Analysis of Tumor-infiltrating Lymphocytes Following CD45 Enrichment

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[Abstract] Measuring antigen-specific T cell responses in the blood and lymphoid organs of vaccinated mice can give us a useful indication of the potency of a vaccine formulation. Unfortunately, systemic or even localized lymphoid T cell responses are not always predictive of the ability of a vaccine to induce tumor protection. Measuring the antigen-specific T cell response within the tumor infiltrating lymphocytes is a more accurate indicator or vaccine efficacy. However, multi-parameter flow cytometric analysis of T cells isolated from tumor tissue can be quite challenging due to the over-whelming number of tumor cells present in relation to the tumor infiltrating lymphocytes (TIL) and to problems associated to the large and adhesive nature of many tumor cell. Here we take advantage of a pre-flow separation of CD45$^+$ leukocytes from the tumor tissue using the MACS magnetic cell sorting system, resulting in a much cleaner cell preparation with which to proceed to flow cytometric staining and analysis.

Materials and Reagents

A. Cell lines

1. The B16.OVA melanoma cell line (Fidler, 1975; Schuler *et al.*, 2008) maintained in cDMEM supplemented with G418-sulphate (geneticin selective antibiotic) (at 1 mg/ml)
   Note: Adherent cells detached using 0.25 % Trypsin-EDTA.

B. Mice

1. All mice used in this protocol were between 6 and 12 weeks of age and were sex and age matched for each individual experiment. Three mice were used per group in each experiment.
2. CD45.1 congenic (B6.SJL-PtprcaPep3b/BoyJArc), bred in-house at the SPF Animal Facility of the UNIL
3. OT-I mice (Hogquist *et al.*, 1994)
4. OT-IIxFoxp3-eGFP mice [referred to as OT-II (Barnden *et al.*, 1998, Wang *et al.*, 2008)] bred in-house at the SPF Animal Facility of the UNIL

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C. Antibodies

1. Va2 and Vb5.1/5.2 antibodies (BD Biosciences)
2. CD45 PE antibody (BD Biosciences)
3. CD45.1 APC-eFluor780 (eBioscience)
4. CD45.2 pacific blue (eBioscience)
5. CD8 eFluor700 (eBioscience)
6. CD4 PE-Texas red (eBioscience)
7. 2.4G2 (Anti-FcgRII monoclonal antibody)

D. Buffers and media

1. Dulbecco's modified Eagle's medium (DMEM), high glucose, GlutaMAX™ supplement (Life Technologies, Gibco®, catalog number: 10566-016)
2. 1 M HEPES (Life Technologies, Gibco®, catalog number: 15630-080)
3. Penicillin-streptomycin (5,000 U/ml) (Life Technologies, Gibco®, catalog number: 15070063)
4. Fœtal bovine serum (FBS). (performance sera with low endotoxin : qualified, US origin) (Life Technologies, catalog number: 26140 or similar)
5. Geneticin® selective antibiotic (G418 Sulfate, 50 mg/ml) (Life Technologies, Gibco®, catalog number: 15630-080)
6. 0.25% Trypsin-EDTA (1x) (phenol red) (Life Technologies, Invitrogen™, catalog number: 25200056)
7. Phosphate buffered saline (PBS) (Laboratorium Dr. Bichsel AG)
8. Collagenase, Type I (Life Technologies, Gibco®, catalog number: 17018-029)
9. DNAse I (Roche Diagnostics, catalog numner: 04536282001)
10. UltraPure™ 0.5 M EDTA (pH 8.0) (Life Technologies, Invitrogen™, catalog number: 15575-020)
11. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
12. Complete Dulbecco's modified Eagle's medium (cDMEM) (see Recipes)
13. Tumor digesting buffer (see Recipes)
14. Flow Buffer (see Recipes)
15. MACS Buffer (see Recipes)

E. Peptides

1. OVA257-264 and OVA323-339 peptides

   Note: Peptides were manufactured by the Protein and Peptide Chemistry Facility (PPCF) of the UNIL.

