Before experiment:

1. Prepare 200ml MACS buffer: 190ml auto MACS rinsing solution, 10ml MACS BSA stock solution, place the buffer on ice
2. Prepare PBS-W: PBS containing 2% FBS, 50ml PBS, 1ml FBS

Harvesting Naïve T from spleen and lymph nodes:

1. Sacrifice mice by CO2
2. Take lymph nodes and spleen, place it into a 1.5-ml tube containing 1ml MACS buffer (MACS buffer: Rinse + 0.5% BSA). Put it on ice.
3. Put a 70-um cell strainer on top of a 50-ml tube.
4. Transfer the organ to the strainer. Use the piston of 5-ml syringe to smash it.
5. During smash, wash the strainer with MACS buffer if necessary.
6. Wash strainer with up to 10ml of MACS buffer.
7. 300g, 5min, RT. Discard the supernatant.
8. Suspend splenocytes with 10ml ACK lysis buffer. Stand the tube at RT for 10min.
9. 300g, 5min, RT. Discard the supernatant.
10. Suspend all cells in total 10ml MACS buffer.
11. Put a 40-um cell strainer on top of a 50-ml tube.
12. Transfer cells to the new 50-ml tubes through the 40-um strainer.
13. Add another 5ml MACS buffer to rinse the original tube, and transfer remained MACS buffer to the new 50-ml tube through the 40-um strainer.  
    (Step11-13 may be skipped If harvesting cells from less than 2 mice. However, when harvesting from more than 4 mice, it’s better to perform step11-13 to avoiding cell clogs in the following steps)
14. Transfer all cells to a new 15-ml tube. Count cell number.
15. 300g, 5min, 4C. Discard the supernatant.
16. Suspend cell in 90ul MACS buffer for per 108 cells.
17. Add 10ul CD4 (L3T4) MicroBeads for per 108 cells.
18. Mix well and incubate 15 min on ice.
19. Add 10 ml MACS buffer for washing.
20. 300 g for 5 min at 4°C, remove supernatant.
21. Suspend in 3 ml MACS buffer.

MACS beads:

1. Balance the column by 3ml MACS buffer.
2. Apply cell into the column
3. Add another 3ml MACS buffer into the tube, and apply the wash fluids to the column
4. Repeat the previous step
5. Discard the flow through and the 15-ml tube. Remove the column from the Magnet stand. Place another new 15-ml tube to collect cells.
6. Apply 7ml MACS buffer into the tube
7. Use piston to push the flow through into the new 15-ml tube
8. Calculate cell number
9. Centrifuge, 300 g for 5 min at 4°C.
10. Suspend cells in PBSW, make the density is 108 cell/ml (107 cell/100ul)

FACS staining:

1. Use those four Abs: #203(APC-CD25), #214(PE-CD62L), #156(BV-CD4), #140(Percp/Cy5-5-CD44)
2. Add 1ul of each Abs for 2E+7 cell
3. Wrap the tube with aluminum foil, Incubate on ice for 1hr
4. Add 10ml PBSW into tube
5. Centrifuge 300g,4C, 5min
6. Discard the supernatant
7. Suspend cell in IMDM-CM, make the density is 2\*107 cell/ml
8. Go to FACS sorting
9. During sorting, put place sorted cells on ice

Anti-CD3 plate pre-coating:

1. Make a 1/200X dilution of anti-CD3 antibody (No.1) in PBS buffer  
   (for 10rxns: add 20ul into 4ml PBS buffer; for 20rxns: add 40ul into 8ml PBS buffer)
2. Add 400ul (24-well plate), 200ul(48-well plate) or 100ul(96-well plate) into each well
3. Incubate at 37C 5% CO2 for 2 hours
4. After incubation, wash three times by 150ul PBS

Cytokines stimulation and cell seeding:

1. Prepare cytokines according to the preparation sheet
2. Centrifuge cell from FACS sorting, 4C, 560g, 5min (Cells are lighter after sorting)
3. Suspend cells and adjust the cell density to 2\*106/ml medium. (for 24-well plate)
4. Incubate cells at 37C, 1hr
5. Add 200ul of cells per well (seeding: 0.4\*106/well)
6. Add 200ul of medium plus cytokines per well
7. Mix gently, incubate cell at 37C for 48 hours  
   -> follow ICS protocol or RNA purification protocol