CDCl_{3/CD_3OD=3/2}$) δ 7.95 (s, 1H, CH), 4.56 (br, 2H, CH_2), 3.88 (br, 2H, CH_2), 3.82 (s, 2H, CH_2), 3.56-3.58 (m, 6H, CH_2), 3.46-3.48 (m, 16H, CH_2), 3.24-3.29 (m, 20H, CH_2), 3.06-3.08 (m, 16H, CH_2), 2.75-2.88 (m, 34H, CH_2), 2.61 (br, 12H, CH_2), 2.40-2.44 (m, 32H, CH_2), 2.15 (t, 4H, *J* = 7.2 Hz, CH_2), 1.56 (br, 4H, CH_2), 1.23 (br, 56H, CH_2), 0.85 (t, 6H, *J* = 6.6 Hz, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.88, 173.56, 173.47, 172.99, 172.72, 142.97, 124.15, 70.10, 69.88, 68.99, 68.60, 52.30, 52.09, 51.92, 49.84, 49.74, 48.77, 48.60, 48.43, 48.26, 48.09, 47.92, 47.74, 41.53, 41.30, 40.63, 40.40, 38.86, 38.57, 37.15, 36.09, 33.47, 33.32, 33.13, 31.58, 29.34, 29.23, 29.07, 29.01, 25.53, 22.31, 13.54. HRMS *m/z* calcd for $C_{125}H_{247}N_{37}O_{20}$, $[M+4H]^{4+}$ 647.9942, found 647.9947.

Supplementary Methods: Isolation of primary immune cells

Isolation of primary CD4$^+$ T cells from human donors.

We have standardized this technique with frozen PBMCs or fresh blood samples that were recovered using either lithium heparin or EDTA at anticoagulants. However, please be aware that the level of CD4$^+$ and HIV-1 susceptibility is varying due to different sample resources and donor-to-donor variation. Therefore, experiments conducted with different donors or sample resources (fresh *versus.* frozen) should not be compared each other. Generally, we use discarded peripheral blood from anonymous, healthy adult donors for isolation of PBMCs and subsequently primary CD4$^+$ T cells cultures. Upon personal request, fresh blood samples are obtained from healthy donors at the City of Hope National Medical Center. In 24 hours of blood extraction, PBMCs are isolated from fresh blood by centrifugation through a Ficoll-Hypaque solution. Best results are obtained if the procedure is performed less than 2 hours after blood collection. Typically, the expected PBMCs yield from whole blood of healthy adult human donors are 0.8 – 3.2 million cells per mL blood. PBMCs are usually frozen right away after the Ficoll isolation and used subsequently in functional assay. However, for functional tests on frozen cells, a resting period (typically overnight) is often recommended after cell thawing.
Primary CD4$^+$ T-cell fractions are purified by negative selection using EasySep™ Human CD4$^+$ T Cell Isolation Kit (StemCell Technologies). This kit contains a combination of monoclonal antibodies in PBS and a suspension of magnetic particle, in which the untouched CD4$^+$ T cells are separated from the unwanted cells that are magnetically labeled by using an EasySep™ magnet. The desired CD4$^+$ T cells are simply poured or pipetted into a new tube and are immediately available for downstream steps. Despite donor-to-donor variations, we typically obtain purities above 95% for CD4$^+$ T cells and yields of around 25% of the raw PBMCs for CD4$^+$ T-cells.

Isolation of PBMCs ● TIMING 1.5 h for Ficoll gradient separation

1. Store blood tubes at room temperature prior to the isolation of PBMCs. Measure the usable whole blood volume within 0.50 mL.
2. Blood dilution: Transfer fresh blood sample from peripheral vein into a sterile 50 mL conical tube and dilute with an equal volume of PBS. Mix gently the blood and PBS.

▲CRITICAL The amount of blood and number of donors depend on how many cells will be used in the assay and experimental design. It is recommended to start with at least 10 mL of

blood to ensure that enough $CD4^+$ T cells are recovered (2-3 million $CD4^+$ T cells from 10 mL of blood).

3. Density gradient cell separation: Gently add Filcoll-PAQUE plus at the bottom of a new tube without touching the side of the tube. Gently overlay the Ficoll with the diluted blood.

