Supporting Online Material

Supplementary Figures

Figure S1. A schematic example of flow cytometry subpopulation frequency measurements used in order to determine TIL profile. (A) Multi color flow cytometry was used for the measurement of multiple subpopulations frequencies within a single sample. By using a series of gates one can count the occurrence of each subpopulation and compute its fraction (i.e. the number of cells in a given subpopulation divided by the total number of cells). (B) Visualization of a TIL's subpopulation frequencies using a color coded vector.

Figure S2. Different subpopulations show a wide range of fraction distributions. Each subplot corresponds to a histogram of subpopulation fractions. The x-axis is the subpopulation fraction (i.e. the number of cells in a TIL that belong to a certain subpopulation divided by the total number of cells in the TIL). The y-axis counts the number of TILs (out of 91) showing each fraction.

Figure S3. Single staining of surface markers reveals strong correlations and anti-correlations. A correlation matrix between different receptor levels is shown (positive and negative correlations are indicated by hot and cold colors respectively). Rows and columns are clustered according to similarity (see Material and Methods). Several clusters emerge. For example, CD152, CD94 and CD85 are all in the same cluster consistent with their common function as inhibitory receptors. CD25 is also in that cluster, which might be explained by its tendency to appear on the surface of regulatory subpopulations.

Figure S4. The subpopulation coefficients of each principle component show distinct patterns. The subpopulation coefficients for the first and second (A and B respectively) principle components are shown (see PCA in Fig 5C). The first principle component is dominated by four types of subpopulation derivatives: CD4+ , CD28- , CD8+ and CD28-, where CD4+ and CD28- have an opposite direction to CD8+ and CD28-. Notably, the first principle component reflects a clear transition from non-reactivity to reactivity (even though the PCA algorithm did not take into account this type of information when computing the principle components coefficients). The second principle component contains a more complex mixture of subpopulations with no apparent phenotypic interpretation.

Figure S5. Rational subpopulation manipulation can cause reactive TILs to become non-reactive and accompanied by a shift in subpopulation signature.

(A) IFN-γ levels of 5 TILs before and after rational subpopulation manipulation that was aimed at reducing TIL reactivity are compared. All 5 TILs show a significant decrease in TIL reactivity ranging from ~10 to ~400 fold decrease. (B) The shift in reactivity can be explained in terms of a shift in subpopulation signature. The subpopulation fractions of the 5 TILs prior and after subpopulation manipulation are shown. Rows and columns correspond to different subpopulations and TILs respectively. Two ways unsupervised clustering was performed on the rows and columns. The 5 reactive TILs prior to manipulation are designated by a blue color and the letter 'P'. The 5 TILs after manipulation are designated by the letter 'A' with red and yellow corresponding to non-reactive and reactive respectively. The shift in reactivity was accompanied by a shift in signature as indicated by the red arrows connecting each corresponding TIL pairs prior and after manipulation. (C) The transformation of a reactive TIL to a non-reactive one

can be described as a path between two points in the subpopulation space. A simple representation of the TIL sub-population space was generated by applying PCA, which is a method for dimensionality reduction (see Material and methods). The data was reduced from 35 to two dimensions. The x and y axes are principle components capturing 67% and 11% of the total variance in the data. The x-axis captures a shift from CD8+ and CD28- enriched subpopulation to CD4+ and CD28+ subpopulations while the y-axis reflects a combination of additional subpopulations. The figure shows a two distinct subspaces that are overpopulated with reactive and non-reactive TILs. The change in reactivity can be visualized as a path from a nonreactive TIL to a TIL that resides in the reactive subspace (for example see dotted arrow).