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F. Adjuvants
1. Poly (I: C) HMW (tlrl-pic) and Imiquimod R837 (tlrl-imq, InvivoGen)
2. CpG-ODN 1826 (Coley Pharmaceuticals. No longer available. CpG-ODN 1826, tlrl-1826 from InvivoGen can be substituted.)
3. Quil A saponin mix from Quillaja saponaria (Brenntag Nordic A/S)

G. Commercial reagents
1. CD45 MACS microbeads, mouse (Miltenyi Biotec, catalog number: 130-052-301)
Datasheet available online:
https://www.miltenyibiotec.com/~/media/Images/Products/Import/0001200/IM0001245.ashx
2. LIVE/DEAD Aqua cell stain (Life Technologies, Invitrogen™, catalog number: L34957)

Equipment

1. Falcon™ 40 µm cell strainer (blue) (Corning, catalog number: 352340)
2. 1ml BD Tuberculin Syringe & 26 g or 27 g x 0.5" BD™ PrecisionGlide Needle (BD, catalog number: 3052111 or 305109)
3. Falcon™ 50 ml conical centrifuge tubes (Corning, catalog number: 352070)
4. Falcon™ 15 ml conical centrifuge tubes (Corning, catalog number: 352099)
5. Falcon™ 6 well, non-treated, flat-bottom tissue culture (Corning, catalog number: 351147)
6. Dissection scissors or scalpel blade and fine-tipped forceps or tweezers
7. 10 ml BD™ syringe (catalog number: 309604) with 18 g or 20 g x 1" BD™ PrecisionGlide needle (BD, catalog number: 305195 or 305175)
8. Haemocytometer
9. AutoMACS automatic cell separator (Miltenyi Biotech)
10. FACSCanto flow cytometers (BD)

Software

1. FlowJo software for Mac, version 9 software (TreeStar)
2. Prism Graphpad software
Note: It was used to perform One-way Anova test combined with the Dunnet's post test for statistical analysis of 3 samples per group, per experiment. Experiments were repeated twice for statistical robustness.

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Procedure

A. Adoptive cell transfer, tumor challenge and immunization

1. Adoptive cell transfers

   Antigen-specific $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells were isolated from spleens of $CD45.2^+$ T cell receptor (TCR)-transgenic mice. Spleens were disrupted over 40 µm cell strainers with the plastic end of a 1 ml tuberculin syringe plunger and collected into 50 ml Falcon tubes. Total cell numbers were determined by counting with a Haemocytometer. The frequency of OT-I and OT-II cells in the spleens was determined by labelling with TCR Va2 and Vb5.1/5.2 antibodies and analysis by flow cytometry before injection of total splenocytes containing the desired number of antigen-specific T cells (Figure 1). Naive CD45.1 recipient mice received 1e6 OT-I cells and 3e6 OT-II cells in 200 µl of DMEM intravenously in the caudal vein.

   Note: T cells can also be transferred by intra-orbital or intra-peritoneal injection if preferred.

2. Tumor challenge

   Mice were challenged the next day with 2e5 B16.OVA tumor cells in 100 µl PBS injected subcutaneously in the left flank. Briefly, mice were restrained using a single-handed hold. About 2/3 of the length of the needle of the tuberculin syringe was inserted into the subcutaneous layer of skin proximal to the joint of the hind leg, at an upward and sideways angle. The correct position of the end of the needle was checked by gently moving the bevel below the skin (just about the hip joint) and confirming it was in loose space. The tumor cells were slowly injected in a single location. The needle was withdrawn carefully and the exit point massaged slightly with the fingertip to prevent escape of the tumor cells.

3. Immunisations

   a. One week later, once tumors were palpable, mice were immunised with 10 µg $OVA_{257-264}$ and 10 µg $OVA_{323-339}$ peptides in 100 µl PBS subcutaneously at the base of the tail with a 1 ml tuberculin syringe. Briefly, mice were restrained in a holding tunnel with an opening at the end to access the base of the tail. About half the length of the needle of the tuberculin syringe was inserted into the subcutaneous layer of skin just above and to the side of the tail. The correct depth was confirmed by visualizing the bevel just below the skin. The vaccine was slowly injected while keeping pressure at the needle entry point.

      Note: A bubble of liquid should become visible under the skin if the correct technique is applied.

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b. Peptides were injected alone or in combination with 50 μg of one of the following adjuvants: CpG-ODN (CpG), HMW Poly (I: C), imiquimod, or Quil A.

