HIT: a versatile proteomics platform for multi-analyte phenotyping of cytokines, intracellular proteins and surface molecules

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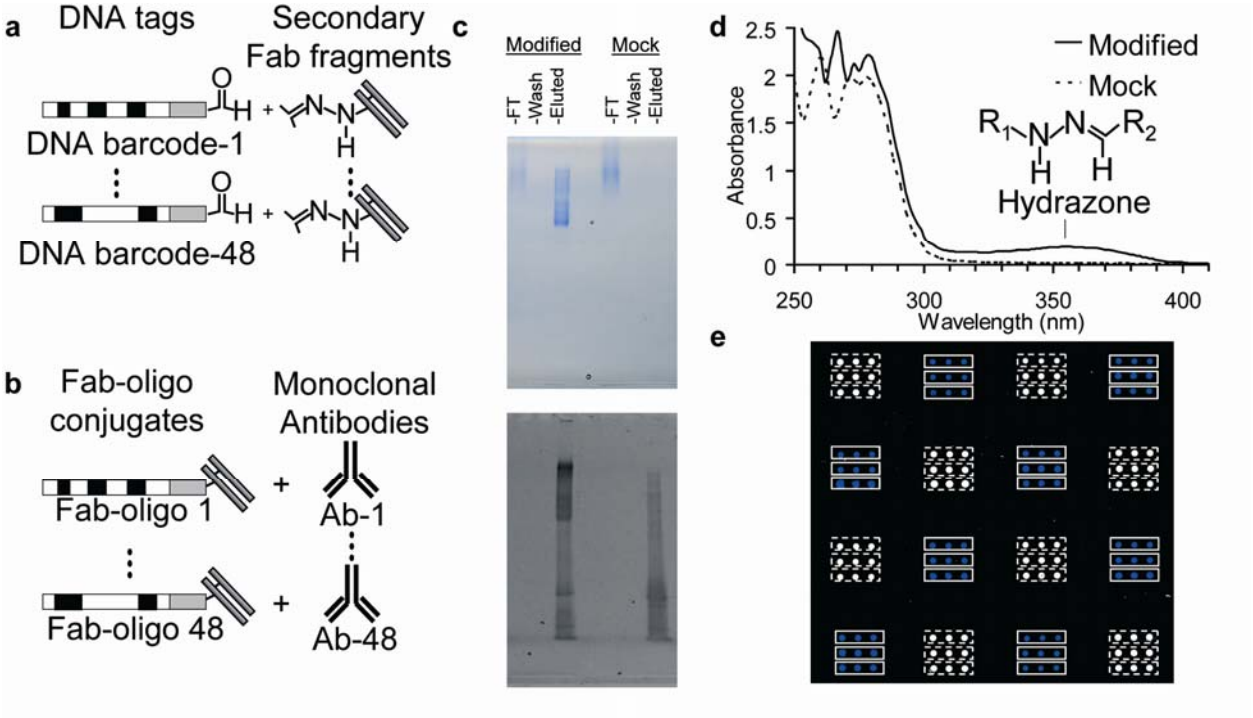
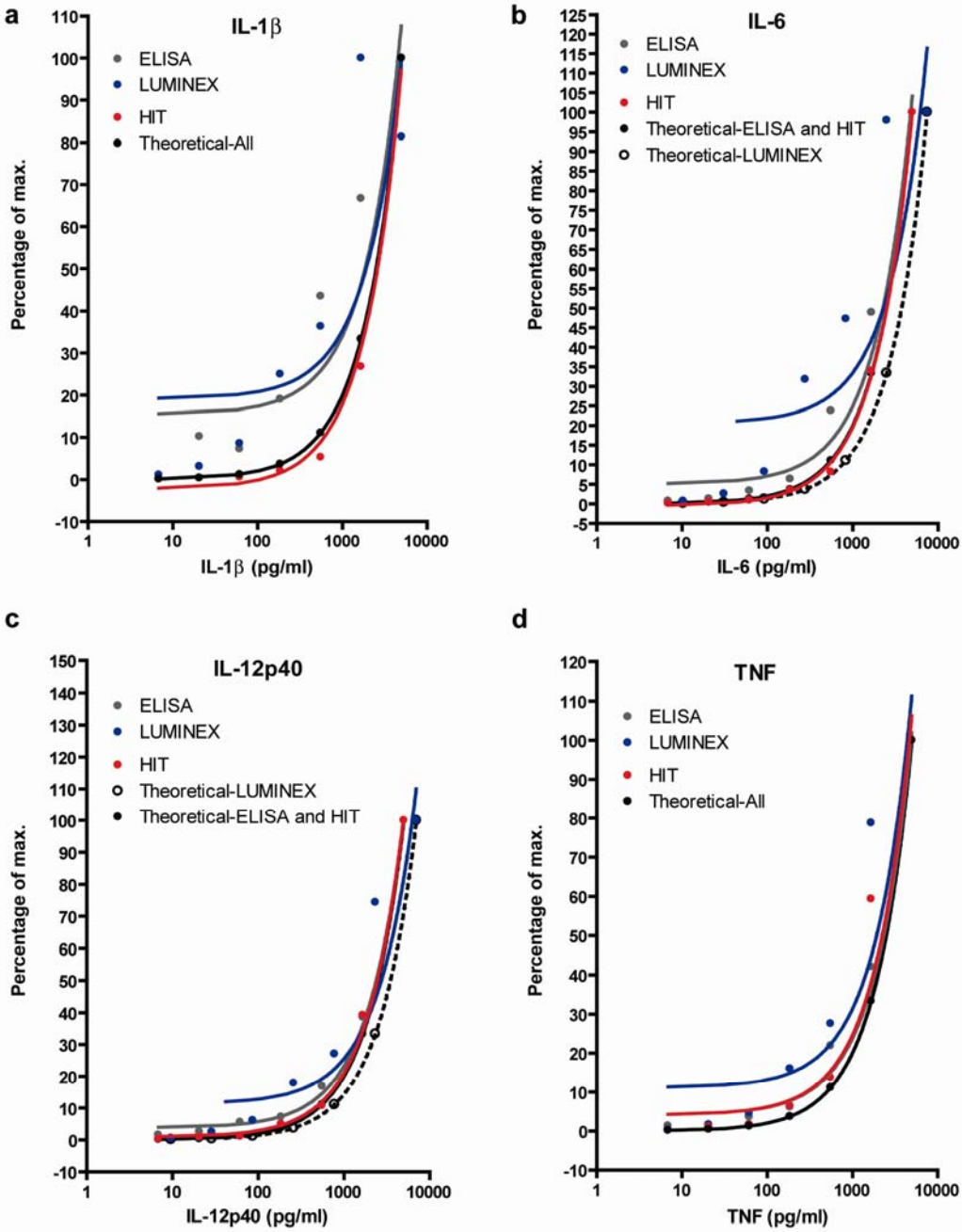
Supplementary Figures