Supplementary Tables

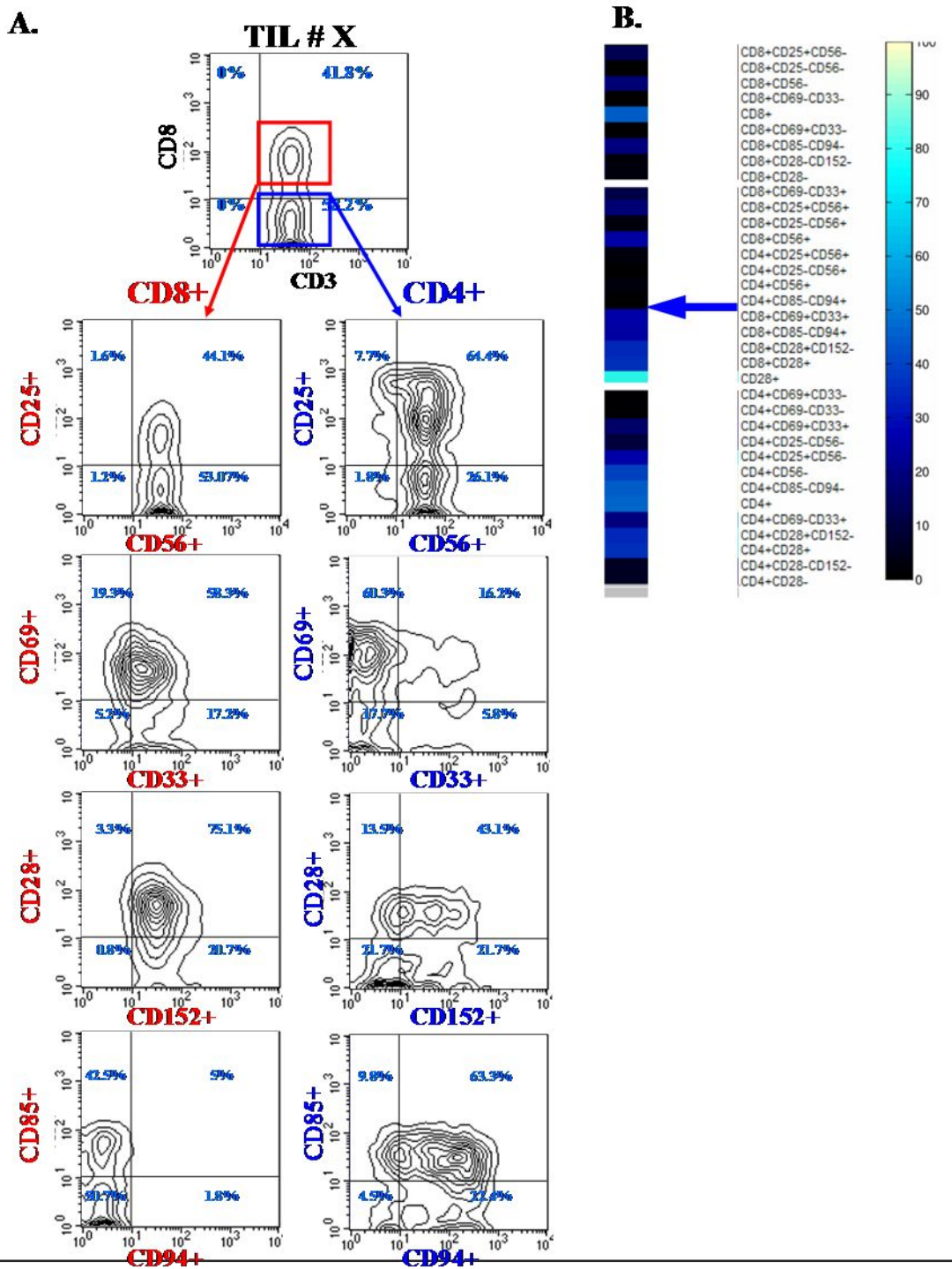
Table SI. TILs information. A tumor mass was removed from 27 patients from each of which two to five TILs were extracted resulting in 91 TILs. Of the 91 TILs, 39 were reactive and 52 non-reactive. The degree of reactivity in terms of IFN-γ secretion as well as reactivity classification is specified for each TIL. Some TILs show different reactivity levels even when extracted from the same patient and same tumor mass.

Table SII. A list of subpopulations used for TIL characterization. (A) The subpopulations composition of each TIL was measured using flow cytometry. The staining included a combination of CD4 and CD8 together with the couples: CD25, CD56 or CD33, CD69 or CD28, CD152 or CD85, CD94. In addition the intracellular levels of perforin and granzymeB were measured in CD3 positive cells. This resulted in 102 features characterizing each TIL (see Excel S1). (B)

Subpopulation filtering procedure (see Material and Methods) resulted in a final data set containing 33 features (see also Excel S2).

Table SIII. Reactivity changes of 12 fresh TILs before and after subpopulation manipulation. Information on IFN-γ levels of 12 fresh TILs prior and after subpopulation manipulation are shown. 10 of the 12 TILs were also characterized in terms of their subpopulation content. The table shows fraction of two major subpopulations, CD4 and CD8, prior and after manipulation. The two remaining TILs (marked ND) did not have sufficient number of cells for such a characterization. The clinical 200 pg/ml threshold on the levels of IFN-γ was used to classify TILs as reactive and non-reactive. 9 of the 12 TILs become reactive after subpopulation manipulation with IFN-γ fold change of up to 106 for TIL #2. We note that the viability of the manipulated cells was above 95%.

