Supplementary Material For:

Effective flow cytometric phenotyping of cells using minimal amounts of antibody

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Materials and methods

Animals

FVB mice and mammary carcinoma susceptible congenic rats harboring a Wistar-Furth genetic background were bred and maintained in an AAALAC-approved facility as previously published (13-14). All animal protocols were approved by the University of Wisconsin Medical School Animal Care and Use Committee (13-14).

Isolation of mouse and rat lymphocytes

Mouse or rat spleens were aseptically removed and placed in sterile dishes containing RPMI 1640 media (Invitrogen, Grand Island, NY, USA). Single-cell suspensions were prepared by gently pressing the spleen through a sterile 100 µm nylon mesh (BD Biosciences, San Jose, CA, USA). Splenocytes were centrifuged and red blood cells were lysed by brief hypotonic shock using 4.5ml of ice cold water for 15 sec. Red blood cell lysis was stopped by adding 500µl 10x PBS (ice cold). Leukocytes were washed and resuspended in RPMI medium. Live cells were counted by trypan blue exclusion.

Isolation of mouse mammary epithelial cells

Mouse mammary glands were harvested and finely scissor-minced over ice. The minced mammary glands were individually digested by horizontal shaking for 6 hours in 1 ml EPICULT-B media (StemCell Technologies, Vancouver, BC, Canada) containing 1x EPICULT-B Supplement, hydrocortisone at 1 mg/ml final concentration, 10% Fetal Bovine Serum (FBS; Hyclone, Thermo Scientific, Waltham, MA, USA), and 1x collagenase/hyaluronidase (StemCell Technologies). The cells were pelleted by centrifugation at 450 x *g* for 5 min. The digestion media and fat were removed by pipetting and the pellet was resuspended in 1 ml of modified Hank's Balanced Salt Solution (StemCell Technologies) containing 2% FBS (HF). Four ml of room temperature ammonium chloride solution (StemCell Technologies) was added to lyse the red blood cells. After 1 minute incubation, red blood cell lysis was stopped by adding 5 ml of HF and immediate centrifugation to pellet the cells. To trypsinize the cells, the pellet was resuspended in 2 ml of a prewarmed (37°C) Gibco Hank's Balanced Salt Solution (Invitrogen), containing 0.2% EDTA (Sigma-Aldrich, St. Louis, MO, USA) and 0.025% of Trypsin (Worthington Biochemical Corp., Lakewood, NJ, USA). Following a 5 min incubation, the reaction was stopped by adding 4 ml of Gibco DMEM/F12 (Invitrogen) containing 10% FBS. The cells were washed in DMEM/F12/10%FBS, passed through a 40 µm nylon filter (BD Biosciences) and stored on ice until antibody staining.

CFSE staining

For in vitro activation, 100 million lymphocytes were stained with 1 µM Carboxy fluorescein diacetate succinimidyl ester (CFSE; Invitrogen) in 1 ml RPMI medium for 8 min at 37°C and washed three times using ice-cold RPMI medium (Invitrogen) containing 10% FBS (Hyclone). Two million lymphocytes were stimulated with 1 µg/ml concanavalin A (Sigma) or plate bound CD3 (5 µg/ml) and soluble CD28 (1 µg/ml) antibodies (BD Biosciences) and were cultured in 2 ml RPMI medium containing 10% FBS for 4 days at 37°C in a 5% CO_2 atmosphere. Unstimulated cells served as a control.

Antibody and Hoechst staining

The antibodies for mouse CD3-APC, CD4-PE-cy5, CD8-APC-cy5, CD24-PE-cy7, CD29-FITC, CD31-APC, CD44-APC-cy7, CD45-APC, CD49f-PE-cy5, CD61-PE, γδTCR-PE and rat CD3-APC, CD4-PE-cy5, CD8-FITC, CD161 α, and γδTCR-PE (BD Biosciences) were used. The CD161α antibody was labeled with Dylight405 dye (Thermo Scientific) following manufacturer's instructions.