Supplementary Figure 1. Antibody-oligo coupling and production of oligonucleotide microarrays. (**a**) Schematic of covalent conjugation of hydrazine-modified Fab fragments and aldehyde-modified DNA oligonucleotides to form 48 separate Fab-oligo conjugates. (**b**) Schematic of Fab-oligo pre-incubation with specific monoclonal antibodies. (**c**) Scanned image of native PAGE of oligo-modified and mock-modified Fab fragments stained for protein (top panel) and nucleic acid (bottom panel). The lanes labeled flow-through (FT), Wash, and Eluted refer to fractions of an ion-exchange spin-column purification. (**d**) Absorbance spectrum of oligo-modified and mock-modified Fab fragments. A schematic of the hydrazone bond indicates the wavelength of peak absorbance. (**e**) Scanned image of an array probed with amplified, fluorescently-labeled tags from a HIT cocktail. In this experiment, one sample contained all 48 of the Fab-oligo conjugates (Cy3, pseudo-colored blue), and the other sample contained only 24 of the Fab-oligo conjugates (Cy5, pseudo-colored yellow). Equal emission in both channels is pseudo-colored white. Triplicate features of the 48 tags are surrounded by boxes; features corresponding to tags from the Cy5 reaction are surrounded by hatched boxes.



e

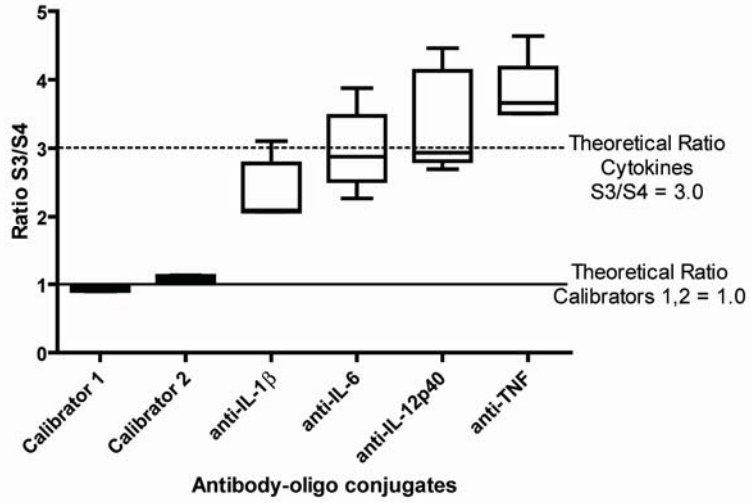
r^2	ELISA	Luminex	HIT
IL-1β	0.8609	0.5641	0.9940
IL-6	0.9700	0.6764	0.9990
IL-12p40	0.9973	0.8598	0.9965
TNF	0.9866	0.8355	0.9407
Mean	0.9537*	0.7340	0.9826*

f

Slope	Theoretical-ELISA and HIT	Theoretical-Luminex	ELISA	Luminex	HIT
IL-1β	0.0200	0.0200	0.019 ± 0.003	0.016 ± 0.006	0.020 ± 0.001
IL-6	0.0200	0.0133	0.020 ± 0.002	0.013 ± 0.004	0.020 ± 0.0003
IL-12p40	0.0200	0.0143	0.019 ± 0.0004	0.014 ± 0.003	0.020 ± 0.0005
TNF	0.0200	0.0200	0.020 ± 0.001	0.020 ± 0.004	0.021 ± 0.002

Supplementary Figure 2. Comparison of ELISA, Luminex®, and HIT to the theoretical curve generated by serial three-fold dilution of IL-1β, IL-6, IL-12p40, and TNF. Recombinant human IL-1β, IL-6, IL-12p40, and TNF were serially diluted in tissue culture media and detected using conventional single-analyte ELISA or HIT. Luminex® data are from a representative standard curve generated in a multiplex assay using the Luminex® standards and bead pairs. For HIT, biotinylated anti-cytokine detection antibodies were coupled to mSA-oligo during pre-incubation. The y-values were expressed as percentage of maximum intensity (Percentage of max.), fit using linear regression, and displayed with cytokine concentration on a $\log_{10}$ scale to better visualize low cytokine concentrations. (**a**–**d**) Graphs of Percentage of max. versus cytokine concentration for (**a**) IL-1β, (**b**) IL-6, (**c**) IL-12p40, and (**d**) TNF. Note: The Luminex® standards for IL-6 and IL-12p40 begin at 7500 and 7000 pg/ml respectively, so the theoretical curves for the Luminex® data sets are displayed separately for these cytokines. (**e**–**f**) Tables of (**e**) r^2 and (**f**) slope for ELISA, Luminex®, HIT, and the theoretical curves. *$p<0.05$ vs. Luminex® by repeated measures one-way ANOVA with Bonferroni multiple comparison post-test. No other pairings were statistically significant.