Figure S1


A.
TIL # X
CD8
CD3
0%
41.8%
0%
58.2%
CD8+
CD4+
CD25+
CD56+
CD69+
CD33+
CD28+
CD152+
CD85+
CD94+
B.
CD8+CD25+CD56-
CD8+CD25-CD56-
CD8+CD56-
CD8+CD69-CD33-
CD8+
CD8+CD69+CD33-
CD8+CD85-CD94-
CD8+CD28-CD152-
CD8+CD28-
CD8+CD69-CD33+
CD8+CD25+CD56+
CD8+CD25-CD56+
CD8+CD56+
CD4+CD25+CD56+
CD4+CD25-CD56+
CD4+CD56+
CD4+CD85-CD94+
CD8+CD69+CD33+
CD8+CD85-CD94+
CD8+CD28+CD152-
CD8+CD28+
CD28+
CD4+CD69+CD33-
CD4+CD69-CD33-
CD4+CD69+CD33+
CD4+CD25-CD56-
CD4+CD25+CD56-
CD4+CD56-
CD4+CD85-CD94-
CD4+
CD4+CD69-CD33+
CD4+CD28+CD152-
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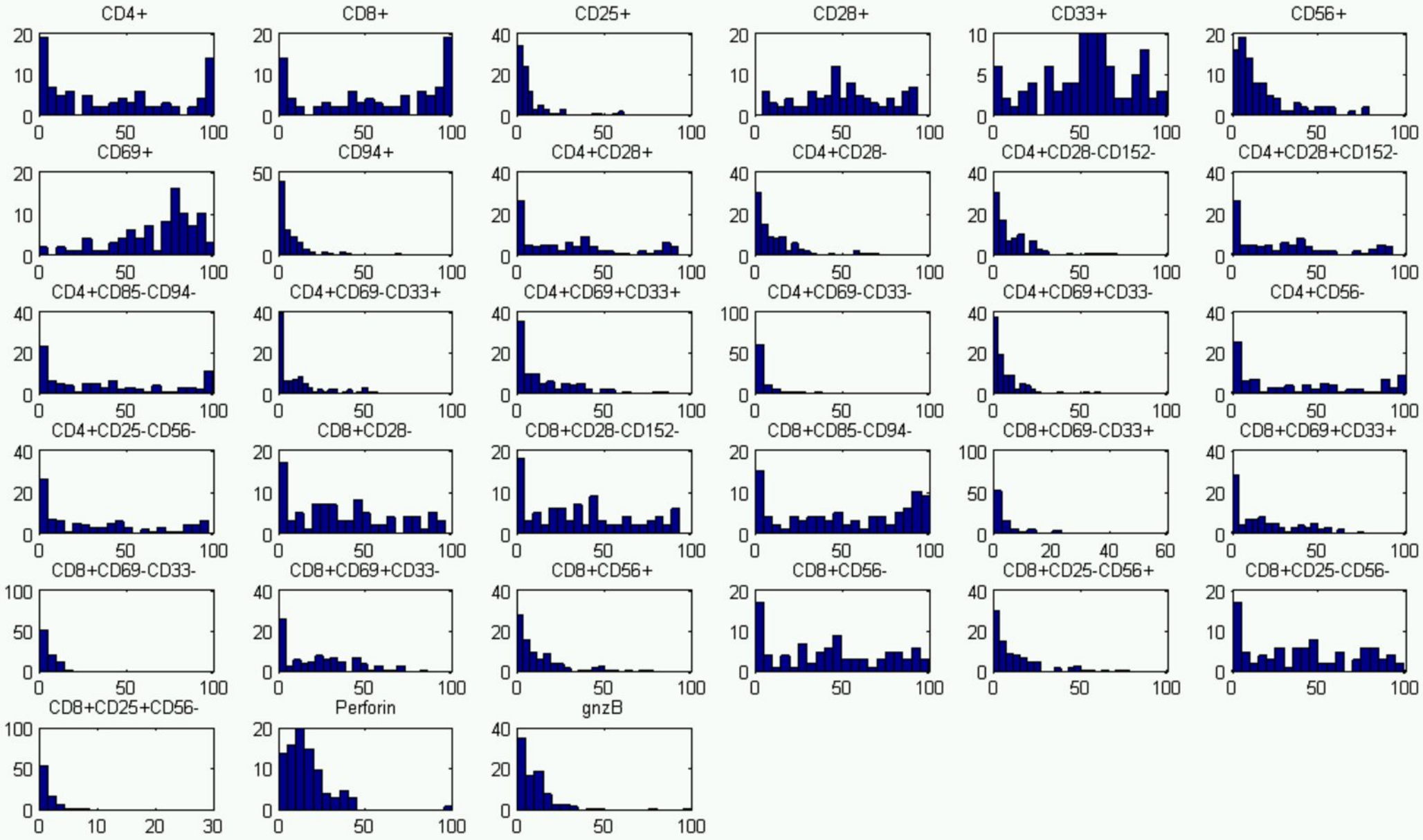
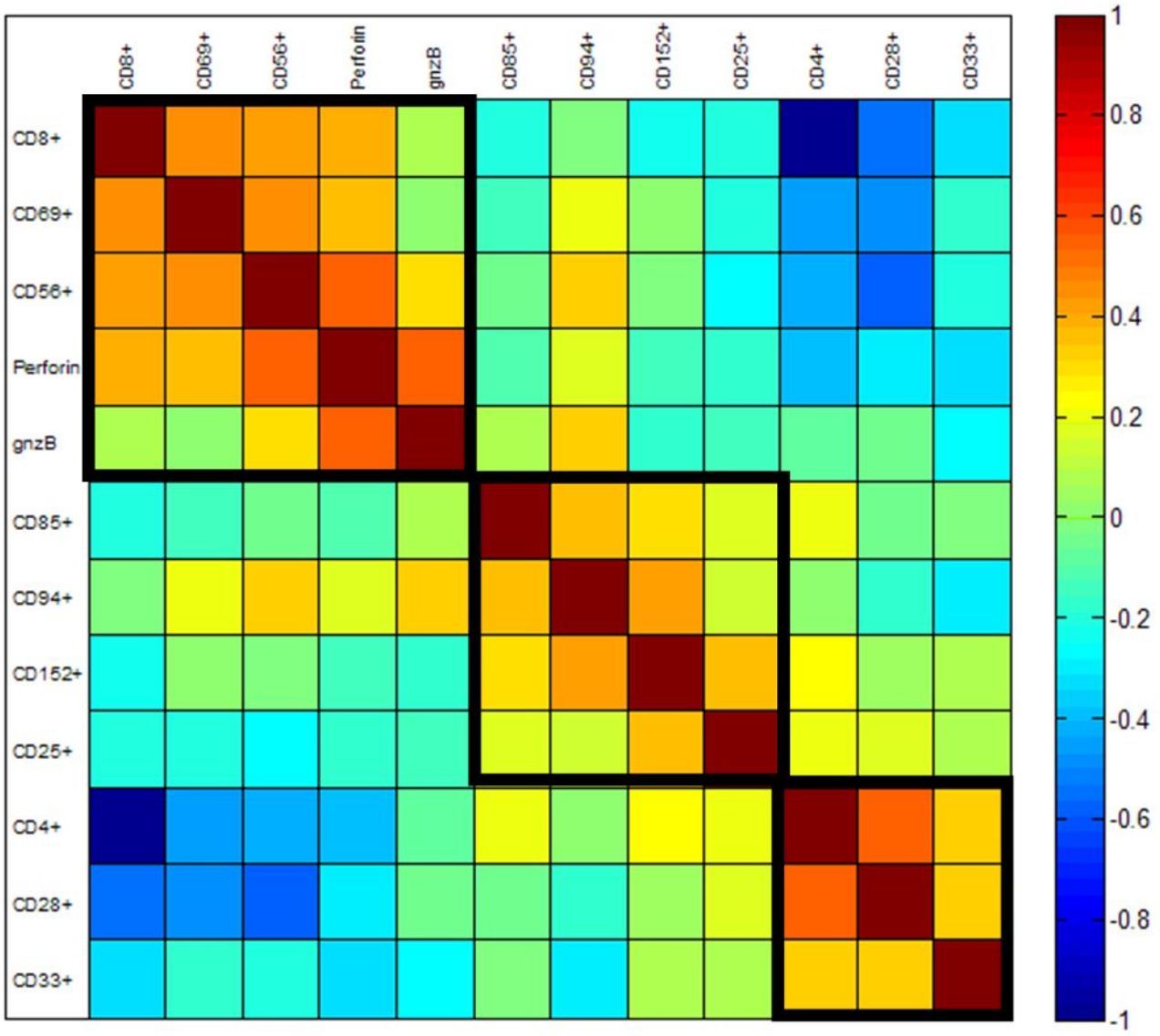
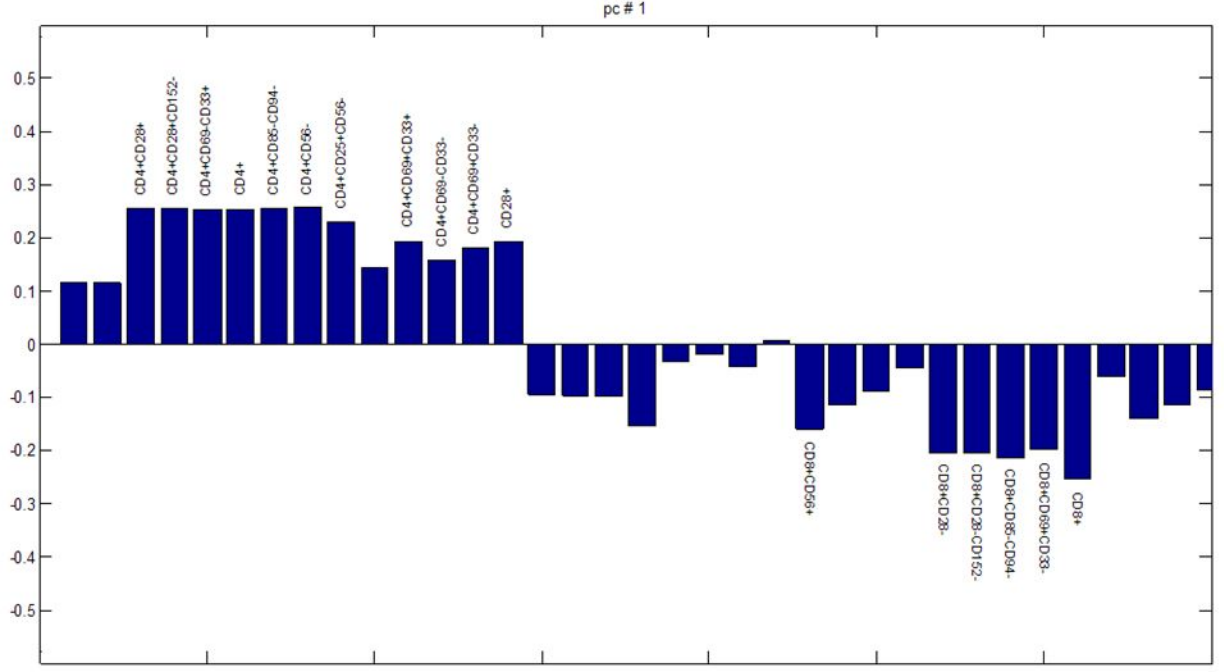