Lymphocytes or mammary epithelial cells (0.5, 1, 2 or 5 million) were resuspended in 1 ml ice-cold RPMI medium containing 10% serum in 1.7 ml Eppendorf tubes. Cells were pelleted by centrifuging at 7000 rpm (5000 x *g*) for 3 min and supernatant was discarded by inverting the tube. The tubes were allowed to stand for 10 sec, following which the residual medium was carefully removed without disturbing the pellet using a 200 µl tip. A master mix containing 1 µg/100 µl final concentration of each (single, five, or six) antibody was prepared and 20 or 100 µl master mix was added to the cells. Alternatively, a master mix containing 0.4 µg/20 µl of each antibody was used. The cell pellet was loosened by gently tapping, vortex mixing for 10 sec, and then incubated on ice for 20 min. Cells were washed twice, resuspended in 500 µl PBS, and fixed in 1% paraformaldehyde for 20 min. Cells were washed with 1 ml PBS, resuspended in 500 µl PBS, and stained with 1 µg/ml final concentration of Hoechst 33342 (Sigma) for 2 h.

Data acquisition and analysis

Cells were acquired on a BD LSR II flow cytometer equipped with 4 lasers (multi-line UV, 405 nm, 488 nm, and 633 nm) using FACS Diva software. Data was stored as FCS3 files and analyzed using FlowJo version 7.6.5 software (Tree Star Inc., Ashland, OR, USA). Live cells were gated based on FSC, SSC, and 2n-4n DNA content. Single cells were gated using FSC and SSC width. Single antibody stained cells and unstained control cells were used for compensation using FlowJo. The data restriction strategy is shown in Figure 3 (upper panel).

