Figure 1

Table 1.

Tumor Type		Fresh Product	Cryopreserved Product	
		post-Harvest	post-Thaw	post-restimulation
Malignant Melanoma (M1053T) REP	**OxPhos (pmol/min)**			
	Basal respiration	17.5 ± 4.4	9.5 ± 4.9	22.5 ± 2.5
	SRC	19.3 ± 9.0	27.5± 12.1	16.8 ± 2.6
	SRC (% of basal)	210.6 ± 51.4	389.8 ± 127.4	174.7 ± 11.3
	Glycolysis (mpH/min)			
	Basal glycolysis	31.1 ± 4.4	22.5 ± 1.2	103.1 ± 5.4
	Glycolytic Reserve	26.4 ± 5.5	20.7 ± 2.2	15.9 ± 7.7
	Glycolytic Res (% of basal)	185.1 ± 17.6	192.0 ± 9.9	115.5 ± 7.4

Figure 2

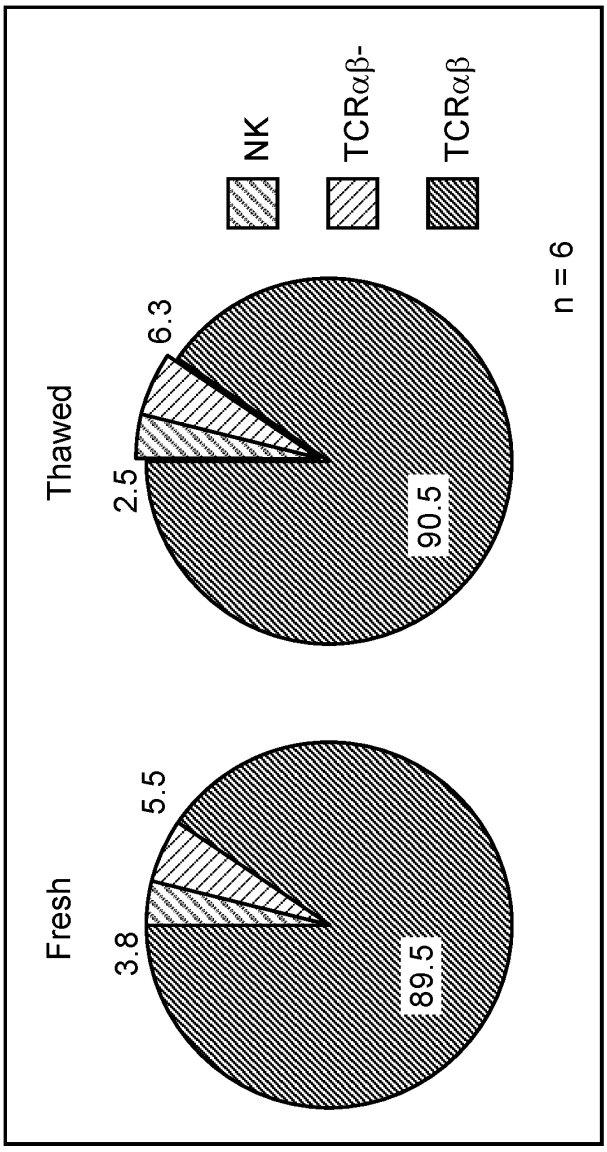


Figure 3

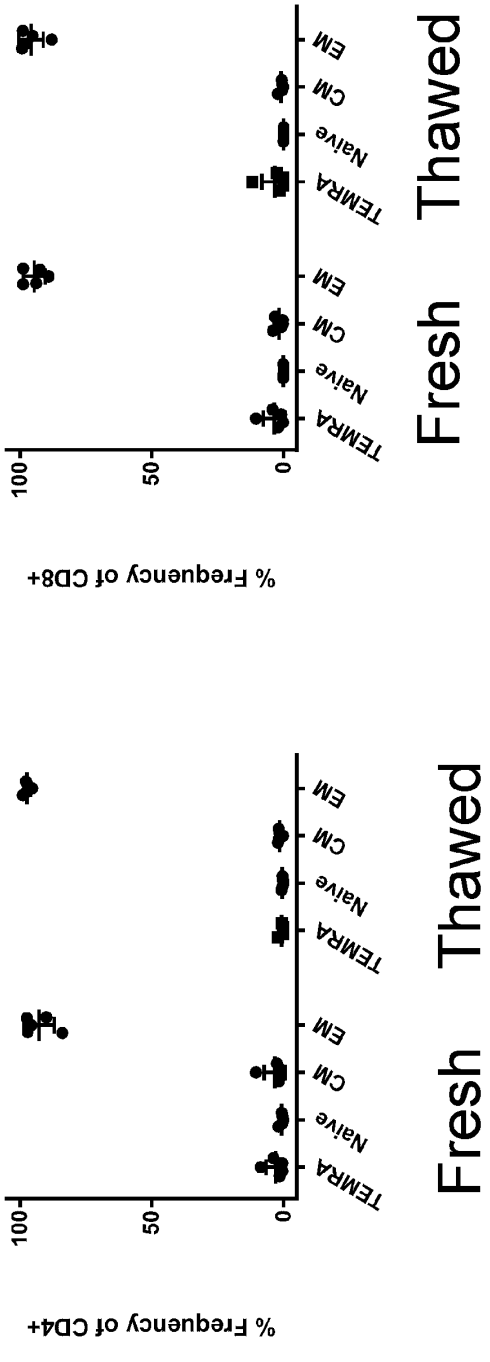
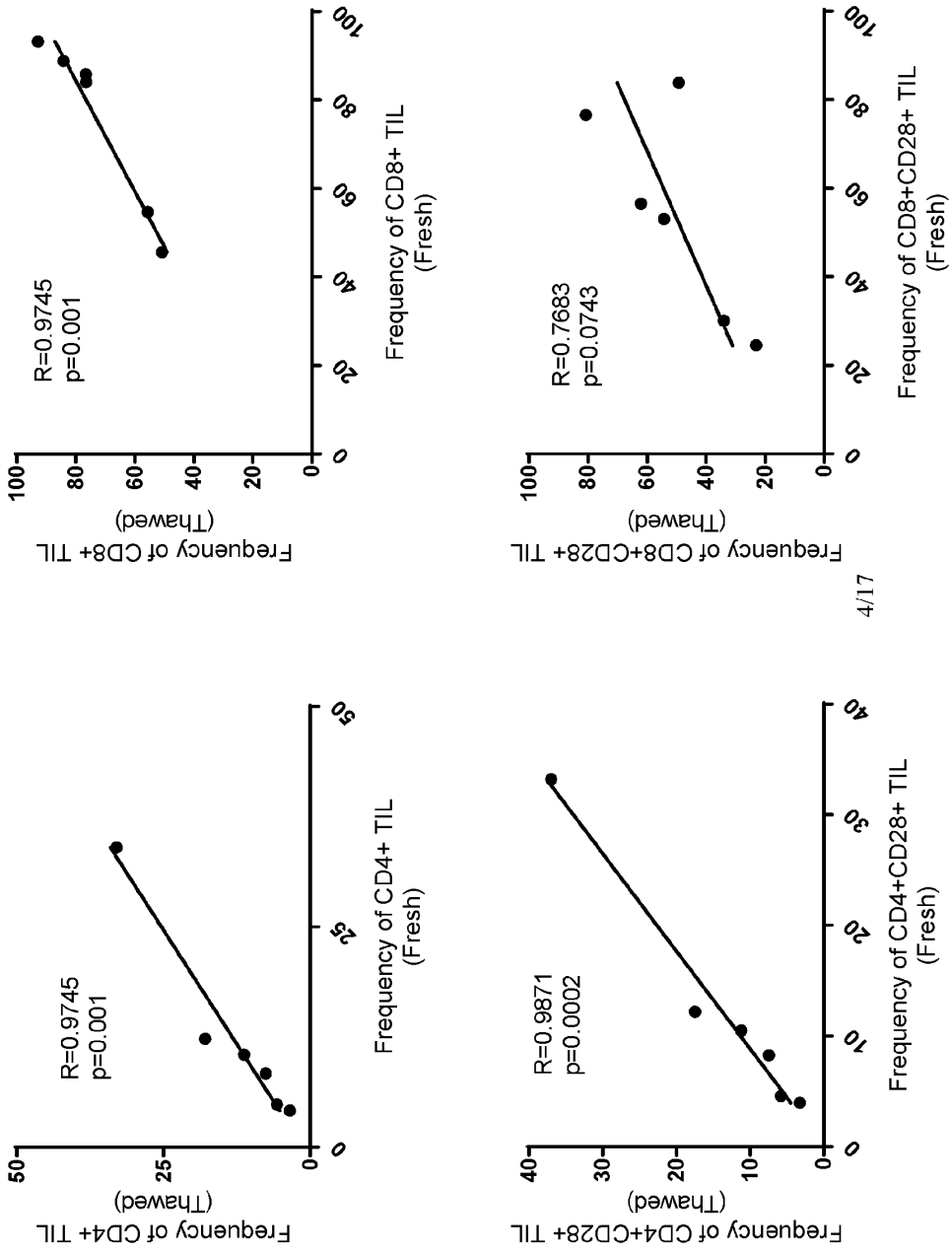

% Frequency of CD8+
100
50
0
TEMRA
Naive
CM
EM
Fresh
Thawed
% Frequency of CD4+
100
50
0
TEMRA
Naive
CM
EM
Fresh
Thawed