CD4+
CD8+
CD25+
CD28+
CD33+
CD56+
CD69+
CD94+
CD4+CD28+
CD4+CD28-
CD4+CD28-CD152-
CD4+CD28+CD152-
CD4+CD85-CD94-
CD4+CD69-CD33+
CD4+CD69+CD33+
CD4+CD69-CD33-
CD4+CD69+CD33-
CD4+CD56-
CD4+CD25-CD56-
CD8+CD28-
CD8+CD28-CD152-
CD8+CD85-CD94-
CD8+CD69-CD33+
CD8+CD69+CD33+
CD8+CD69-CD33-
CD8+CD69+CD33-
CD8+CD56+
CD8+CD56-
CD8+CD25-CD56+
CD8+CD25-CD56-
CD8+CD25+CD56-
Perforin
gnzB

Figure S3


CD8+
CD69+
CD56+
Perforin
gnzB
CD85+
CD94+
CD152+
CD25+
CD4+
CD28+
CD33+
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1


pc # 1
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
CD4+CD28+
CD4+CD28+CD152-
CD4+CD69-CD33+
CD4+
CD4+CD85-CD94-
CD4+CD56-
CD4+CD25+CD56-
CD4+CD69+CD33+
CD4+CD69-CD33-
CD4+CD69+CD33-
CD28+
CD8+CD56+
CD8+CD28-
CD8+CD28-CD152-
CD8+CD85-CD94-
CD8+CD69+CD33-
CD8+