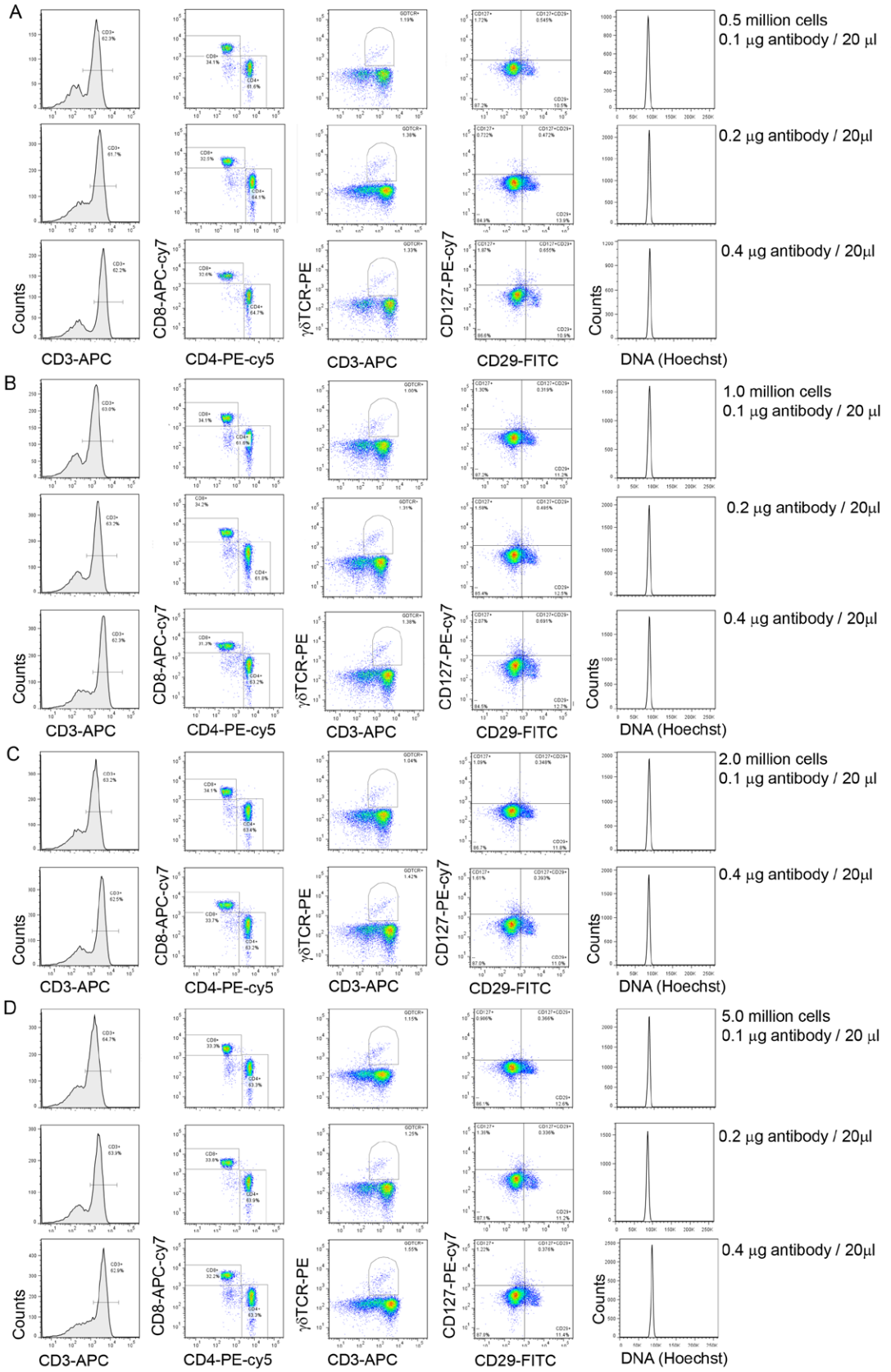

Figure S1. Seven-color flow cytometric analysis of freshly isolated lymphocytes by the classical and low-volume staining methods. Cells were stained with 0.1 μg (upper panel), 0.2 μg (middle panel), or 0.4 μg (lower panel) of CD3, CD4, CD8, CD29, CD127, and γδTCR antibodies followed by Hoechst. The histograms of CD3, pseudo color plots of CD4/CD8, CD3/γδTCR, CD29/CD127, and histograms of DNA content (Hoechst) in (A) $0.5x10^6$, (B) $1x10^6$, (C) $2x10^6$ and (D) $5x10^6$ cells are shown. Data shown are from one of three samples and two such independent experiments were carried out.

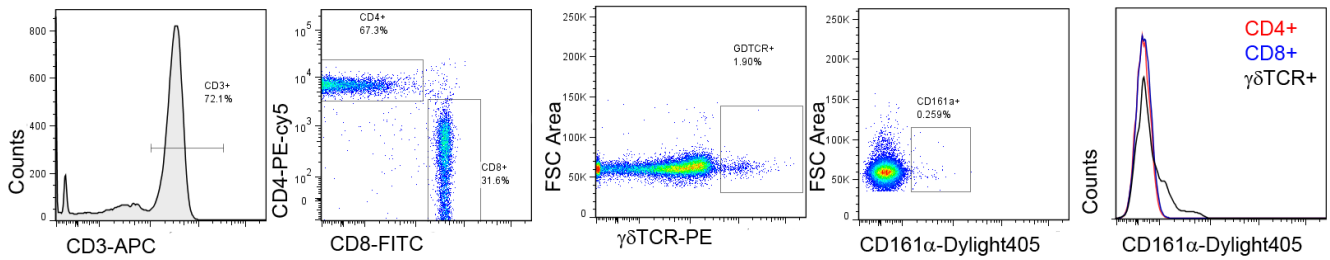

Figure S2. Flow cytometric phenotyping of rat lymphocyte subpopulations by a modified staining method. Lymphocytes ($2x10^6$) from rat inguinal lymph nodes were stained with 0.2 µg of CD3-APC, CD8-FITC, CD4-PE-cy5, γδTCR-PE, and CD161α-Dylight405 antibodies in 20 µl staining volume. CD3+ cells (histogram), CD4+ and CD8+ cells within CD3+ population (pseudo color plot), CD3+γδTCR+ cells (pseudo color plot) and CD161α+ NK cells (pseudo color plot) are shown. Overlaid histograms show CD161α expression in CD4+ (red line), CD8+ (blue line), and γδTCR+ (black line) T cells. Data shown are from one of three samples and two such independent experiments were carried out.

Table S1. Z scores for different cell populations identified by flow cytometry using titrating amount of antibodies and varying number of cells.