▲CRITICAL Use 4 mL Filcoll-PAQUE plus for 6-10 mL diluted blood in a 15 mL conical tube. Use 20 mL Filcoll-PAQUE plus for 20-35 mL diluted blood in a 50 mL conical tube. Please be gently. Allow the diluted blood to Flow down the side of tube and pool on top of the density gradient media surface without breaking surface plane.

4. Gradient centrifugation: Centrifuge at 350 g for 20 min at room temperature (25 °C) with the breaks OFF.

▲CRITICAL Please turn off breaks since the deceleration disrupts the density gradient. Weigh and balance precisely all the tubes.

5. PBMCs collection: Discard as much the top plasma phase (yellow) as possible through gentle aspiration with a 10 mL pipette. Collect the mononuclear cell-containing interface (white, the plasm/Ficoll interface) with a 5 mL pipette and transfer to a new sterile conical 50 mL tube.
6. PBMCs wash: Add PBS to fill up the tube, and centrifuge at 350 g for 10 min at room temperature. Remove carefully the supernatant by tilting the tube and pipetting off the supernatant without touching the pellet. Loosen the cell pellet by gentle pipetting in 1.0 mL PBS and fill the tube with PBS. Repeat PBS wash twice.

▲CRITICAL To ensure the best results, minimize the time that the cells remain in a pellet or in contact with the Ficoll. Because cell pellet is loose due to red blood cells, wash the cells carefully after the Ficoll.

7. PBMCs count and viability: After the last wash, pipet off the supernatant and loosen the pellet by adding 1.0 mL PBS and gently resuspend cells with the 1.0 mL pipette. Mix 10 µL cells with 10 µL Trypan Blue Stain 0.4% and count cells in a hemocytometer. Determine the live cell concentration and the percentage of live cells.

▲CRITICAL Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer. If cells take up trypan blue (turn blue), they are considered non-viable. Fresh whole blood should show a fresh PBMCs yield above 0.80 million per mL whole blood and viability above 95%. Long processing time and poor technique may adversely affect the yield and viability.

■PAUSE POINT The isolated PBMCs can be frozen right away after the Ficoll isolation or immediately used for $CD4^+$ T cells isolation. However, for functional tests on frozen cells, a resting period (typically overnight) is often recommended after PBMCs thawing.

Isolation and activation of $CD4^+$ T cells ● TIMING 0.5 h for $CD4^+$ T cell isolation, and 2-3 days for the activation of $CD4^+$ T cells.

8. Concentrate PBMCs at 400 g for 5 min. Resuspend cells with chilled separation buffer at 5.0×10^7 cells per mL within the volume range (0.25- 2.0 mL) in 5 mL polystyrene round-bottom tube.
9. Negative selection of $CD4^+$ T cells: Gently mix 50 µL isolation cocktail per mL cells, and incubate the mixture at room temperature for 5 min. Vortex RapidSpheres™ for 30 seconds to evenly disperse the particles in the solution. Immediately, add RapidSpheres™ to cells (50 µL per mL cells). Add separation buffer to top up the sample to the 2.5 mL. Mix by gently pipetting up and down 2-3 times. Place the tube (without lid) into the EasySep™ magnet and incubate at room temperature for 3 min. Finally, pick up the magnet with tube, and carefully transfer the enrich cell suspension in a new tube by simply pouring off or pipetting.
10. Activation of $CD4^+$ T cells: Remove supernatant and resuspend cell pellet in activation medium to a final concentration of 1 million cells per mL. Place cells into a 6-well plate (2.0 million per 2.0 mL medium in each well) and culture them for 2-3 days at 37 °C, under 5% CO_2 in a humidified atmosphere.

HIV-1 challenge of $CD4^+$ T cells ● TIMING 2 h for HIV-1 infection, 1 h for washing at 24 h post-infection, and totally 5 days for HIV-1 replication.