Figure 4


R=0.9745
p=0.001
Frequency of CD8+ TIL (Thawed)
Frequency of CD8+ TIL (Fresh)
R=0.7683
p=0.0743
Frequency of CD8+CD28+ TIL (Thawed)
Frequency of CD8+CD28+ TIL (Fresh)
R=0.9745
p=0.001
Frequency of CD4+ TIL (Thawed)
Frequency of CD4+ TIL (Fresh)
R=0.9871
p=0.0002
Frequency of CD4+CD28+ TIL (Thawed)
Frequency of CD4+CD28+ TIL (Fresh)

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Figure 5

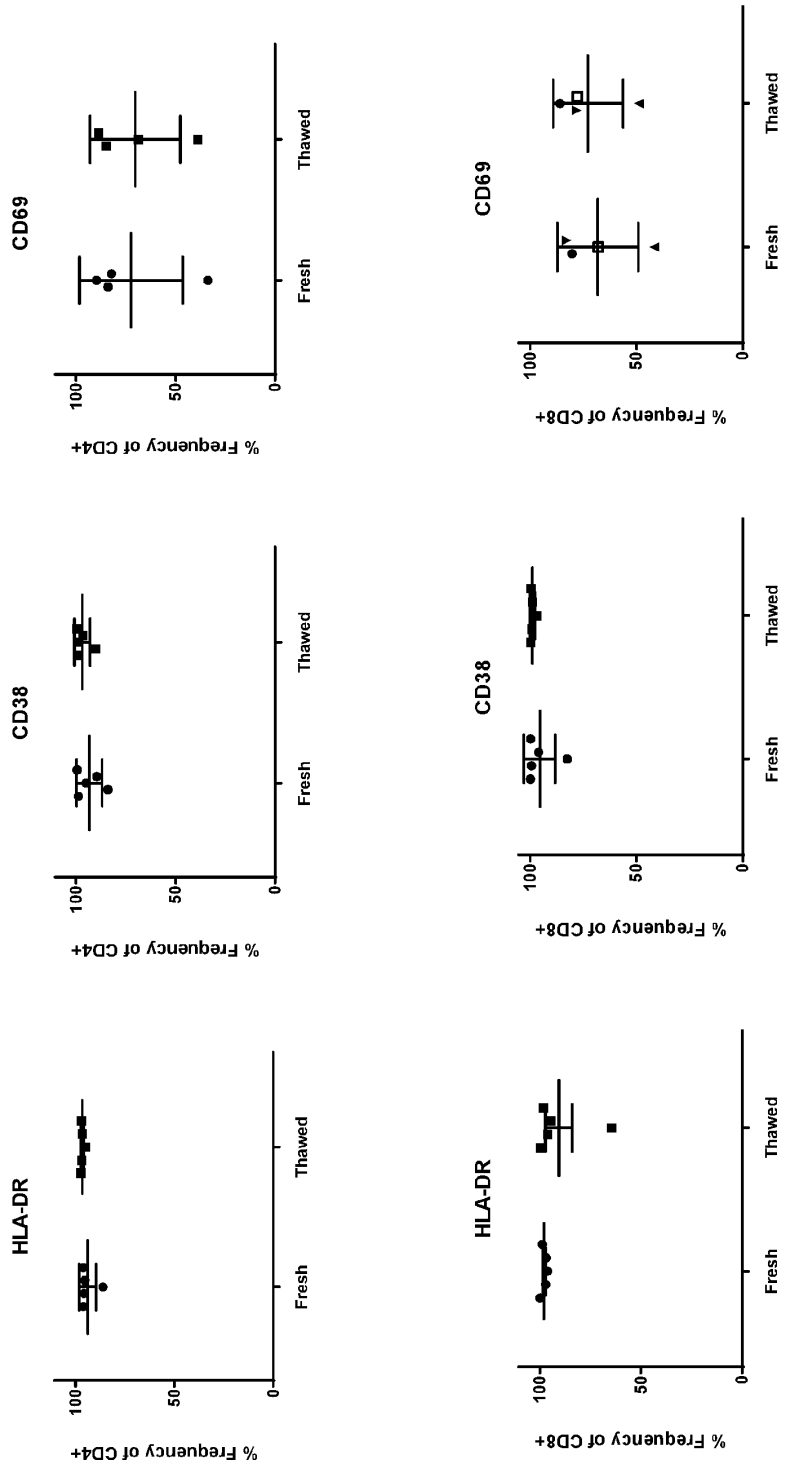

CD69
% Frequency of CD4+
100
50
0
Fresh
Thawed
CD69
% Frequency of CD8+
100
50
0
Fresh
Thawed
CD38
% Frequency of CD4+
100
50
0
Fresh
Thawed
CD38
% Frequency of CD8+
100
50
0
Fresh
Thawed
HLA-DR
% Frequency of CD4+
100
50
0
Fresh
Thawed
HLA-DR
% Frequency of CD8+
100
50
0
Fresh
Thawed