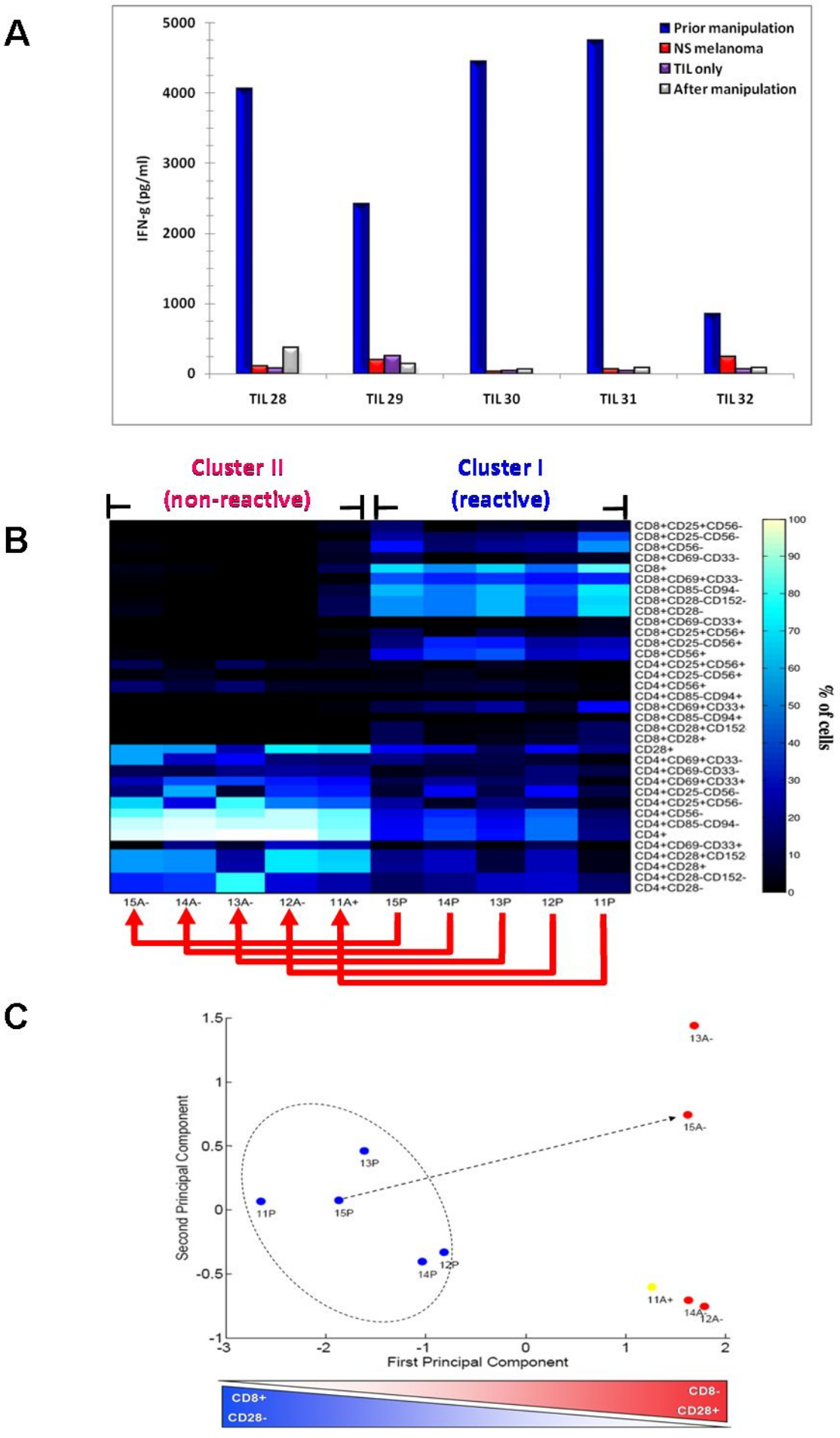

A
5000
4000
3000
2000
1000
0
IFN-g (pg/ml)
Prior manipulation
NS melanoma
TIL only
After manipulation
TIL 28
TIL 29
TIL 30
TIL 31
TIL 32
B
Cluster II
(non-reactive)
Cluster I
(reactive)
CD8+CD25+CD56-
CD8+CD25-CD56-
CD8+CD56-
CD8+CD69-CD33-
CD8+
CD8+CD69+CD33-
CD8+CD85-CD94-
CD8+CD28-CD152-
CD8+CD28-
CD8+CD69-CD33+
CD8+CD25+CD56+
CD8+CD25-CD56+
CD8+CD56+
CD4+CD25+CD56+
CD4+CD25-CD56+
CD4+CD56+
CD4+CD85-CD94+
CD8+CD69+CD33+
CD8+CD85-CD94+
CD8+CD28+CD152
CD8+CD28+
CD28+
CD4+CD69+CD33-
CD4+CD69-CD33-
CD4+CD69+CD33+
CD4+CD25-CD56-
CD4+CD25+CD56-
CD4+CD56-
CD4+CD85-CD94-
CD4+
CD4+CD69-CD33+
CD4+CD28+CD152
CD4+CD28+
CD4+CD28-CD152-
CD4+CD28-
100
90
80
70
60
50
40
30
20
10
0
% of cells
15A-
14A-
13A-
12A-
11A+
15P
14P
13P
12P
11P
C
1.5
1
0.5
0
-0.5
-1
-3
-2
-1
0
1
2
Second Principal Component
First Principal Component
13A-
15A-
13P
11P
15P
14P
12P
11A+
14A-
12A-
CD8+
CD28-
CD8-
CD28+