B. Tissue harvest and processing

1. One week after vaccination, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Tumors were exposed by cutting through the skin of the flank and excised by carefully grasping with fine-tipped forceps and snipping away the surrounding skin and connective tissue using sharp dissection scissors. Tumors were then placed in pre-weighed 15 ml Falcon tubes containing 2 ml PBS.
2. Tubes containing tumors were weighed again and the initial weight of the tube and buffer subtracted to determine tumor mass.
3. Tumors larger than 500 μg were cut down to ~500 μg for processing, to avoid overloading of MACS column and as the number of cells obtained in larger tumors are in excess of requirement for flow cytometric analysis.
4. Tumors were cleaned of connective tissue and each tumor was placed in a 6 well plate in 1 ml of fresh Tumor digesting buffer. Tumors were dissected by chopping with scissors or by slicing repeatedly with a scalpel blade to produce pieces of ~2 mm^2 or less. A further 9 ml of Tumor digesting buffer was added to each well and mixed with the tissue by pipetting gently.
5. Tumor tissue was then incubated at 37 °C for one hour to allow digestion, swirling the plate or pipetting the suspension up and down after 30 min to mix.
6. At the end of the incubation the tissue pieces were aspirated several times through a 10 ml syringe with a large bore needle (18-20 gauge) to break up remaining tissue and obtain a single cell suspension.
7. Cells were washed 2-3 times in 50 ml cDMEM and centrifuged at 1,500 rpm for 10 min to remove excess melanin.
8. A rough total cell count was performed using a haemocytometer to determine the amount of MACS beads required.

C. $CD45^+$ cell selection by magnetic cell sorting with the AutoMACS

1. Tumor cells were transferred to 50 ml Falcon tubes and resuspended in 90 μl MACS buffer per 10^7 cells, as per the manufacturer's protocol.
2. 10 μl anti-CD45 beads were added per 10^7 cells and the cell and bead mixture was incubated on ice for 15 min with mixing by inversion every 5 min.
3. Cells were then washed twice in 50 ml MACS buffer to remove excess beads.
4. Cells were then resuspended in 500 μl MACS buffer by gentle pipetting and passed over a 40 μm cell strainer to remove any remaining clumps.

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5. $CD45^+$ cells were purified by positive selection using magnetic cell separation (MACS) beads and the AutoMACS automatic cell separator. Cell supensions were mixed by vortex at low speed just before passing them through the autoMACS system using a POSSEL program.
6. The positive fraction was counted using a haemocytometer to determine the total TIL number before proceeding to FACS staining of the cells.

   Notes:

   a. *The POSSEL program passes the sample through the autoMACS using a single pass over one magnetic column and is designed to obtain a particular cell population by positive selection from a sample in which a normal to high frequency of the cells express the antigen of interest.*
   b. *If an autoMACS machine is not available then manual MACS columns (Miltenyi Biotech) may be used instead. The manual separation procedure can be found in the CD45 MACS microbead datasheet (see section G).*
   c. *The purity and yield of the sort can be checked by labeling a small aliquot of the pre-sort and post-sort positive and negative fractions with a CD45 PE antibody and analysis by flow cytometry (Figure 2). After the first 2 experiments, yielding >90% $CD45^+$ cells in the positive fraction, purity testing was no longer performed before the global FACS analysis.*

D. Flow cytometry

1. ~5 x 10^6 cells (or the whole sample if less than this number were obtained) were transferred to a 96 well u-bottom plate and washed with PBS.
2. Cells were resuspended in 100 µl Live/dead Aqua cell stain at a 1/250 dilution in PBS and incubated for 20 min before washing twice with PBS.
3. 25 µl of supernatant from a 2.4G2 (anti-FcgRII monoclonal antibody) producing hybridoma (grown in-house) was added to the samples and incubated on ice for 5-10 min to inhibit nonspecific antibody binding.
4. A mix of fluorescent antibodies at 2x final concentration was made in Flow buffer and 25 µl of the mix was added to the cells without first removing the 2.4G2.
5. Cells were incubated for a further 20-30 min and the plate was then washed twice with 200 µl per well of Flow buffer.
6. Cells were resuspended in 200 µl of Flow buffer and kept on ice until analysis.
7. Samples were acquired using LSR-II and FACSCanto flow cytometers.
8. Lymphocytes were gated on the basis of forward scatter and side scatter properties and LIVE/DEAD Aqua cell stain was used to exclude dead cells.
9. Flow data was analyzed using FlowJo software (Figure 3).

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E. Multi-colour staining panel
1. Live/dead dye Aqua cell stain.
2. CD45.1 APC-eFluor780, CD45.2 Pacific Blue, CD8 eFluor700, CD4 PE-Texas Red (optional labeling with Va2 APC and Vb5.1/5.2 PE for more precise identification of TCR transgenic T cells).
3. Foxp3 eGFP (endogenous expression).
4. As well as the multi-colour staining mix used to label the samples, single stains were made for each fluorochrome (and blood from a Foxp3-eGPF mouse was extracted by nicking of the caudal vein for the GFP single stain).
5. The auto compensation option in the BD Diva software was used to compensate the spectral overlap between all the different fluorochromes.