Figure 6A

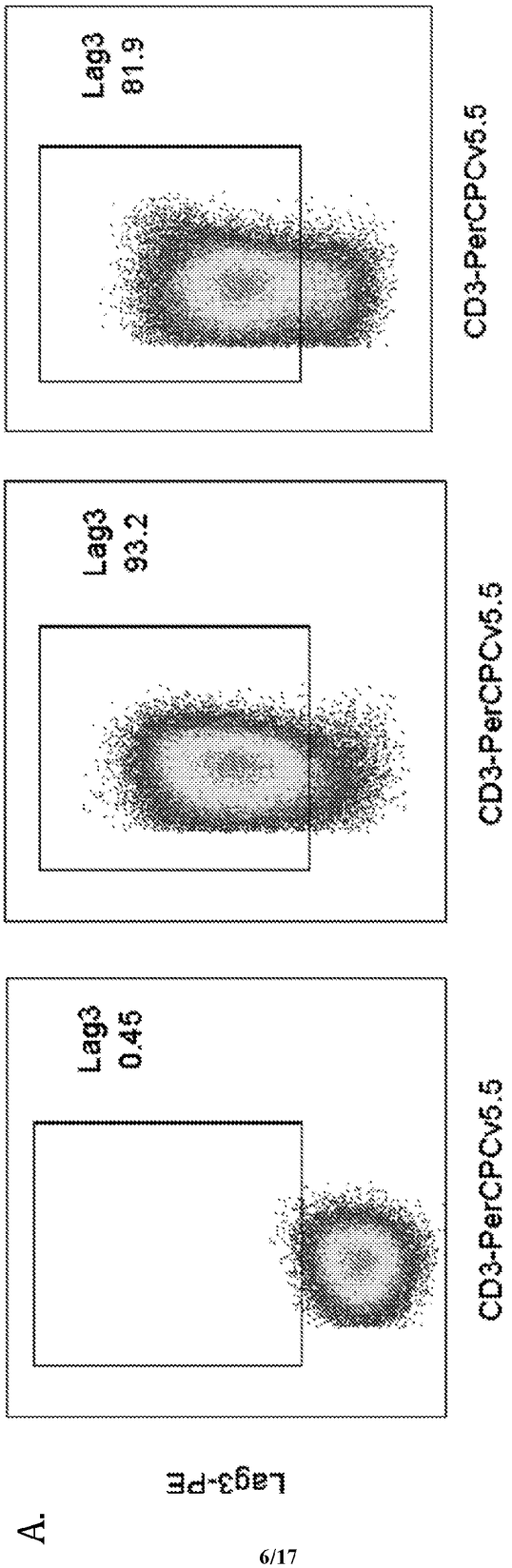
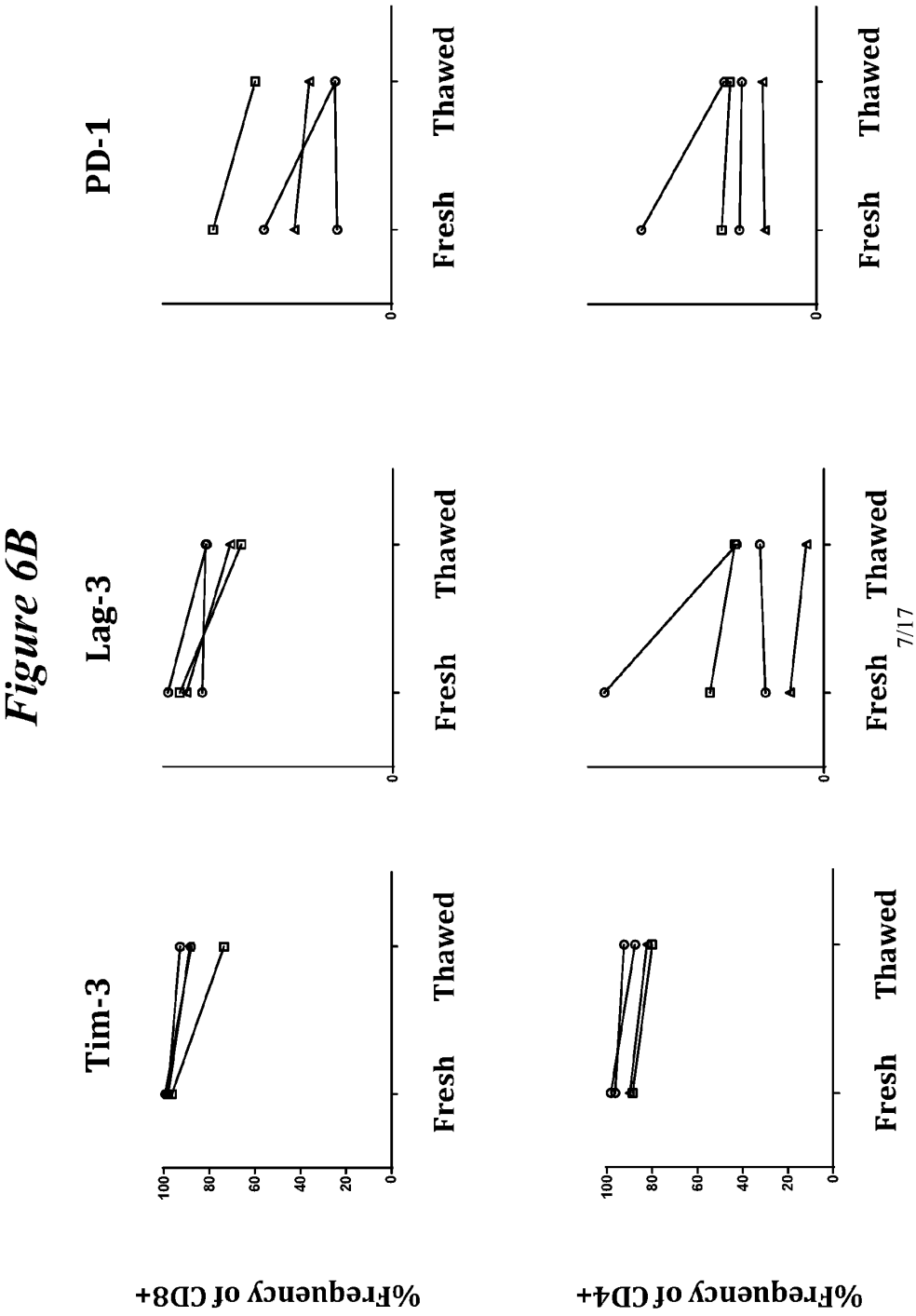