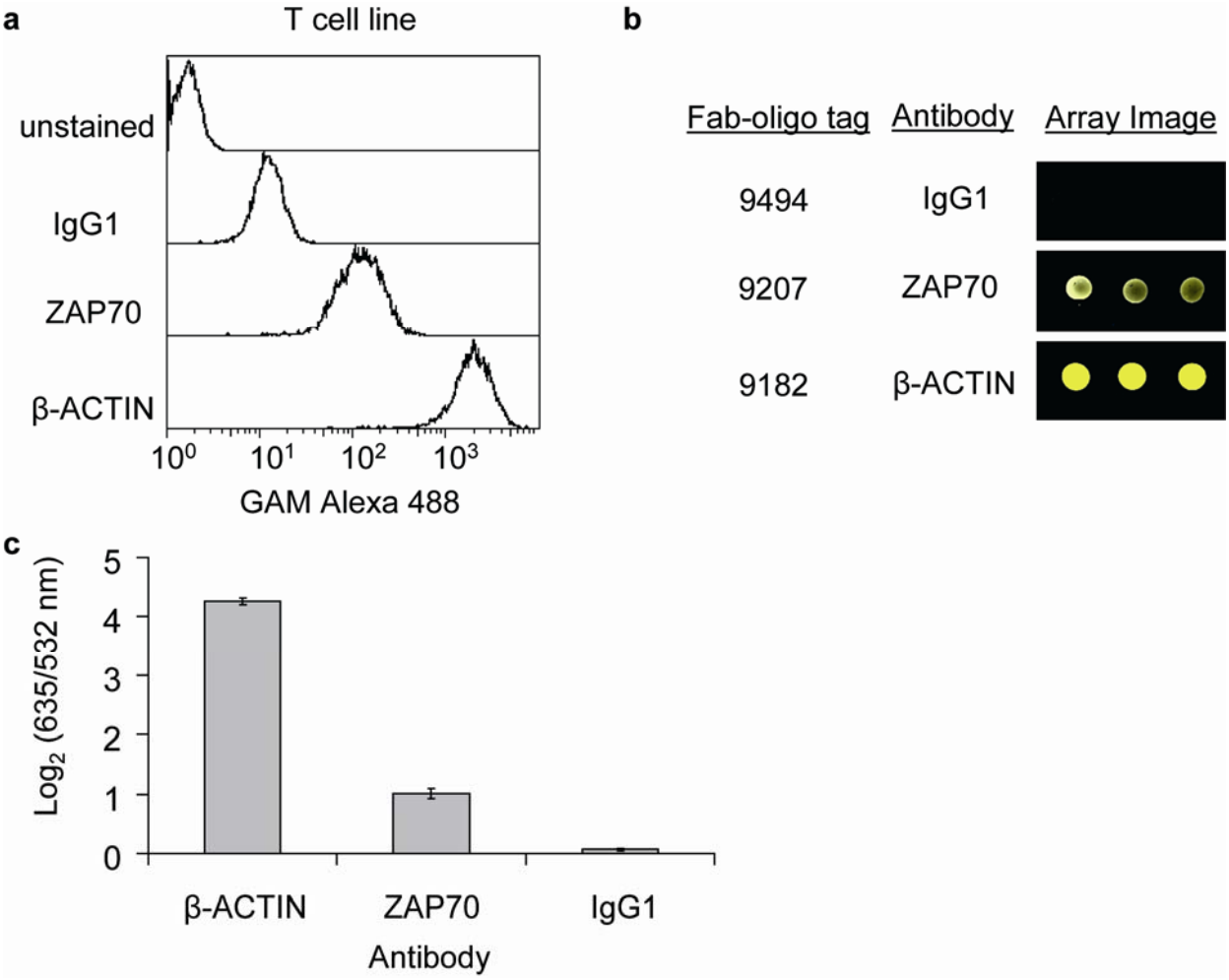
a



b

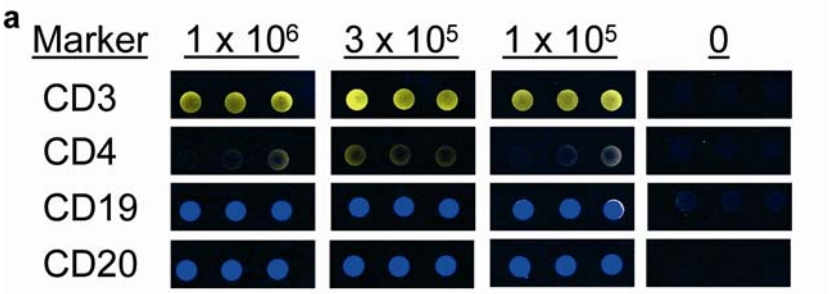
	Calibrator 1	Calibrator 2	IL-1β	IL-6	IL-12p40	TNF
Theoretical ratio	1	1	3	3	3	3
Measured ratio (mean)	0.93	1.08	2.36	2.97	3.36	3.81
%CV	3.7%	4.2%	18.9%	19.7%	22.1%	12.4%

Supplementary Figure 3. HIT %CV for 5 replicate measurements of cytokine standards. Five independent replicates of the third and fourth standard (S3 and S4, respectively), from the three-fold serial dilution curve in **Supplementary Fig. 2** were measured using HIT. (**a**) Box and whiskers plot of S3/S4 ratio measured by HIT. The theoretical ratio is 3.0 (hatched line). The calibrators Calibrator 1 and Calibrator 2 are control tags spiked equally into all wells prior to amplification. The theoretical ratio for the calibrator tags is 1.0 (continuous line). (**b**) Table of mean measured ratio, theoretical ratio, and coefficient of variation (%CV) for each marker.



Supplementary Figure 4. HIT detects intracellular proteins. Fixed and permeabilized Jurkat T cells were stained separately with two HIT cocktails. The positive control cocktail (Cy5-UTP, pseudo-colored yellow) contained an IgG1 isotype control antibody, anti-ZAP70, and anti-β-ACTIN conjugated to 9494, 9207, or 9182, respectively. The negative control cocktail (Cy3-UTP, pseudo-colored blue) contained all tags conjugated to IgG1. (**a**) Histogram plots of fluorescent intensity after staining Jurkat T cells with anti-ZAP70, anti-β-ACTIN**,** or an isotype control. (**b**) Scanned images and (**c**) Log_2 (635nm/532nm) of tags 9494, 9207, or 9182.