Representative data

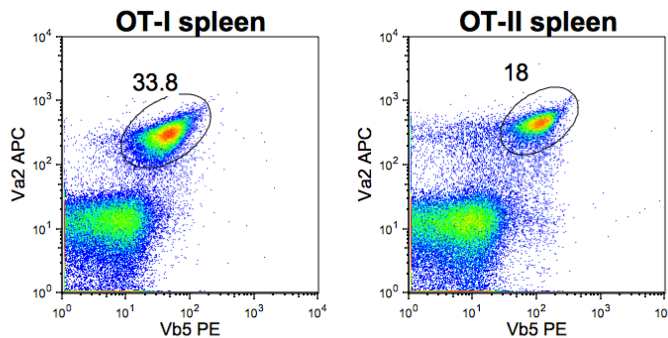

Figure 1. Determining TCR transgenic T cell frequency by Flow cytometry prior to adoptive T cell transfer (lymphocyte population gated based on forward and side scatter properties)

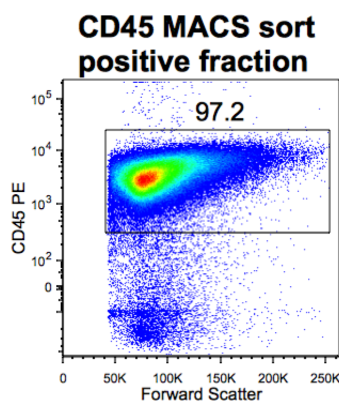

Figure 2. CD45 cell purity after MACS positive selection (single cells first gated based on forward scatter height vs area and live cells detected using Live/dead dye Aqua cell stain)

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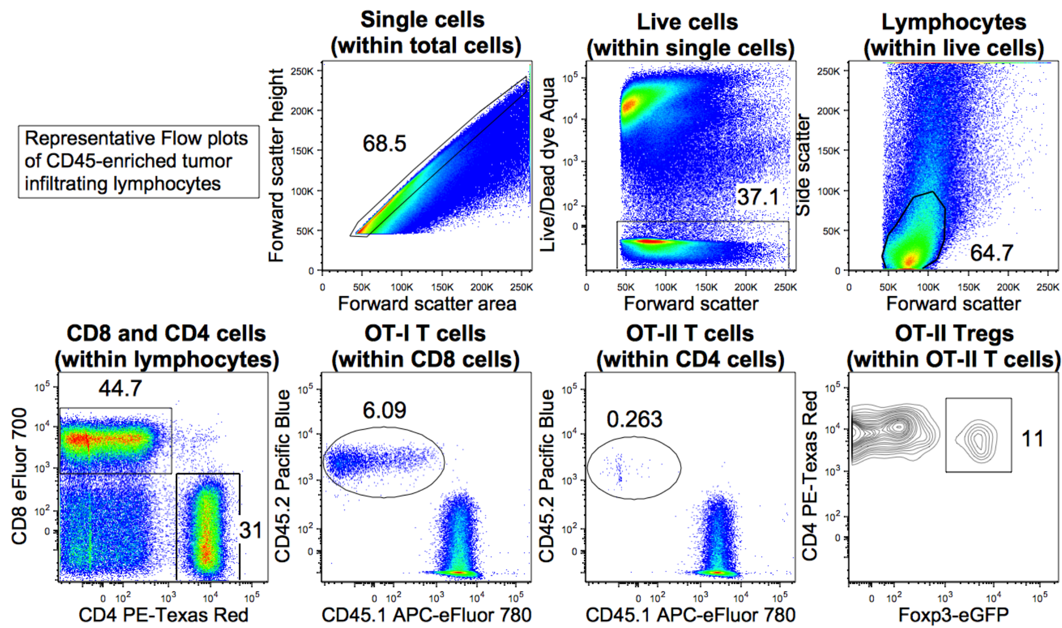

Figure 3. Representative Flow plots showing progressive gating strategy for analysis of tumor infiltrating lymphocytes in CD45 MACS-enriched tumor samples

Recipes

1. Complete medium (cDMEM)
   DMEM
   5% FBS
   100 U/ml penicillin & 100 µg/ml streptomycin
   25mM Hepes
   55 µM 2-ME
2. Tumor digesting buffer
   DMEM
   4.4 mg/ml Collagenase I
   10 µg /ml DNase I
3. Flow buffer
   PBS
   2% FBS
4. MACS buffer
   PBS
   1% BSA
   10 mM EDTA

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