Dexamethasone-thymus *in vivo* efferocytosis assay

- Sacrifice the mice via isoflurane inhalation and measure their body weight
- Collect blood from the mice via heart puncture in tubes filled with 20 µL of 0.5 M EDTA
    - Bring 30 µL of whole blood to the core for CBC, spin the rest down for plasma collection (12,000 g for 10 min at 4°C)
- Remove the thymus, and weigh the entire thymus on an analytical balance
    - Cut the thymus into 2 pieces down the middle
    - Cut one lobe into 2 pieces again and put a part in a cassette in 10% buffered formalin and another part in liquid N_2
    - Take the second lobe and place it into a 40 µm strainer in a 60 mm dish and add 500 µL PBS (**on ice!**)
    - Dissociate the cells mechanically using a syringe plunger (**on ice!**)
    - Add 1000 µL of PBS to collect the all the cells and place it into 15 mL tubes (**on ice!**)
    - Fill up the tube to 10 mL ice-cold PBS in total and leave the cells **on ice**
- After all the thymi are collected, spin the tubes down (800 g 5 min at 4°C)
- Remove supernatant and add 10 mL of RBC lysis buffer (Sigma, #R7757-100ML)
- Incubate for 3 min and afterwards spin the tubes down (800 g 5 min at 4°C)
- Remove lysis buffer and resuspend the cells in 4 ml of PBS
- Count the cells by using the automated cell counter, and multiply the values by (4x) 2 to get the cellularity of the entire thymus (both lobes)

Stain a part of the cells for Annexin V on the same day:

- Take ¼ of the cells (1 mL; <1 M cells) for Annexin V staining directly
- Also take a little bit of cells from each tube (50 µL) as an unstained control sample (spin down and add Ca^+ Mg^+ buffer without Annexin V antibody)
- Put 1 mL of cells into 1.5 mL tubes and spin down (800 g 5 min at 4°C)
- Resuspend each sample in 200 µL Ca^+ Mg^+ buffer (Annex V Binding Buffer, Biolegend, #422201) containing a 1:20 dilution of Annexin V antibody (Biolegend, #640906)
    - For 19+1 samples, add 200 µL Ab to 3.8 mL Annex V Binding Buffer
- Incubate for 10 min on ice, and then place the sample in flow tubes for analysis

Fix the other cells for F4/80 and optional additional stainings the next day

- After successfully obtaining the Annexin V data, spin the tubes with the remainder of the cells down (800 g 5 min at 4°C)
- Fix the cells with 500 µL 4% PFA for 15 min, then add 10 mL of PBS and spin them down again (800 g 5 min at 4°C)
- Aspirate, resuspend in 1 mL of FACS buffer (with sodium azide because the cells are fixed)
- Store the cells in 1.5 mL tubes in the fridge until staining the next day

- The next day, take ½ of the cells for an F4/80 staining, combined with any other preferred targets
- Take a little bit of each sample (50 µL) as unstained control, and for single F4/80 and any other stainings for compensation control
- Spin the cells down (800 g 5 min at RT) and resuspend into 100 µL FACS buffer containing a 1:200 dilution of Fc Block (TruStain FcX antibody, BioLegend, #101320)
    - For 21+1 samples, add 11 µL Ab to 2189 µL FACS Buffer
    - (19 samples plus 2 compensation control samples plus 1 surplus)
- Incubate for 30 min on ice, and then directly add 200 µL solution with F4/80 antibody (Biolegend, #123119) combined with optional additional Abs, at a 1:50 dilution for a final dilution of 1:100
    - For 19+1 samples, add 40 µL of (each) Abs to 1.960 mL FACS Buffer
    - (19 samples plus 1 surplus)
    - For the compensation controls, add 2 µL of a single Ab to 98 µL FACS buffer
- After 1 h incubation on ice, add 1 mL of FACS buffer and spin down (800 g 5 min at 4°C)
- Aspirate and resuspend in 1 mL of FACS buffer to wash, and spin down (800 g 5 min at 4°C)
- Resuspend in fresh FACS buffer (200-300 µL per sample) and perform flow analysis
    - Analyze all 19 samples including the F4/80 only (FITC) compensation control (and any other compensation controls) and the unstained control

Calculations for i.p. injections of dexamethasone

- Purchase 25 mg dexamethasone from Sigma (D4902-25MG)
- Reconstitute this to 25 mg/mL by adding 1 mL of DMSO (stock solution)
- Then dilute this further into PBS: 250 µg in 250 µL per mouse, so 1 mg/mL solution (25x diluted)
- For each cage of 5 mice + 1 surplus, add 60 µL stock solution to 1.44 mL PBS
- Make this solution fresh and mix very well before injecting each cage
- Inject 250 µL of this working solution i.p. in mice, using BD sciences 1 mL TB syringe 26G x 3/8 Slip Tip with intradermal bevel needle