11. Transfer the activated $CD4^+$ T cells (2.0 mL per a well of 6-well plate) from wells to 15 mL conical tubes with 5 mL pipette. Add 5 mL pre-warmed cell culture medium. Centrifuge at 400 g for 5 min at room temperature. Gently aspirate supernatant and resuspend cell pellets in 1.0 mL of IL-2 medium.
12. The activated $CD4^+$ T cells count and viability: Count cells with a hemocytometer as described. Adjust concentration to 1.0 million per mL with IL-2 medium and place cells into a 6-well plate (2.0 million per 2.0 mL medium in each well).
13. HIV-1 infection of the activated $CD4^+$ T cells: Dilute viral stocks to the desired concentration and directly add viral suspension to the cells at MOI = 0.001. Incubate plate for 24 h at 37 °C in a humidified incubator under 5% CO_2.

!CAUTION HIV-1 is a class 3 human pathogen and all the HIV-1 related procedures (HIV-1 infection, cell transfection and samples isolation) should be handled in a BSL2/3 level facility. No Sharps and glass are allowed in BSL2/3 level facility. Suction is not allowed for BL2/3 practice. Samples for centrifugation must be prepared in the hood with screw-capped leak-proof tubes and all the tubes should be loaded in the sealed buckets inside of the hood. All liquid waste including culture medium, supernatants and other liquid waste should be collected in a proper container and decontaminated with bleach or wescodyne for 30 min.

▲CRITICAL For HIV-1 challenge, both primary and lab-adapted strains can be used. We routinely assay the HIV-1 infection in parallel against an R5 HIV-1 (Bal) and an X4 HIV-1 (IIIB). The results obtained with both HIV-1 strains are generally comparable. As our previous experience, the use of a multiplicity of infection (MOI) = 0.001 of HIV-1 Bal or IIIB for 5-day infection in primary $CD4^+$ T cells regularly yields between 10^5-10^6 pg per mL of HIV-1 p24 antigen at the peak of viral replication. A value below 10^5 pg per mL may not allow a good discrimination between high-efficient and low-efficient siRNA candidates, and infections producing above 10^6 pg per mL of HIV-1 p24 in culture supernatants may be too strong even for most high-efficient siRNA candidates.

14. Washing off free virus: At day 1 after infection, carefully transfer the HIV-1 infected $CD4^+$ T cells from wells to 15 mL conical tubes with 1.0 mL pipette. Add 5.0 mL pre-warmed cell culture medium. Centrifuge at 400 g for 5 min at room temperature and gently remove supernatant. Repeat washing step twice. After the last wash resuspend cell pellets with an equal volume of IL-2 medium. Place cells into a new 6-well plate and incubate for additional 4 days at 37 °C in a humidified incubator under 5% CO_2.

▲CRITICAL After centrifugation, the HIV-1-infected cells are concentrated at the bottom of the tube. For aspiration, tilt the tube and place the tips on the wall of the tube, well above the cell pellet, to avoid loss of cells.

15. Washing off free virus: At day 5 after infection, carefully transfer the HIV-1 infected $CD4^+$ T cells from wells to 15 mL conical tubes with 1.0 mL pipette. Wash cells three time with IL-2 medium.

Isolation of murine NK cells

Two-month-old male C57BL/6 mice were anesthetized and decapitated. Spleen were removed and cut into small pieces and single-cell suspension was achieved in Hank's balanced salt solution (HBSS). The suspension was applied to a 70-μm cell strainer. Cells were processed

immediately for MACS MicroBead separation (Miltenyi Biotec - NK cells isolation kit II cat. No. 130-096-892). This produce has been modified by the users according to the Manufacturer's protocol. Briefly, total cells were magnetically labelled with Biotin-Antibody Cocktail MicroBeads. The cell suspension was loaded onto a MACS Column placed in the magnetic field of a MACS Separator and the negative fraction, corresponding to NK cells, was collected. Live NK cells were assessed by immunofluorescence and flow cytometry (FACS). After sorting, the purity of NK cells is about 95%. NK cells were seeded on culture dishes and used for the experiments after 24 h.

Isolation of human NK cells from healthy donors

Peripheral blood mononuclear cells (PBMCs), freshly isolated by lymphoprep (Nycomed AS, Oslo, Norway) were frozen and stored at -80 °C for up to 2 months. The day before the experiment, cells were thawed and kept in culture o/n for recovering. Then, cells were sorted with NK cell subset specific antibodies (Miltenyi Biotec – human NK cells isolation kit cat. No. 130-092-657) and seeded on culture dishes. Human NK cells were used 24 h after seeding for the experiments.

