Ex vivo Human Antigen-specific T Cell Proliferation and Degranulation

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[Abstract] Proliferative capacity and degranulation are important features of antigen-specific $CD8^+$ T cells. By combining tetramer staining with a CFSE staining, we were able to enumerate the total number of antigen-specific T cells, as well as their number of divisions upon antigen-specific stimulation during a week. In addition, we performed restimulation of these cells, to analyze their ability to secrete cytolytic granules, visualized by CD107a staining.

Materials and Reagents

1. Mouse-anti-human CD107a PE (clone H4A3) (BD Biosciences, catalog number: 555801)
2. Mouse-anti-human CD3 PE-Cy7 (clone UCHT1) (Biolegend, catalog number: 300420)
3. Mouse-anti-human CD8 Alexa-Fluor-700 (clone 3B5) (Life Technologies, Invitrogen™, catalog number: MCHD0824)
   Note: The above antibodies have been tested by the author and may be substituted with the antibodies desired by users.
4. CFSE (Molecular Probes Europe BV)
5. Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Invitrogen™)
6. Heat-inactivated fetal calf serum (FCS) (Integro)
7. Human serum (HS) (Sanquin Blood Bank)
8. Recombinant human IL-2 (Proleukin, Chiron)
9. Recombinant human IL-15 (Immunotools, catalog number: 11340155)
10. Antigen-specific peptide (*For example* MiHA or CMV-peptide, Thinkpeptides)
11. APC-conjugated antigen-specific tetramer (Kindly provided by Prof. dr. J. H. F. Falkenburg, Leiden University Medical Centre, the Netherlands)
12. Sytox blue (Life Technologies, Invitrogen™, catalog number: S34857)
13. Mature monocyte-derived DC [cultured following the protocol described in Hobo *et al.* (2010)]
14. FACS buffer (see Recipes)

Equipment

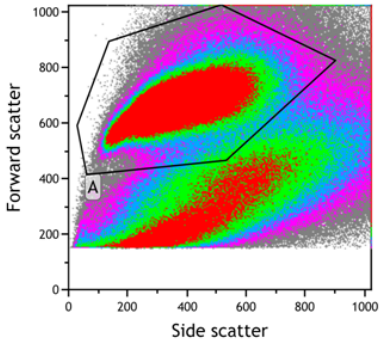
15. Beckman Coulter Navios flow cytometer
16. 24-wells plate
17. 37 °C incubator

Procedure

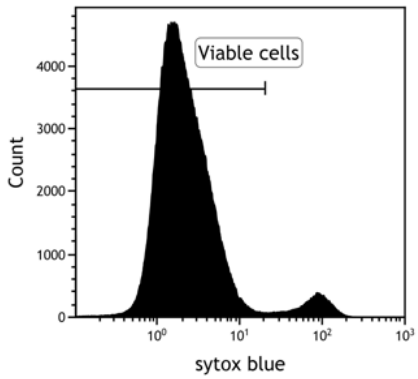
A. Antigen-specific T cell proliferation and degranulation
   1. Wash PBMC containing low percentages of antigen-specific T cells with 14 ml PBS.
   2. Centrifuge the cells for 5 min at 500 *x g* at room temperature (RT).
   3. Resuspend the cells in PBS to a concentration of 20 x 10^6 cells/ml, and add 2.5 µM CFSE at a 1:1 ratio (so in case of 500 µl cell suspension, add 500 µl 2.5 µM CFSE). The end concentration of CFSE will be 1.25 µM.
   4. Incubate the cells for 10 min at RT.
   5. Then, add pure FCS at a 1:1 ratio (so in case of 1 ml CFSE-cell suspension, add 1 ml FCS).
   6. After 2 min, wash the PBMC twice with 14 ml of IMDM/10% HS (centrifuge the cells for 5 min at 500 *x g* at RT), and count the cell number.
   7. Resuspend the PBMC to a concentration of 2 x 10^6 cells/ml in IMDM/ 10% HS.
   8. Plate 500 µl PBMC suspension per well of a 24-wells plate.
   9. Use mature monocyte-derived DC expressing the correct HLA-molecule for stimulation.
   10. Wash the DC with 14 ml PBS.
   11. Centrifuge the cells for 5 min at 500 *x g* at RT.
   12. Resuspend the DC subsequently in 500 µl IMDM containing 10 µM antigen-specific peptide.
   13. Incubate the DC for 30 min at 37 °C in the incubator.
   14. Add 14 ml PBS to the DC.
   15. Centrifuge the cells for 5 min at 500 *x g* at RT.
   16. Resuspend the DC in IMDM/10% HS to a concentration of 0.2 x 10^6 cells/ml.
   17. Add 500 µl DC suspension to each well containing CFSE-labeled PBMC.
   18. Culture the cells in the incubator at 37 °C, 5% CO_2 and 95% humidity.
   19. After 5 days, remove from each well 500 µl supernatant and add 500 µl IMDM/10% HS containing 100 U/ml IL-2 and 10 ng/ml IL-15, so the end concentration in the wells will be 50 U/ml IL-2 and 5 ng/ml IL-15. Supernatants can be used for cytokine analysis.