Figure S5

Table SI

Patient ID	no.	TIL I.4	IFN-γ pg/ml	TIL reactivity
1	1	1.1	2709	Reactive
	2	1.2	513	Reactive
	3	1.3	0	Non-Reactive
	4	1.4	50	Non-Reactive
2	5	2.1	5984	Reactive
	6	2.2	5234	Reactive
	7	2.3	155	Non-Reactive
	8	2.4	188	Non-Reactive
3	9	3.1	235	Reactive
	10	3.2	1742	Reactive
	11	3.3	180	Non-Reactive
	12	3.4	155	Non-Reactive
4	13	4.1	1837	Reactive
	14	4.2	704	Reactive
	15	4.3	0	Non-Reactive
	16	4.4	0	Non-Reactive
5	17	5.1	209	Reactive
	18	5.2	1412	Reactive
	19	5.3	0	Non-Reactive
	20	5.4	50	Non-Reactive
6	21	6.1	778	Reactive
	22	6.2	1608	Reactive
	23	6.3	0	Non-Reactive
	24	6.4	226	Reactive
7	25	7.1	142	Non-Reactive
	26	7.2	113	Non-Reactive
	27	7.3	134	Non-Reactive
	28	7.4	0	Non-Reactive
8	29	8.1	10000	Reactive
	30	8.2	6488	Reactive
	31	8.3	3851	Reactive
	32	8.4	174	Non-Reactive
9	33	9.1	0	Non-Reactive
	34	9.2	119	Non-Reactive
	35	9.3	112	Non-Reactive
	36	9.4	134	Non-Reactive
10	37	10.1	50	Non-Reactive
	38	10.2	0	Non-Reactive
11	39	11.1	256	Reactive
	40	11.2	712	Reactive
	41	11.3	125	Non-Reactive
	42	11.4	125	Non-Reactive
12	43	12.1	1878	Reactive
	44	12.2	4352	Reactive
	45	12.3	1753	Reactive
	46	12.4	0	Non-Reactive
	47	12.5	0	Non-Reactive
13	48	13.1	131	Non-Reactive
	49	13.2	99	Non-Reactive
14	50	14.1	0	Non-Reactive
	51	14.2	0	Non-Reactive
15	52	15.1	1354	Reactive
	53	15.2	6532	Reactive
	54	15.3	0	Non-Reactive
	55	15.4	0	Non-Reactive
16	56	16.1	1612	Reactive
	57	16.2	3011	Reactive
	58	16.3	0	Non-Reactive
	59	16.4	0	Non-Reactive
17	60	17.1	50	Non-Reactive
	61	17.2	50	Non-Reactive
18	62	18.1	0	Non-Reactive
	63	18.2	145	Non-Reactive
19	64	19.1	2659	Reactive
	65	19.2	6000	Reactive
	66	19.3	0	Non-Reactive
20	67	20.1	10000	Reactive
	68	20.2	0	Non-Reactive
	69	20.3	0	Non-Reactive
21	70	21.1	1159	Reactive
	71	21.2	3542	Reactive
	72	21.3	0	Non-Reactive
	73	21.4	0	Non-Reactive
22	74	22.1	950	Reactive
	75	22.2	1621	Reactive
	76	22.3	0	Non-Reactive
23	77	23.1	288	Reactive
	78	23.2	2141	Reactive
24	79	24.1	0	Non-Reactive
	80	24.2	0	Non-Reactive
25	81	25.1	784	Reactive
	82	25.2	704	Reactive
	83	25.3	75	Non-Reactive
	84	25.4	84	Non-Reactive
26	85	26.1	168	Non-Reactive
	86	26.2	1351	Reactive
	87	26.3	1245	Reactive
	88	26.4	0	Non-Reactive
27	89	27.1	4750	Reactive
	90	27.2	142	Non-Reactive
	91	27.3	135	Non-Reactive