Isolation of human PBMCs ● TIMING 1.5 h for Ficoll gradient separation

Please refer the step **1-7**.

Dissociation of mouse spleen tissue ● TIMING 10 min for tissue dissociation

16. Collect the isolated spleen from mouse into a sterile culture dish containing ice-cold NK cell Culture medium.
17. Prepare a single-cell suspension applying pressure on the pores of a 70 μm filter (pre-wet with culture medium) with the plunger of a 1.0 mL syringe in a new culture dish.

▲CRITICAL Do not allow the tissue to dry during this step, work fast and keep cells cold.

Magnetic isolation of murine NK cells from spleen or human NK cells from human PBMCs ● TIMING 45 min for cells magnetic labeling and separation, and 1 day for cell recover.

18. Count cell number.

▲CRITICAL Generally work with 10^8 total cells. When working with higher cell numbers, it could create some non-specific labeling, and it is recommended to scale up all reagent volumes accordingly.

19. Centrifuge single cell suspension at 1000 g for 10 min.
20. Discard the supernatant and resuspend the cell pellet in 200 µL of MACS buffer per 10^8 total cells.
21. Add 50 µL of NK cell Biotin-Antibody Cocktail, mix well with vortex.
22. Incubate for 5 min in the dark in the refrigerator (2-8 °C).

▲CRITICAL Timing and temperature of incubation with the antibody are important to avoid non-specific labeling.

23. Add 2.0 mL of MACS buffer and centrifuge the cells at 1000 g for 10 min.
24. Discard totally the supernatant and resuspend the cell pellet in 400 µL of MACS buffer.
25. Add 100 µL of Anti-Biotin Microbeads, mix well with vortex.
26. Incubate for an additional 10 min in the dark in the refrigerator (2-8 °C).
27. Place LS large column in the magnetic field of MACS Separator.
28. Rinsing the LS column with 3.0 mL of MACS buffer.

▲CRITICAL For all the step including column, wait until the solution in the column is empty before proceeding. Do not allow the column to dry out.

29. Place a suitable collection tube under the column to collect the flow-through containing the NK cell.
30. Apply the single-cell suspension in the column.
31. Wait until the column reservoir is empty and wash the column with 3.0 mL of MACS buffer. The flow-through represent the unlabeled NK cells.
32. Centrifuge the cells 1000 g for 10 min and resuspended the cell pellet in the culture medium. Seed the cells on culture dishes appropriately.
33. Maintain the culture for 1 day before proceeding with the transfection.

Isolation of murine bone marrow derived macrophages (BMDM)

Primary macrophages can be obtained from various organs of the mouse. Here, we use bone marrow, because it is the initial source of macrophages which yields large quantities of cells in all strains of mice. We recommend using young mice for better yields; with both tibias and femur of a 5-8 week-old C57BL/6 mice, we obtain $5.0 – 7.0 \times 10^7$ monocytes. In presence of Macrophage Colony Stimulating Factor (M-CSF), monocytes differentiate into macrophages.

The cell line L929 secreted M-CSF, thus we use its culture medium as a source of M-CSF. Usually, with 4 – 5 × 10^6 monocytes seeded in 10 cm Petri dish, we obtain 8 – 10 × 10^6 differentiated macrophages composed of 95 – 98% cells expressing F4/80 and CD11b markers at their surface with 96 – 98% live cells.

Preparation of L929 conditioned medium as a source of natural M-CSF for macrophage culture ● TIMING 21-30 days to obtain one batch of L929 conditioned medium

34. Plate 5 × 10^5 L929 cells in a T75 flask in 20 mL of BMDM base medium and incubate at 37°C in a humidified incubator under 5% CO_2.
35. Split the cells every 2 or 3 days.

▲CRITICAL Daily check the cell proliferation under microscope. Once reach 90% confluence, split the cells. The expanded cells can be harvested and frozen for future use.