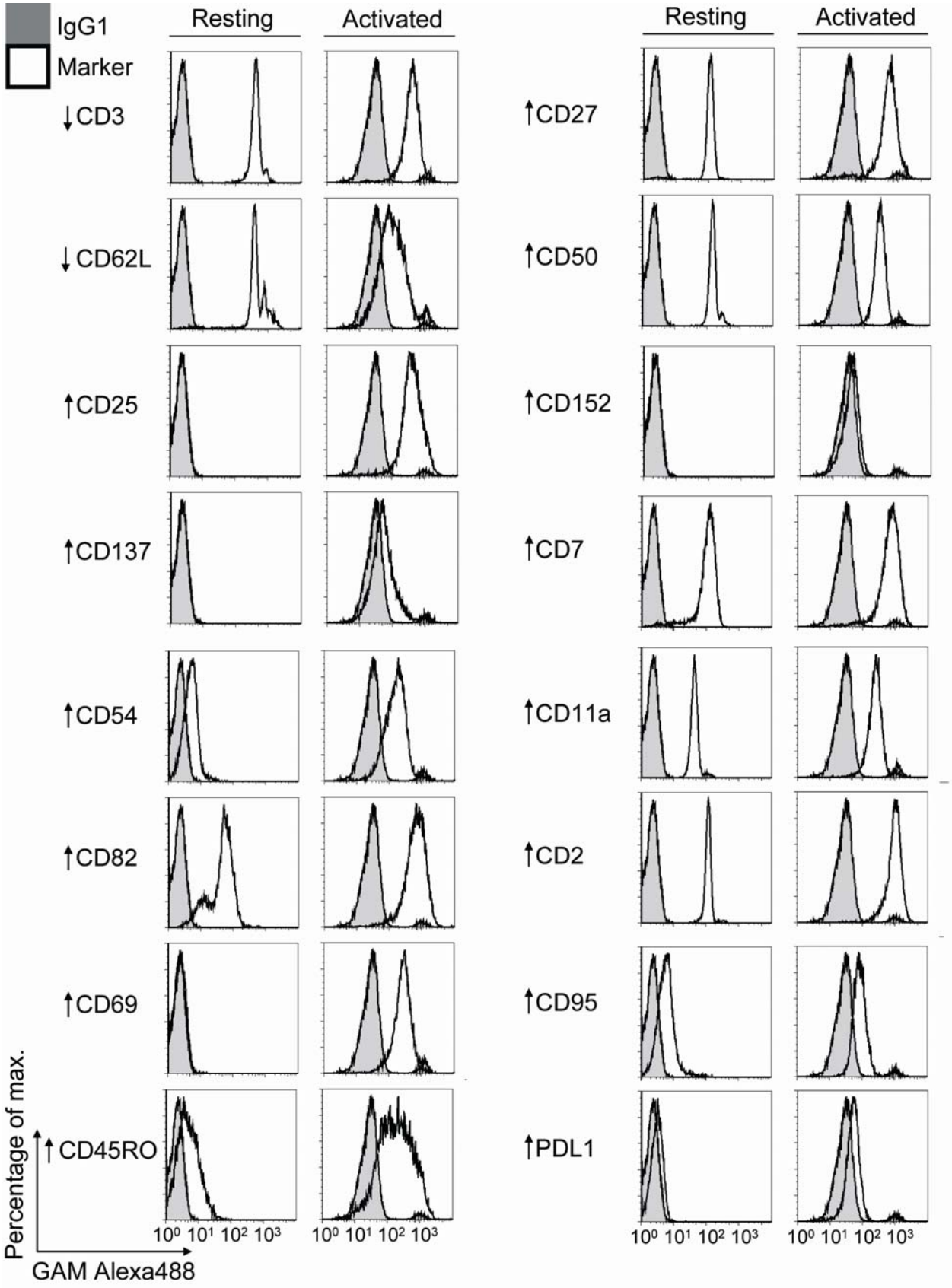
a



b

Marker	1 x 10^6	3 x 10^5	1 x 10^5	0
CD3	5.0	4.4	4.9	-0.7
CD4	1.9	2.0	2.0	-0.3
CD19	-5.9	-5.9	-5.2	0.4
CD20	-6.4	-6.5	-5.5	-0.5

Supplementary Figure 5. Dilution of cells for surface marker detection. Dilutions of Jurkat T cells and OCI B cells were stained with a HIT cocktail containing anti-CD3, anti-CD4, anti-CD19, and anti-CD20 antibodies. During T7 polymerase amplification, Cy5-UTP was added to the T cell reaction and Cy3-UTP was added to the B cell reaction. (**a**) Scanned images and (**b**) Log_2 (635nm/532nm) of array features corresponding to tags linked to anti-CD3, anti-CD4, anti-CD19, and anti-CD20. Cy5 is pseudo-colored yellow, Cy3 is pseudo-colored blue, and equivalent Cy5 and Cy3 intensity is pseudo-colored white.


IgG1
Marker
Resting
Activated
↓CD3
↓CD62L
↑CD25
↑CD137
↑CD54
↑CD82
↑CD69
↑CD45RO
↑CD27
↑CD50
↑CD152
↑CD7
↑CD11a
↑CD2
↑CD95
↑PDL1
Percentage of max.
GAM Alexa488
10⁰ 10¹ 10² 10³

Supplementary Figure 6. Validation of T cell activation markers by flow cytometry. Purified $CD4^{+}CD45RO^{-}CD25^{-}$ human T cells were either rested or activated for 48 h with anti-CD3/anti-CD28 coated magnetic beads. Shown are representative histogram plots of fluorescent intensity from one donor after staining 1-2 x 10^5 resting or activated T cells with primary antibodies directed against each marker listed and an IgG1 isotype negative control, followed by secondary detection with a goat anti-mouse IgG antibody conjugated to Alexa488 (GAM Alexa488). An arrow indicates whether the HIT screen (**Fig. 4**) identified the marker as being up- or down-regulated upon activation.