Table SII

A

Single staining	Double staining	Triple staining
CD4+	CD4+CD28+	CD4+ CD8- CD28- CD152-
CD4 - (~CD8+)	CD4+CD28-	CD4+ CD8- CD28- CD152+
CD8+	CD4+CD152+	CD4+ CD8- CD28+ CD152-
CD8 - (~CD4+)	CD4+CD152-	CD4+ CD8- CD28+ CD152+
CD25+	CD8+CD28+	CD4- CD8+ CD28- CD152-
CD25 -	CD8+CD28-	CD4- CD8+ CD28- CD152+
CD28+	CD8+CD152+	CD4- CD8+ CD28+ CD152-
CD28 -	CD8+CD152-	CD4- CD8+ CD28+ CD152+
CD33+	CD4+CD25+	CD4+ CD8- CD25- CD56-
CD33 -	CD4+CD25-	CD4+ CD8- CD25- CD56+
CD45R0+	CD4+CD56+	CD4+ CD8- CD25+ CD56-
CD45R0 -	CD4+CD56-	CD4+ CD8- CD25+ CD56+
CD45RA+	CD8+CD25+	CD4- CD8+ CD25- CD56-
CD45RA -	CD8+CD25-	CD4- CD8+ CD25- CD56+
CD56+	CD8+CD56+	CD4- CD8+ CD25+ CD56-
CD56 -	CD8+CD56-	CD4- CD8+ CD25+ CD56+
CD69+	CD4+CD69+	CD4+ CD8- CD33- CD69-
CD69 -	CD4+CD69-	CD4+ CD8- CD33- CD69+
CD85+	CD4+CD33+	CD4+ CD8- CD33+ CD69-
CD85 -	CD4+CD33-	CD4+ CD8- CD33+ CD69+
CD94+	CD8+CD69+	CD4- CD8+ CD33- CD69-
CD94 -	CD8+CD69-	CD4- CD8+ CD33- CD69+
CD152+	CD8+CD33+	CD4- CD8+ CD33+ CD69-
CD152 -	CD8+CD33-	CD4- CD8+ CD33+ CD69+
	CD4+CD85+	CD4+ CD8- CD85- CD94-
	CD4+CD85-	CD4+ CD8- CD85- CD94+
	CD4+CD94+	CD4+ CD8- CD85+ CD94-
	CD4+CD94-	CD4+ CD8- CD85+ CD94+
	CD8+CD85+	CD4- CD8 CD85- CD94-
	CD8+CD85-	CD4- CD8 CD85- CD94+
	CD8+CD94+	CD4- CD8 CD85+ CD94-
	CD8+CD94-	CD4- CD8 CD85+ CD94+
	CD4+CD45RA+	CD4+ CD8- CD45R0- CD45RA-
	CD4+CD45RA-	CD4+ CD8- CD45R0- CD45RA+
	CD4+CD45R0+	CD4+ CD8- CD45R0+ CD45RA-
	CD4+CD45R0-	CD4+ CD8- CD45R0+ CD45RA+
	CD8+CD45RA+	CD4- CD8+ CD45R0- CD45RA-
	CD8+CD45RA-	CD4- CD8+ CD45R0- CD45RA+
	CD8+CD45R0+	CD4- CD8+ CD45R0+ CD45RA-
	CD8+CD45R0-	CD4- CD8+ CD45R0+ CD45RA+

B

Single staining	Double staining	Triple staining
CD4+	CD4+ CD28+	CD4+ CD85- CD94-
CD8+	CD4+ CD28-	CD4+ CD69- CD33+
CD25+	CD4+ CD56-	CD4+ CD69+ CD33+
CD28+	CD8+ CD28-	CD4+ CD69- CD33-
CD33+	CD8+ CD56+	CD4+ CD69+ CD33-
CD56+	CD8+ CD56-	CD4+ CD25- CD56-
CD69+		CD8+ CD85- CD94-
CD94+		CD8+ CD69- CD33+
Perforin		CD8+ CD69+ CD33+
gnzB		CD8+ CD69- CD33-
		CD8+ CD69+ CD33-
		CD8+ CD25- CD56+
		CD8+ CD25- CD56-
		CD8+ CD25+ CD56-
		CD4+ CD28- CD152-
		CD4+ CD28+ CD152-
		CD8+ CD28- CD152-

Table SIII

			Prior manipulation			After manipulation			Fold change (Prior/After)		
	Before	After	IFN-γ (pg/ml)	CD8(%)	CD4(%)	IFN-γ (pg/ml)	CD8(%)	CD4(%)	FC IFN-γ	FC CD8	FC CD4
TIL1	non reactive	reactive	125	20.72	79.28	4055	71.44	28.56	32.4	3.4	0.4
TIL2	non reactive	reactive	38	42.45	57.55	4020	88.49	11.51	105.8	2.1	0.2
TIL3	non reactive	reactive	113	51.63	48.37	3478	89.49	10.51	30.8	1.7	0.2
TIL4	non reactive	reactive	71	90.85	9.15	3308	79.26	20.74	46.6	0.9	2.3
TIL5	non reactive	reactive	110	6.42	93.58	2886	52.05	47.95	26.2	8.1	0.5
TIL6	non reactive	reactive	0	ND	ND	1388	ND	ND	ND	ND	ND
TIL7	non reactive	reactive	36	87.45	12.55	605	87.08	12.92	16.8	1.0	1.0
TIL8	non reactive	reactive	57	73.19	26.81	560	92.98	7.02	9.8	1.3	0.3
TIL9	non reactive	reactive	30	56.78	43.22	295	71.65	28.35	9.8	1.3	0.7
TIL10	non reactive	non reactive	8	3.56	96.44	73	48.98	51.02	9.1	13.8	0.5
TIL11	non reactive	non reactive	25	98.77	1.23	24	99.01	0.99	1.0	1.0	0.8
TIL12	non reactive	non reactive	23	ND	ND	0	ND	ND	ND	ND	ND