36. Maintain and expand cells until sixteen T75 flasks. Subsequently, transfer these cells into new sixteen T175 flasks and incubate for additional 3 days in 30 mL of BMDM base medium.
37. Once the cells are confluent, replace with 50 mL of fresh BMDM base medium and incubate for another 7 days.
38. Collect ~50 mL of culture medium from each flask and store in a sterile container for further processing.
39. Meanwhile add 50 mL of fresh BMDM base medium to each flask and incubate cells at 37°C in a humidified incubator under 5% CO_2 for another 7 days. Typically, at least 2 batches of L929 conditioned medium can be collected from the procedures described in Step 37 and 38.
40. Filter the L929 conditioned medium collected in Step 37 and 38 through a 0.45 µM filter.

■PAUSE POINT The filtered L929 conditioned medium is now ready for BMDM culture. The medium can be aliquoted (~40 mL per 50 mL conical tube) and stored at -80°C for up to 1 year.

Isolation of mice bone marrow cells from tibia and femur ● TIMING 1 h

41. Sacrifice a mouse by cervical dislocation. Harvest long bones (tibia and femur), remove muscle tissue and place the bones immediately in a 35 mm Petri dish containing DPBS-.

▲CRITICAL In order to avoid contamination, from now on all the process is carried out under the tissue culture hood under sterile conditions.

42. Clean each bone using a compress (Non-Woven sterile swabs) and place them on a plate containing sterile DPBS.
43. Cut the head of each bone using a sterile scissor and flush the marrow with DMEM base medium using a 10 mL syringe fitted to a 25 G needle into a 50 mL conical tube.
44. Gently pass the bone marrow cells 3-4 times through a 18 G needle to break the lumps.
45. At the end pass the culture medium containing bone marrow cells through a cell strainer to get rid of any small piece of bone. Wash the strainer with another 5 mL of BMDM base medium.
46. Centrifuge the cells at 400 *g* for 5 min and discard the supernatant, resuspend the cell pellet in 1 mL of RBC lysis buffer and immediately transfer the conical tube to ice for 4 min.
47. At the end of 4 min, add 10 mL of DMEM base medium to limit the lysis reaction.
48. Meanwhile, prepare BMDM complete medium by supplementing the BMDM base medium with L929 conditioned medium 20% (vol/vol).
49. After centrifugation, discard the supernatant and resuspend the cells in 5 mL BMDM cell culture complete medium; count the cells using a Countess™ II FL Automated Cell Counter using Trypan blue as a viability dye. This cell preparation will serve as source of monocytes.
50. Seed the monocyte cells at a density of 5.0 x 10^6 cells / 10 cm bacteriological plates containing 12 mL of BMDM cell culture complete medium and culture the cells at 37 °C in a humidified incubator under 5% CO_2.

▲CRITICAL Use sterile bacteriological plates for culture because if you use tissue culture plates, it is impossible to recover living cells due to their strong attachment to plastic dishes.

51. On day 4, remove half of the culture medium and replace it with fresh BMDM cell culture complete medium.

▲CRITICAL Do not remove the whole volume because there is autocrine signaling in the culture, thus removal of whole culture medium may lead to poor BMDM differentiation.

52. On day 7 culture, remove medium and wash the plates gently with sterile $DPBS^-$ to remove all the serum proteins; add 2-3 mL of accutase to each Petri dish and incubate at 37 °C in a humidified incubator under 5 % CO_2 for 5-7 minutes.
53. Once the cells are detached, add 10 mL of $DPBS^-$ to each Petri dish and wash gently to take out all the macrophages; collect the $DPBS^-$ in a 50 mL conical tube.
54. At the end, centrifuge cells, discard the supernatant, resuspend the cells in BMDM base medium and count the cells using a Cell Counter using Trypan blue as a viability dye. The cells are now ready for further experiments.

Isolation and culture of primary microglial cells

Microglial primary cultures are prepared from the brains of Wistar rat pups or C57BL/6J mouse pups at postnatal day P0 – P2 as described.[4] The pups are rapidly decapitated and the brain is removed from the cranium. Cells isolated from dissociated cerebral cortices are plated in culture flasks. Culture conditions enable the formation of a mixed primary glial cell culture. After 8 – 10 days, microglial cells, which are loosely adherent on the top of confluent cell culture monolayers are collected by gentle shaking and centrifugation. The cells are checked for viability and seeded on culture dishes appropriately. Typically, more than 96% of the isolated cells are positive for isolectin B4, a specific microglial marker. The average expected yield is 0.30 or 1.0 million cells per one mouse or rat brain, respectively.