Supplementary Tables

Tag ID	Sequence
99	5'-AAAAAAAAAATTCAACCTCATCCGAGTGGCTCCAATAGGACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
9802	5'-AAAAAAAAAACTTCGCGGAGTGCGATCTAAAGTAGACTGACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
9640	5'-AAAAAAAAAAGAAGTATTCCTCGAGGGGATCAGCGTGATACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
9494	5'-AAAAAAAAAAGTGGTTTGCTAATGCCAGAAATGACCCGCACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
9207	5'-AAAAAAAAAAAGCGGATACTATGCCTTCTGAGCAGCTCAACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
9182	5'-AAAAAAAAAAAGCACAGGAGTTACTAGCTAAGGCGTTTCCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
9165	5'-AAAAAAAAAAAATGCAGTACAAGGACAACGGGTCGGTCTTTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
8877	5'-AAAAAAAAAACCAGTGCAGCTGTTCCCAGCCGCACCGATGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
8567	5'-AAAAAAAAAATGGATGTCGGCTAACGTAGCCGAATCACTACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
8552	5'-AAAAAAAAAAAGCGTAGGCCAGGGCCTGTAGAAAATATTGTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
8430	5'-AAAAAAAAAAAGACGCTCAGGGTTGGGTGCATTAGAATACCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
8226	5'-AAAAAAAAAATCGCGGAACCGGAGTCAATGTACAACTTTGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
8122	5'-AAAAAAAAAACGCGTTGCAAAGGGACCCGTTTACCATTAACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
7794	5'-AAAAAAAAAAATCTACTCAAGCAGAGACTGAGACGTTTGGGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
7553	5'-AAAAAAAAAAAGTCAGTTTCGGCCGTAGCTAATGAAGCAGACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
7425	5'-AAAAAAAAAAAGCTGATTTCAGTGATGGCCAGAGATAGCCACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
7130	5'-AAAAAAAAAAAATGGCGAGAATGCGAAGCTTCCCTATGTAGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
6991	5'-AAAAAAAAAAAAGGCTCGATGATTTACACAGAGCATTGGCCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
6861	5'-AAAAAAAAAAATACACGATACCAGATTCATAGGTTGCCGGCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
6491	5'-AAAAAAAAAAATCAGTTTAACTAGCAGTCCGTCGGCAAGACCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
641	5'-AAAAAAAAAATTAAGTGTCTACAACCTGACCACCACTCGCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
5896	5'-AAAAAAAAAAATGAACTGGCGATAGATGATGGCACGTTGAGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
5891	5'-AAAAAAAAAAAGTGATATAAATCGCGCCCACATTTCGCAGGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
5757	5'-AAAAAAAAAAACCAGAGGCATTGCGGAACACTGCTGTAATTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
5509	5'-AAAAAAAAAAACTCGAACCAACAACTGTGTGGGATTGCATGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
5104	5'-AAAAAAAAAAATACCTATCAGAACAGATTGGCTGGGCGCTACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
5062	5'-AAAAAAAAAAATGCGTTATCAGAGCCCTAACCCCAATTAGCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
4820	5'-AAAAAAAAAAACAGGGATAATTCTCCCAGGTCATCACTGAGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
4810	5'-AAAAAAAAAAACCTATGGACAGTCGGTAAAAGCTACCCTGTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
4227	5'-AAAAAAAAAAAACGTCATTATAGGCAGGCTGGATCAACTCCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
4174	5'-AAAAAAAAAAAGCACTTAGTATATATCGCGGGGGGGAAAGTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
4118	5'-AAAAAAAAAAACATCGAACTCGGTGAGATAGGGGTGGTAATCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
3767	5'-AAAAAAAAAAAAATGGTAGGCCGATATTGCCTACTACGGACCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
3381	5'-AAAAAAAAAAAATTCCCGCCAGGTGACAGTTTGCACTAAGACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
3297	5'-AAAAAAAAAAAACTTGGTAGTGAGAGGTGATAATCCACCCCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
3218	5'-AAAAAAAAAAATGAATTACCCACACTAGGAGTCGGTAGTCGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
3171	5'-AAAAAAAAAAGAAAGATGTTGTGCGAAATGTCCAGCCTGGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
2347	5'-AAAAAAAAAAAAAACCTGAGTACGCTCGCAGGTTATCGTGACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
2233	5'-AAAAAAAAAAGGAAAATTTTCAGCCCCATGGGATGGACGTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
2202	5'-AAAAAAAAAAAACAACTTACCGTTACACACGTGGGTGGAGTCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
2186	5'-AAAAAAAAAAATGCCCGCGCCACTACTTGTGGTCGAGGGCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1967	5'-AAAAAAAAAAATCATAATACCGGAGGGCATGATTCAGTGGGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1698	5'-AAAAAAAAAAGCTATGGACCGGCGGCAATTTTATGAGAACCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1606	5'-AAAAAAAAAAAATGTGAAGAGTGTTCAGCTCGACGGACTACCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1326	5'-AAAAAAAAAAGAGCCTTAAGGCTATTGGTTGACCACCGAACCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1247	5'-AAAAAAAAAAGGTAGAGGGCTAACCCGCATTTTGGTAAAGCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1064	5'-AAAAAAAAAACAGAACAGATGTTTTCGGACGTAGCTGAGCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'
1040	5'-AAAAAAAAAATACCCGAATAGGGGCTCAAAGGTCATGTTCCCCTATAGTGAGTCGTATTAGGAATTCCAT-3'

Supplementary Table 1. Oligonucleotide barcode template strand sequences.