B. Overnight restimulation

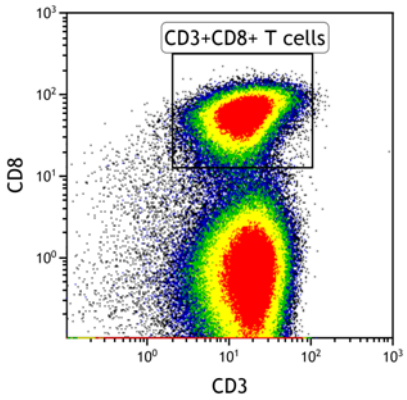
20. At day 7 of culture, 500 µl supernatant was removed.
21. Add to each well 500 µl IMDM/10% HS containing 10 µM antigen-specific peptide, so the end concentration is 5 µM Antigen-specific peptide.
22. In addition, add 6.5 µl CD107a-PE antibody to each well.
23. Culture the cells overnight in the incubator at 37 °C, 5% CO_2 and 95% humidity.
24. The following day, harvest the cultured cells and count the cell numbers.
25. Centrifuge the cells for 5 min at 500 *x g* at room temperature.
26. Resuspend the cells in 100 µl IMDM/10% HS and add 1 µl APC-conjugated antigen-specific tetramer.
27. Incubate the cells for 20 min in the dark, at RT.
28. Then (without prior washing!), add 2.5 µl CD3 PE-Cy7 and 1 µl CD8 Alexa-Fluor-700.
29. Incubate the cells for 30 min at ice, in the dark.
30. Subsequently, wash cells with 1 ml FACS buffer.
31. Centrifuge the cells for 5 min at 500 *x g* at RT.
32. Resuspend the cells in 300 µl FACS buffer containing 0.1% Sytox blue solution.
33. Analyze the samples on the Navios flow cytometer.
34. Flow cytometrical analysis:
    a. Gate on the leukocytes (Forward/ Side scatter)



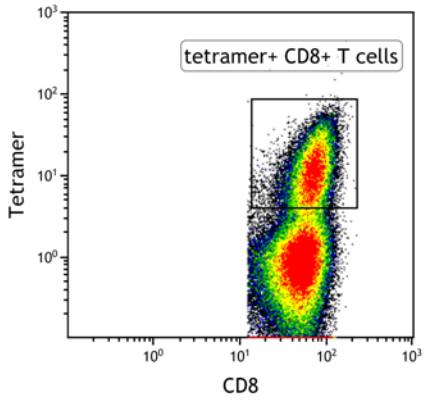
    b. Gate on viable cells (Sytox blue negative)



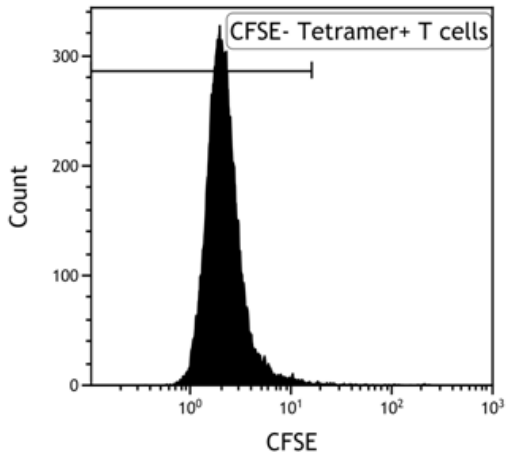
c. Gate on cytotoxic T cells (CD3 positive, CD8 positive)



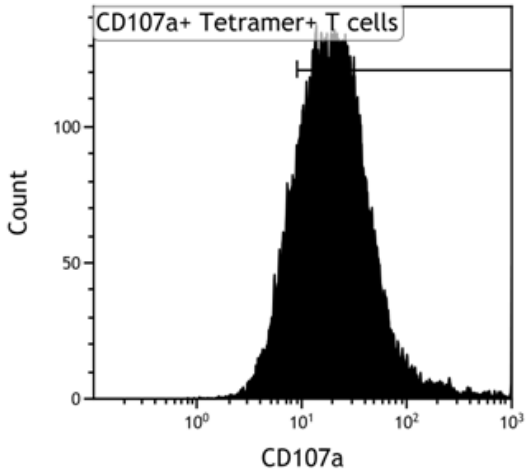
d. Gate on tetramer-positive $CD8^+$ T cells



e. Analyze CFSE dilution pattern of tetramer-positive $CD8^+$ T cells (NB. Use tetramer-negative cells for setting the gate of non-divided cells)



f. Analyze CD107a-positivity within the tetramer-positive $CD8^+$ T cells (NB. Use tetramer-negative cells for setting the gate of CD107a- cells)



Recipes

1. FACS buffer (0.5 L)
   2.5 g BSA in 500 ml PBS

Acknowledgments

This protocol has been adapted from the publication by Hobo *et al.* (2012). We thank Rob Woestenenk for assistance in flow cytometry and Michel Kester for providing us with tetramers.

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References

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