A.
Lag3-PE
FMO Control
Lag3
0.45
CD3-PerCPCv5.5
Fresh
Lag3
93.2
CD3-PerCPCv5.5
Thawed
Lag3
81.9
CD3-PerCPCv5.5

Figure 6B



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Figure 7

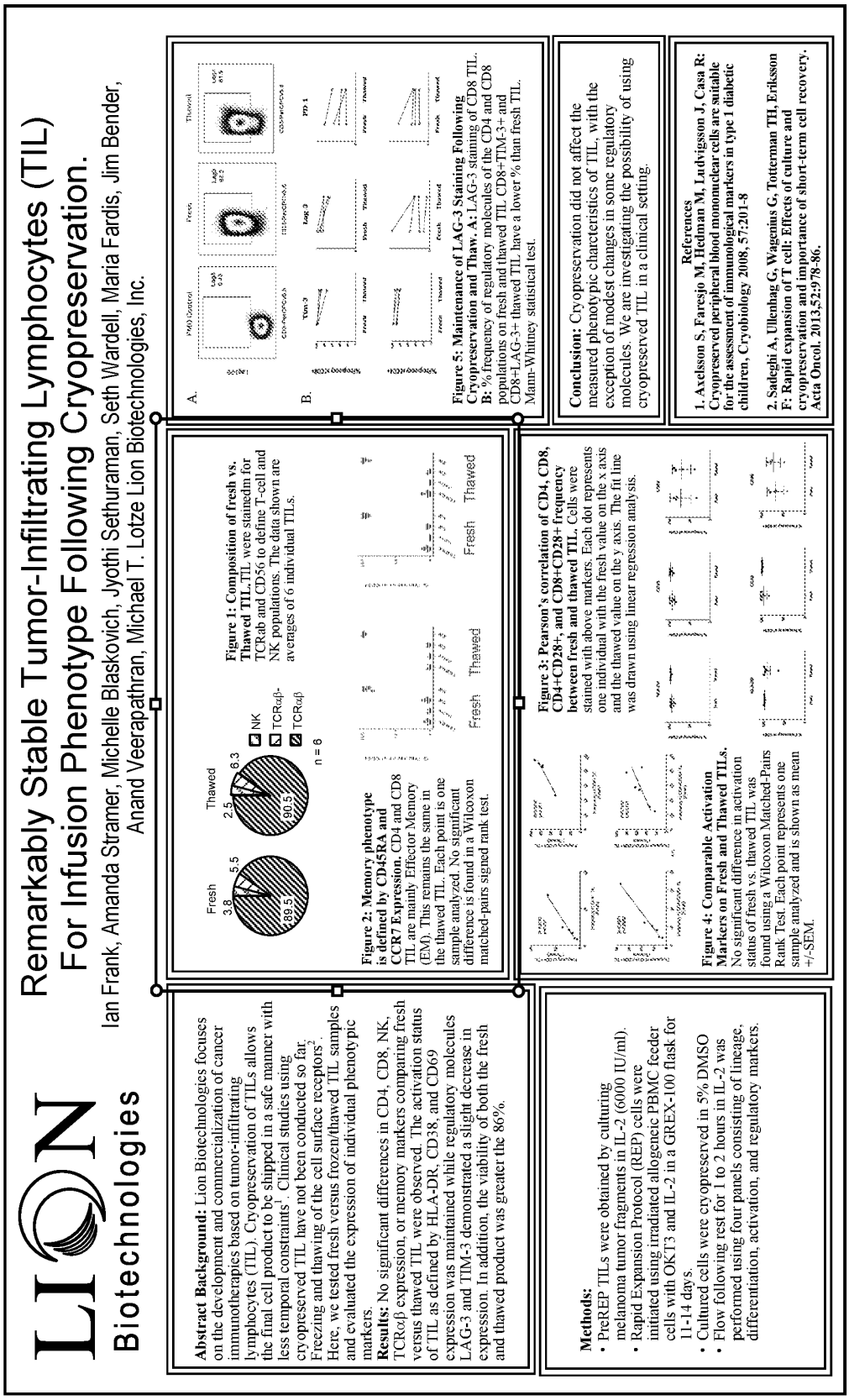

LION
Biotechnologies
Remarkably Stable Tumor-Infiltrating Lymphocytes (TIL) For Infusion Phenotype Following Cryopreservation.
Ian Frank, Amanda Stramer, Michelle Blaskovich, Jyothi Sethuraman, Seth Wardell, Maria Fardis, Jim Bender, Anand Veerapathran, Michael T. Lotze Lion Biotechnologies, Inc.
Abstract Background: Lion Biotechnologies focuses on the development and commercialization of cancer immunotherapies based on tumor-infiltrating lymphocytes (TIL). Cryopreservation of TILs allows the final cell product to be shipped in a safe manner with less temporal constraints[1]. Clinical studies using cryopreserved TIL have not been conducted so far. Freezing and thawing of the cell surface receptors[2]. Here, we tested fresh versus frozen/thawed TIL samples and evaluated the expression of individual phenotypic markers.
Results: No significant differences in CD4, CD8, NK, TCRαβ expression, or memory markers comparing fresh versus thawed TIL were observed. The activation status of TIL as defined by HLA-DR, CD38, and CD69 expression was maintained while regulatory molecules LAG-3 and TIM-3 demonstrated a slight decrease in expression. In addition, the viability of both the fresh and thawed product was greater the 86%.
Methods:
PreREP TILs were obtained by culturing melanoma tumor fragments in IL-2 (6000 IU/ml).
Rapid Expansion Protocol (REP) cells were initiated using irradiated allogeneic PBMC feeder cells with OKT3 and IL-2 in a GREX-100 flask for 11-14 days.
Cultured cells were cryopreserved in 5% DMSO
Flow following rest for 1 to 2 hours in IL-2 was performed using four panels consisting of lineage, differentiation, activation, and regulatory markers.
Fresh
Thawed
NK
TCRαβ-
TCRαβ
n = 6