Tag ID	Sequence
99	5’-TTCAACCTCATCCGAGTGGCTCCAATAGGA-3’
9802	5’-CTTCGCGGAGTGCGATCTAAAGTAGACTGA-3’
9640	5’-GAAGTATTCCTCGAGGGGATCAGCGTGATA-3’
9494	5’-GTGGTTTGCTAATGCCAGAAATGACCCGCA-3’
9207	5’-AGCGGATACTATGCCTTCTGAGCAGCTCAA-3’
9182	5’-AGCACAGGAGTTACTAGCTAAGGCGTTTCC-3’
9165	5’-ATGCAGTACAAGGACAACGGGTCGGTCTTT-3’
8877	5’-CCAGTGCAGCTGTTCCCAGCCGCACCGATG-3’
8567	5’-TGGATGTCGGCTAACGTAGCCGAATCACTA-3’
8552	5’-GCGTAGGCCAGGGCCTGTAGAAAATATTGT-3’
8430	5’-AGACGCTCAGGGTTGGGTGCATTAGAATAC-3’
8226	5’-TCGCGGAACCGGAGTCAATGTACAACTTTG-3’
8122	5’-CGCGTTGCAAAGGGACCCGTTTACCATTAA-3’
7794	5’-TCTACTCAAGCAGAGACTGAGACGTTTGGG-3’
7553	5’-GTCAGTTTCGGCCGTAGCTAATGAAGCAGA-3’
7425	5’-GCTGATTTCAGTGATGGCCAGAGATAGCCA-3’
7130	5’-ATGGCGAGAATGCGAAGCTTCCCTATGTAG-3’
6991	5’-AGGCTCGATGATTTACACAGAGCATTGGCC-3’
6861	5’-TACACGATACCAGATTCATAGGTTGCCGGC-3’
6491	5’-TCAGTTTAACTAGCAGTCCGTCGGCAAGAC-3’
641	5’-TTAAGTGTCTACAACCTGACCACCACTCGC-3’
5896	5’-TGAACTGGCGATAGATGATGGCACGTTGAG-3’
5891	5’-GTGATATAAATCGCGCCCACATTTCGCAGG-3’
5757	5’-CCAGAGGCATTGCGGAACACTGCTGTAATT-3’
5509	5’-CTCGAACCAACAACTGTGTGGGATTGCATG-3’
5104	5’-TACCTATCAGAACAGATTGGCTGGGCGCTA-3’
5062	5’-TGCGTTATCAGAGCCCTAACCCCAATTAGC-3’
4820	5’-CAGGGATAATTCTCCCAGGTCATCACTGAG-3’
4810	5’-CCTATGGACAGTCGGTAAAAGCTACCCTGT-3’
4227	5’-ACGTCATTATAGGCAGGCTGGATCAACTCC-3’
4174	5’-GCACTTAGTATATATCGCGGGGGGGAAAGT-3’
4118	5’-CATCGAACTCGGTGAGATAGGGGTGGTAAT-3’
3767	5’-AATGGTAGGCCGATATTGCCTACTACGGAC-3’
3381	5’-ATTCCCGCCAGGTGACAGTTTGCACTAAGA-3’
3297	5’-ACTTGGTAGTGAGAGGTGATAATCCACCCC-3’
3218	5’-TGAATTACCCACACTAGGAGTCGGTAGTCG-3’
3171	5’-GAAAGATGTTGTGCGAAATGTCCAGCCTGG-3’
2347	5’-AAACCTGAGTACGCTCGCAGGTTATCGTGA-3’
2233	5’-GGAAAATTTTCAGCCCCATGGGATGGACGT-3’
2202	5’-ACAACTTACCGTTACACACGTGGGTGGAGT-3’
2186	5’-ATGCCCGCGCCACTACTTGTGGTCGAGGGC-3’
1967	5’-TCATAATACCGGAGGGCATGATTCAGTGGG-3’
1698	5’-GCTATGGACCGGCGGCAATTTTATGAGAAC-3’
1606	5’-ATGTGAAGAGTGTTCAGCTCGACGGACTAC-3’
1326	5’-GAGCCTTAAGGCTATTGGTTGACCACCGAA-3’
1247	5’-GGTAGAGGGCTAACCCGCATTTTGGTAAAG-3’
1064	5’-CAGAACAGATGTTTTCGGACGTAGCTGAGC-3’
1040	5’-TACCCGAATAGGGGCTCAAAGGTCATGTTC-3’

Supplementary Table 2. Oligonucleotide barcode microarray sequences. For microarray printing, a 5’ primary amine with a six carbon spacer was added during synthesis.

Antibody Target	Clone	Commercial Source	Catalog Number
ABCG2 (Bcrp1, MXR)	5D3	eBioscience	14-8888-80
αβ-TCR	IP26	eBioscience	14-9986-80
B7-DC(PD-L2)	MIH18	eBioscience	14-5888-80
B7-H1(PD-L1)	MIH1	eBioscience	14-5983-80
B7RP-1 (B7h, B7-H2, GL50, ICOS-L)	MIH12	eBioscience	14-5889-80
BAMBI (BMP and activin membrane-bound inhibitor)	4E8	eBioscience	14-5826-80
BLyS (BAFF, TALL-1, THANK)	1D6	eBioscience	14-9017-80
CCR7(EBI-1, CD197)	CCR7.6B3	eBioscience	14-9977-80
CD10 (CALLA)	SJ5-1B4	eBioscience	14-0107-80
CD103	B-Ly7	eBioscience	14-1038-80
CD116	4H1	eBioscience	14-1169-80
CD117 (c-Kit)	YB5.B8	eBioscience	14-1179-80
CD11a	HI111	eBioscience	14-0119-80
CD11b	ICRF44	eBioscience	14-0118-80
CD11c	3.9	eBioscience	14-0116-80
CD123	6H6	eBioscience	14-1239-80
CD124	X2/45-12	eBioscience	14-1249-80
CD131	1C1	eBioscience	14-1319-80
CD134 (OX-40)	ACT35 (ACT-35)	eBioscience	14-1347-80
CD135 (Flk2/Flt3)	BV10A4H2	eBioscience	14-1357-71
CD138	DL-101	eBioscience	14-1389-80
CD150 (SLAM)	A12 (7D4)	eBioscience	14-1509-80
CD152 (CTLA-4)	14D3	eBioscience	14-1529-80
CD154	24-31	eBioscience	14-1548-80
CD16a (FcγRIII Receptor)	MEM-154	eBioscience	14-0169-80
CD18(ß2 integrin)	CLB-LFA-1/1	eBioscience	14-0187-80
CD180 (RP105)	MHR73-11	eBioscience	14-1809-80
CD1a	HI149	eBioscience	14-0019-80
CD2	RPA-2.10	eBioscience	14-0029-80
CD205 (DEC-205)	MG38	eBioscience	14-2059-80
CD24 (HSA)	SN3	eBioscience	14-0247-80
CD26	2A6	eBioscience	14-0269-80
CD27	O323	eBioscience	14-0279-80
CD28	CD28.2	eBioscience	14-0289-80
CD33 (GP67, P67)	WM-53	eBioscience	14-0338-80
CD38	HIT2	eBioscience	14-0389-80
CD40	5C3	eBioscience	14-0409-80
CD43	L10	eBioscience	14-0437-80
CD45	HI30	eBioscience	14-0459-80
CD45RA	JS-83	eBioscience	14-9979-80
CD45RB	MEM-55	eBioscience	14-7457-80
CD45RO	UCHL1	eBioscience	14-0457-80
CD49d	9F10	eBioscience	14-0499-80
CD5	UCHT2	eBioscience	14-0059-80
CD50 (ICAM-3)	TP1/25.1	eBioscience	14-0507-80
CD54 (ICAM-1)	HA58	eBioscience	14-0549-80
CD62L (L-selectin)	DREG-56	eBioscience	14-0629-80
CD64 (FcγRI)	10.1	eBioscience	14-0649-80
CD69 (VEA)	FN50	eBioscience	14-0699-80
CD7	CD7-6B7	eBioscience	14-0077-80
CD79b	SN8	eBioscience	14-0797-71
CD80 (B7-1)	2D10.4	eBioscience	14-0809-80
CD83	HB15e	eBioscience	14-0839-80
CD86 (B7-2)	IT2.2	eBioscience	14-0869-80
CD9	MM2/57	eBioscience	14-0099-80
CD94	DX22	eBioscience	14-0949-80
CD95 (Fas, Apo-1)	DX2	eBioscience	14-0959-80
Fas-L (CD95-L) Ligand	NOK-1	eBioscience	14-9919-80
CD99 (E2 antigen, MIC2)	3B2/TA8	eBioscience	14-0997-71
CXCR4 (CD184, Fusin, LESTR, or HUMSTR)	12G5	eBioscience	14-9999-80
DcR1 (TRAIL-R3, TRID, and LIT)	DJR3	eBioscience	14-6238-80
DcR2 (TRAIL-R4, TRUNDD)	DJR4-1	eBioscience	14-6239-80
DR3 (Apo-3, WSL-1, TRAMP, LARD, DDR3, and TR3)	JD3	eBioscience	14-6603-80
DR4 (TRAIL-R1)	DJR1	eBioscience	14-6644-80
TWEAK-R(Fn14)	ITEM-1	eBioscience	14-9019-80
AITR (GITR)	T11-621	eBioscience	14-5877-80
AITRL (GITRL)	EB11	eBioscience	14-5876-80
ICOS (H4)	ISA-3	eBioscience	14-9948-80