No of cells ($x10^6$)	Staining volume (µl)	Amount of antibody (µg)	CD3+ cells (%)	Z score	CD3+CD4+ cells (%)	Z score	CD3+CD8+ cells (%)	Z score	γδTCR+ cells (%)	Z score	CD3+ CD29+ cells (%)	Z score	CD3+ CD127+ cells (%)	Z score
0.5	20	0.1	60.6	1.62	61.8	-0.84	34.1	-1.23	1.2	1.44	10.7	-0.23	1.67	-1.29
0.5	20	0.2	64.4	0.00	63.4	-1.87	34	-1.13	1.29	0.96	12	-3.21	1.48	-0.68
0.5	20	0.4	66.1	-0.73	63.7	-2.06	32.9	-0.09	1.57	-0.53	11.2	-1.38	1.84	-1.84
1	20	0.1	64.3	0.04	60.9	-0.26	33.9	-1.04	0.996	2.52	11	-0.92	1.06	0.68
1	20	0.2	65.1	-0.30	61.9	-0.90	34	-1.13	1.22	1.33	11.4	-1.83	1.45	-0.58
1	20	0.4	62.5	0.81	63.1	-1.68	31.1	1.60	1.31	0.85	11.2	-1.38	1.6	-1.06
2	20	0.1	61.5	1.24	63.1	-1.68	34.4	-1.51	0.955	2.74	11.6	-2.29	1.11	0.52
2	20	0.2	62.8	0.68	61.2	-0.45	33.1	-0.28	1.32	0.80	10.5	0.23	1.4	-0.42
2	20	0.4	61.8	1.11	64	-2.26	32.9	-0.09	1.44	0.16	11.2	-1.38	1.71	-1.42
5	20	0.1	65.2	-0.34	62.8	-1.48	33.4	-0.57	1.08	2.07	12.5	-4.36	0.946	1.05
5	20	0.2	61	1.45	61.9	-0.90	32.7	0.09	1.27	1.06	11.2	-1.38	0.888	1.23
5	20	0.4	64	0.17	62.4	-1.23	33.1	-0.28	1.65	-0.96	12	-3.21	1.19	0.26
2	100	1.0	64.4	0.00	60.5	0.00	32.8	0.00	1.47	0.00	10.6	0.00	1.27	0.00

Cells (0.5-5 million) were stained with 0.1, 0.2 or 0.4 µg antibody and CD3+, CD4+, CD8+, γδ-T cell receptor+, CD29+ or CD127+ cells were gated using FlowJo software. Cellular DNA content was measured by Hoechst 33342 staining. The data from each sample was compared with that from 2 million cells stained with these antibodies in 100 µl volume. Z score was calculated using formula $z=(x-\mu)/\sigma$ where x=mean of test sample, µ= mean of sample stained by classical method and σ= standard deviation. Data shown is from mean of three samples and two such independent experiments were carried out.

Table S2. Coefficient of variation of different populations identified by flow cytometry using titrating amount of antibodies and varying number of cells.

No of cells ($x10^6$)	Staining volume (µl)	Amount of antibody (µg)	CV of DNA content in CD3+ cells	CV of width of CD3+ cells	CV of CD3+CD4+ cells	CV of CD3+CD8+ cells	CV of γδTCR+ cells	CV of CD3+ CD29+	CV of CD3+ CD127+ cells
0.5	20	0.1	3.54	50.1	23.4	24.6	66.8	78.5	290
0.5	20	0.2	3.46	42.3	21.1	22.8	78.3	66.1	149
0.5	20	0.4	3.20	39.3	21.0	22.7	87.1	55.8	274
1.0	20	0.1	3.33	50.7	23.0	24.6	60.2	53.7	360
1.0	20	0.2	3.47	45.7	22.0	24.3	57.4	70.6	288
1.0	20	0.4	3.42	42.7	22.0	23.9	65.5	76.9	116
2.0	20	0.1	3.93	48.4	22.4	24.6	77.1	75.9	329
2.0	20	0.2	3.40	47.7	21.9	23.5	70.4	50.9	210
2.0	20	0.4	3.31	40.8	23.4	23.0	66.7	53.9	213
5.0	20	0.1	3.41	51.1	20.8	23.9	65	56.4	288
5.0	20	0.2	3.45	45.6	20.6	22.8	79.6	63.1	242
5.0	20	0.4	3.20	43.2	23.0	23.4	87.1	58.0	320
2.0	100	1.0	3.40	40.3	21.3	23.0	80.7	67.4	245

Cells (0.5-5 million) were stained with 0.1, 0.2 or 0.4 µg antibody and CD3+, CD4+, CD8+, γδ-TCR+, CD29+ or CD127+ cells were gated using FlowJo software. Cellular DNA content was measured by Hoechst 33342 staining. Mean CV for each individual gated population from three replicates is shown and two independent experiments were carried out.