Figure 1: Composition of fresh vs. Thawed TIL.TIL were stainedm for TCRab and CD56 to define T-cell and NK populations. The data shown are averages of 6 individual TILs.
Figure 2: Memory phenotype is defined by CD45RA and CCR7 Expression. CD4 and CD8 TIL are mainly Effector Memory (EM). This remains the same in the thawed TIL. Each point is one sample analyzed. No significant difference is found in a Wilcoxon matched-pairs signed rank test.
Figure 3: Pearson's correlation of CD4, CD8, CD4+CD28+, and CD8+CD28+ frequency between fresh and thawed TIL. Cells were stained with above markers. Each dot represents one individual with the fresh value on the x axis and the thawed value on the y axis. The fit line was drawn using linear regression analysis.
Figure 4: Comparable Activation Markers on Fresh and Thawed TILs. No significant difference in activation status of fresh vs. thawed TIL was found using a Wilcoxon Matched-Pairs Rank Test. Each point represents one sample analyzed and is shown as mean +/-SEM.
A.
PMA/Ionomycin
Fresh
Thawed
B.
Tim-3
Lag-3
PD-1
Figure 5: Maintenance of LAG-3 Staining Following Cryopreservation and Thaw. A: LAG-3 staining of CD8 TIL. B: % frequency of regulatory molecules of the CD4 and CD8 populations on fresh and thawed TIL CD8+TIM-3+ and CD8+LAG-3+ thawed TIL have a lower % than fresh TIL. Mann-Whitney statistical test.
Conclusion: Cryopreservation did not affect the measured phenotypic charcteristics of TIL, with the exception of modest changes in some regulatory molecules. We are investigating the possibility of using cryopreserved TIL in a clinical setting.
References
1. Axelsson S, Faresjö M, Hedman M, Ludvigsson J, Casas R: Cryopreserved peripheral blood mononuclear cells are suitable for the assessment of immunological markers in type 1 diabetic children, Cryobiology 2008, 57:201-8
2. Sadeghi A, Ullenhag G, Wagenius G, Totterman TH, Eriksson F: Rapid expansion of T cell: Effects of culture and cryopreservation and importance of short-term cell recovery. Acta Oncol. 2013,52:978-86.