IL-18Ra (IL-1Rrp)	H44	eBioscience	14-7183-80
PD-1	J116	eBioscience	14-9989-80
TLR1	GD2.F4	eBioscience	14-9911-80
TLR4	HTA125	eBioscience	14-9917-80
TRAIL	RIK-2	eBioscience	14-9927-80
TWEAK	CARL-1	eBioscience	14-9915-80
TLR2	T2.5	eBioscience	14-9024-80
B7-S1 (B7-H4, B7x)	H74	eBioscience	14-5949-80
CD29	MEM-101A	eBioscience	14-0297-80
CD82	53H5	eBioscience	14-0829-80
CD3	UCHT1	eBioscience	16-0038
CCR4	1G1	BD Pharmingen	551121
CD137 (4-1BB)	4B4-1	BD Pharmingen	555955
CD58	1C3	BD Pharmingen	555919
IgG1 Isotype Control	P3	eBioscience	14-4714-85
IgG2a Isotype Control	HOPC-1	Southern Biotech	0103-01
IgG2b Isotype Control	A-1	Southern Biotech	0104-01
IgG3 Isotype Control	B10	Southern Biotech	0105-01
CD4	OKT4	eBioscience	14-0048
CD3	OKT3	eBioscience	14-0037-82
CD19	HIB19	eBioscience	14-0199-82
CD20	2H7	eBioscience	14-0209-82
HSP70	C92F3A-5	Stressgen	SPA-810B
ZAP70	29	BD Biosciences	610239
Ovalbumin	OVA-14	Sigma-Aldrich	A 6075
IL-1β capture	CRM56	eBioscience	14-7018
IL-6 capture	MQ2-13A5	eBioscience	16-7069-85
IL-12p40 capture	C8.3	eBioscience	14-7127
TNF capture	MAb1	eBioscience	14-7348
IL-1β biotinylated detector	CRM57	eBioscience	13-7016
IL-6 biotinylated detector	MQ2-39C3	eBioscience	13-7068
IL-12p40 biotinylated detector	C8.6	eBioscience	13-7129
TNF biotinylated detector	MAb11	eBioscience	13-7349

Supplementary Table 3. Commercial sources of antibodies.

Supplementary Methods

Production of microarrays, hybridization, and scanning. We spotted amine-modified 30mer oligonucleotides (**Supplementary Table 2**) onto NHS-ester coated Codelink slides (GE Healthcare) at a concentration of 50 μM in 50 mM sodium phosphate buffer pH 8.5 (Sigma-Aldrich) using a BioRad VersArray Compact Microarrayer (BioRad) and processed the slides according to the manufacturer's instructions. For hybridization, purified tags were used directly after elution or placed in a microcentrifuge tube in a DNA 120 SpeedVac concentrator (Thermo Scientific) until dry. Pellets were resuspended in 16 μl hybridization buffer [20μg salmon sperm DNA (Invitrogen), 25μg yeast tRNA (Invitrogen), 4X SSC, 0.1% SDS], boiled for 1 min at 95 ^{o}C, and centrifuged for 5 min prior to hybridization. Alternatively, we concentrated the RNA tags using 10,000 NMWL centrifugal ultrafiltration columns (Millipore) in place of SpeedVac concentration. We then pipetted the sample onto a HybriSlip coverslip (Grace Bio-Labs) and placed on the microarray. The slide was placed in a microarray hybridization chamber (Corning) in a 47.5 ^{o}C circulating water bath (GE Healthcare) for 16-20 h. Slides were washed three times for 5 min in 1x TNT wash buffer [(100 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 0.05% Tween-20 (Sigma Aldrich), 2 mM EDTA], washed once for 5 min in 0.5x TNT wash buffer, rinsed for <1 s in ddH_2O and centrifuged to dryness at 600 x g for 5 min. For indirect labeling experiments (**Figure 2, Supplementary Figures 2, 3**), after hybridization slides were washed three times for 5 min in 1x TNT, and then stained for 90 min at 4 ^{o}C in 5% BSA 1xTNT containing 25 mM EDTA, 25 μg/ml salmon sperm DNA, 25μg/ml yeast tRNA, and either 1μg/ml Streptavidin Alexa 647 (Invitrogen) or 2μg/ml anti-biotin or 2μg/ml anti-digoxin (Jackson ImmunoResearch) conjugated to Alexa555 or Alexa647 (Invitrogen), respectively. The slides were then washed again as described above. Slides were vacuum sealed until scanning on the GenePix 4000 scanner (Molecular Devices). The GenePix Pro Version 5.0 software (Molecular Devices) was used to analyze the images.