Dissociation of brain tissue and setting the primary glial culture ● TIMING 3 h for tissue dissociation and starting the culture, and 8-10 days for the maintenance of culture

55. Collect the isolated brains into a sterile culture dish containing ice-cold Opti-MEM and peel away the meninges. Dissected cerebral cortices, pool all tissue in a new culture dish and chop with a razor blade.

▲CRITICAL Do not allow the tissue to dry during this step.

56. Add 1/10 of the final volume of trypsin solution (0.025% trypsin in HEPES; final volume 10 mL per 1 brain) to the minced tissue and transfer it to a glass bottle. Add the remaining volume of trypsin solution to the bottle and incubate at 37 °C for 20 min with vigorous swirling at 110-150 rpm.
57. Terminate the trypsinization by adding trypsin inhibitor solution (0.20 mL per brain). Swirl gently for 2 min at room temperature. Avoid excessive foaming.
58. Add the $MgSO_4$ solution (0.20 mL per brain, final concentration of $MgSO_4$ 3.0 mM) and swirl gently. Next, add the DNase solution (0.10 mL per brain, final concentration of DNase 20 µg/mL) and incubate at room temperature for 5 min with occasional swirling to digest extracellular DNA.
59. Distribute the cell suspension evenly to conical 50 mL tubes and centrifuge.

▲CRITICAL Centrifuge briefly at 750 g and allow the centrifuge to deaccelerate without breaking. Discard the supernatant gently using the serological pipet.

60. Resuspend the pellet in HEPES (5.0 mL per 50 mL tube) supplemented with DNase solution (final concentration of DNase 20 µg/mL) and triturate 15-25 times to create a single cell suspension.

▲CRITICAL This step is critical for final yield. Triturate gently using serological pipette, not pipette tips to avoid excessive cell shearing.

61. Allow settling of the non-dissociated tissue chunks for 5 min, then collect the resulting single cell suspension and transfer it to a new conical tube.

▲CRITICAL Gently, the pellet is very loose! Remove upper ca. 4.0 mL of suspension. Use separate 50 mL conical tubes.

62. Pool the remaining pellets into one tube and add DNase solution in HEPES up to 5.0 mL of total volume. Triturate the suspension, allow it settle for 5 min as before and collect the cell suspension. Distribute it evenly to previous conical 50 mL tubes already containing 4.0 mL of the cell suspension.

63. Underlay the cell suspension with BSA solution (4.0% BSA in HEPES, 2.0 mL per tube) to form a distinct phase gradient and centrifuge the tubes (8 min, 100 g).

64. Resuspended the cell pellet in culture medium and seed at the density of 3.0×10^5 cells/cm^2 on poly-L-lysine coated 75-cm^2 culture flasks.

▲CRITICAL Culture flasks should be coated with poly-L-lysine for 1 h at room temperature and just before seeding of the cells, poly-L-lysine should be removed and the flask washed once with 5.0 mL of PBS. Prepare 1 flask per 1.5 mouse brain and 1 rat brain.

65. Maintain the culture for 8-10 days. Culture medium is changed after 3 days and then twice a week.

Isolation of microglia from the primary mixed glial culture ● TIMING 1 h for microglia isolation by gentle shaking, 30 min for cell counting and seeding

66. After 8-10 days, microglial cells are loosely adherent on the top of confluent mixed glial cell culture monolayers. Isolate the cells by gentle shaking (1 h at 100 rpm at 37 °C) and collect by centrifugation (10 min, 90 g).

▲CRITICAL Make sure that the centrifuge is at room temperature. Too low temperature during processing may adversely affect the yield and viability.

67. Count the number of viable cells using an automatic cell counter (eg. Nucleocounter) following the manufacturer's protocol or in the hemocytometer after trypan blue staining (follow the protocol described for Isolation of PBMC). Seed the cells on culture dishes appropriately. Microglial cultures are used for experiments 48 h after seeding.

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