Figure 8

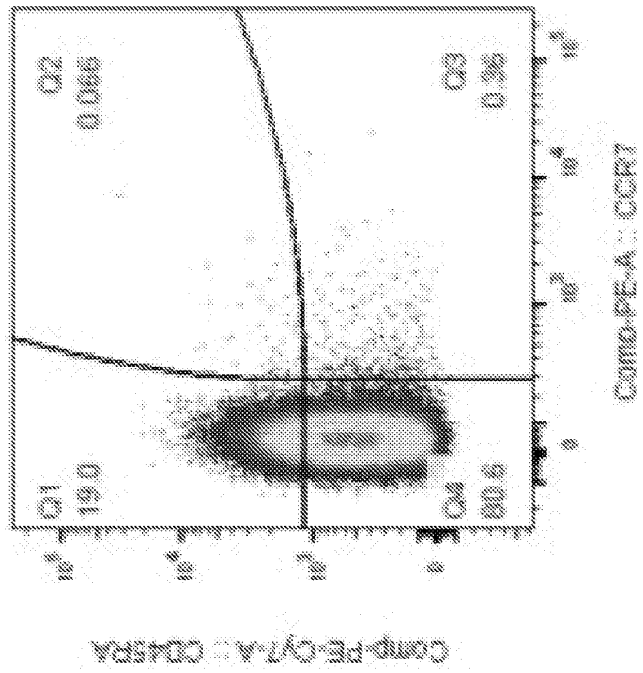


Figure 9

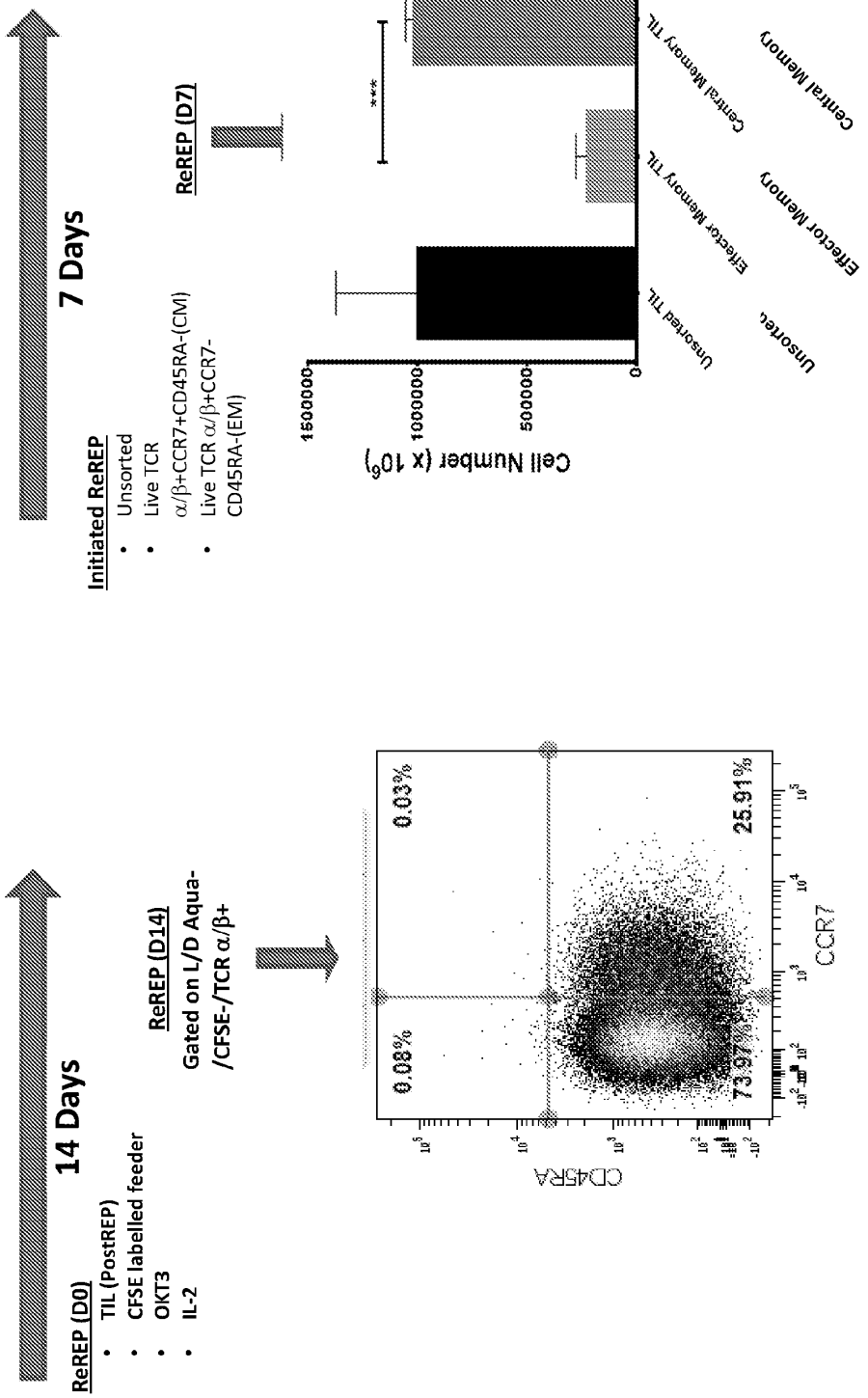

14 Days
ReREP (D0)
TIL (PostREP)
CFSE labelled feeder
OKT3
IL-2
ReREP (D14)
Gated on L/D Aqua-/CFSE-/TCR α/β+
0.08%
0.03%
73.97%
25.91%
CD45RA
CCR7
7 Days
Initiated ReREP
Unsorted
Live TCR α/β+CCR7+CD45RA-(CM)
Live TCR α/β+CCR7-CD45RA-(EM)
ReREP (D7)
Cell Number (x 10^6)
1500000
1000000
500000
0
***
Unsorted TIL
Effector Memory TIL
Central Memory TIL
Unsorted
Effector Memory
Central Memory

Figure 10A

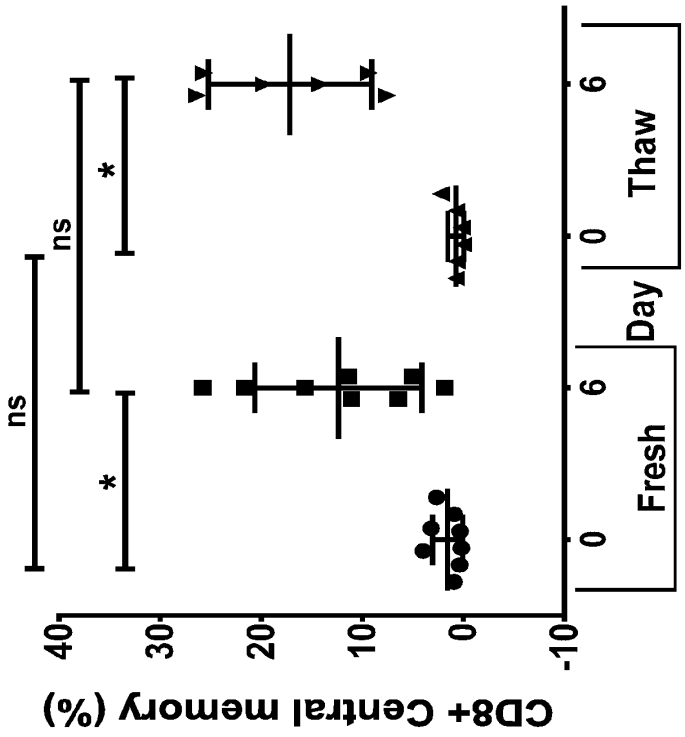