Selection of DNA barcode sequences. Candidate oligonucleotide barcode sequences were generated by creating 20,000 random 30mers with equal nucleotide weights so that an average GC content of 0.5 was obtained. Each sequence was then subjected to a BLAST search against the T7 promoter sequence and all other candidate 30mers. Sequences which exhibited similarity with any other candidate or the T7 promoter sequence were excluded from final consideration. The secondary structure free energy[1] and the free energy of binding the perfect complement of each candidate were then calculated for all remaining candidates. Sequences were sorted based on the calculated binding energy and the number of thymines (T) in each sequence. A set of 48 random 30mer sequences were chosen with similar binding energies, an equal number of thymines, and no appreciable secondary structure.

Cell lines and naïve T cell isolation, culture, and flow cytometry. We cultured Jurkat T cells (clone E6-1) and OCI B cells[2] in complete RPMI-1640 [RPMI-1640 supplemented with 10% heat-inactivated FCS (Omega Scientific), 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, and 100 U/ml of penicillin streptomycin]. We obtained de-identified buffy coats from the Stanford Blood Center from normal volunteer donors without any interaction between ourselves and the donors, and without any means of re-identifying donors. Therefore, the IRB determined the research to be exempt from human subjects research approval. We enriched for $CD4^+$ T cells using RosetteSep Human $CD4^+$ T Cell Enrichment (StemCell Technologies Inc). Naïve $CD4^+$ cells were then purified to >95% purity using a Naive $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec), CD25 microbeads (Miltenyi Biotec), and an LD column (Miltenyi Biotec) which depleted non-CD4 cells, $CD45RO^+$, and $CD25^+$ cells. Cells were cultured in complete X-VIVO 15 media (Lonza) [X-VIVO 15 supplemented with 10% heat-inactivated FCS (Omega Scientific), 100 units/ml penicillin/streptomycin (Invitrogen), 14.3 μM β–mercaptoethanol (Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen)]. For T cell activation, anti-CD3/anti-CD28 coated beads (Miltenyi Biotec) were added at a bead-to-cell ratio of 1:1 and a final cell density of 1 x 10^6 cells/ml. For FOXP3-induction by TGF-β, cells were cultured with 5 ng/ml of TGF-β (R&D Systems) at a density of 2 x 10^6 cells/ml with 1 x 10^6 anti-CD3/anti-CD28/anti-CD2 coated beads/well (Miltenyi

Biotec) in 24-well plates (Falcon). Cells were first transferred to a 12-well plate (Falcon) at 72 h, and then to small culture flasks at 96 h, and finally analyzed at 120 h. Prior to surface marker analysis, beads were removed using a Dynal MPC-S magnet (Invitrogen). For analysis by flow cytometry, cell were stained with 0.5 μg of antibody in FACS buffer [2% FCS, 2mM EDTA, PBS] for 30 min. at 4°C, washed, and then stained with 0.5ug of Alexa488 goat anti-mouse IgG (Invitrogen). Cells were then washed and fixed in 1.6% paraformaldehyde in FACS buffer and analyzed on a FACScan flow cytometer (BD). Validation of markers on TGF-β treated cells was performed by pre-incubating each primary antibody with FITC conjugated monovalent GAM Fab fragments (Jackson ImmunoResearch) at a 3:1 Fab:Antibody molar ratio prior to staining and fixation. FOXP3-PE (eBioscience) was used according to the manufacturer's protocol. CD25-PECy5, CD4-FITC**,** CD45RA-PECy5, CD45RO-PECy5, and CD62L-PECy5 from eBioscience were used as indicated. Flow cytometry data were analyzed using FlowJo software.

Conventional ELISA and Luminex® bead-based cytokine assays. For HSP70, ZAP70, and Ova ELISAs or HIT assays, we coated 96-well Nunc Maxisorp plates (Nalgene Nunc) with recombinant HSP70 (Nventa), recombinant ZAP70 (Sigma-Aldrich Corp.), and purified Ovalbumin (Sigma-Aldrich Corp.) in pH 9.6 coating buffer [100 mM sodium carbonate] for 1 h at 21-23 °C. Plates were washed four times with PBS 0.05% Tween-20 (Sigma Aldrich) containing 2mM EDTA (PBST). Plates were then blocked with 1% BSA PBST for 1 h at 21-23 °C, probed with a 1:1,000 dilution of primary antibody in 1% BSA PBST for 45 min, washed six times in PBST, probed with a 1:5,000 dilution of Goat anti-mouse IgG HRP-conjugated secondary antibody (Jackson ImmunoResearch) in 1% BSA PBST for 1 h, and washed four times in PBST. ELISAs were developed using tetramethylbenzidine (TMB) substrate (Pierce), stopped with 2 M sulfuric acid and scanned at 450 nm using a SpectraMax 190 (Molecular Devices). For IL-1β, IL-6, IL-12p40, and TNF ELISAs, capture antibodies (**Supplementary Table 3**) were coated at 1 μg/ml in PBS overnight at 4 °C. The plates were then washed 4 times in PBST, blocked with 3% FCS PBST and then probed with three-fold serial dilutions of recombinant IL-1β, IL-6, IL-12p40, and TNF (eBioscience) spiked into complete X-VIVO 15 media. Wells were then probed by conventional ELISA and HIT. For HIT analysis the detection antibodies were pre-incubated with mSA-oligo conjugates and processed as described above. Luminex® cytokine standards were analyzed by multiplex bead-based arrays using the Beadlyte Human 22-Plex Multi-Cytokine Detection System (Upstate-Cell Signaling Solutions) and analyzed on the Luminex® 200 instrument according to the manufacturer's protocol.

References

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2. Tweeddale, M.E. et al. The presence of clonogenic cells in high-grade malignant lymphoma: a prognostic factor. *Blood* **69**, 1307-14 (1987).