Figure 10B

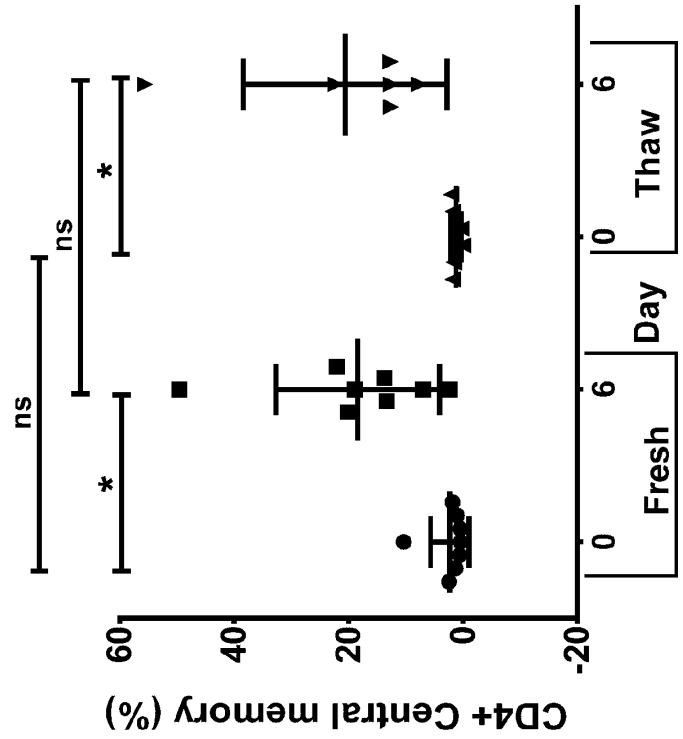


Figure 11

Process 1C : 43-55 Days for Steps A - E

1. **STEP A**

Obtain Patient Tumor Sample

2. **STEP B**

Fragmentation and First Expansion
11 to 21 days

3. **STEP C**

First Expansion to Second Expansion Transition
Optional Storage until Selection

4. **STEP D**

Second Expansion
IL-2, OKT-3, antigen-presenting feeder cells
Optionally repeated one or more times

5. **STEP E**

Harvest TILS from Step D

6. **STEP F**

Final Formulation and/or Transfer to Infusion Bag

Figure 12

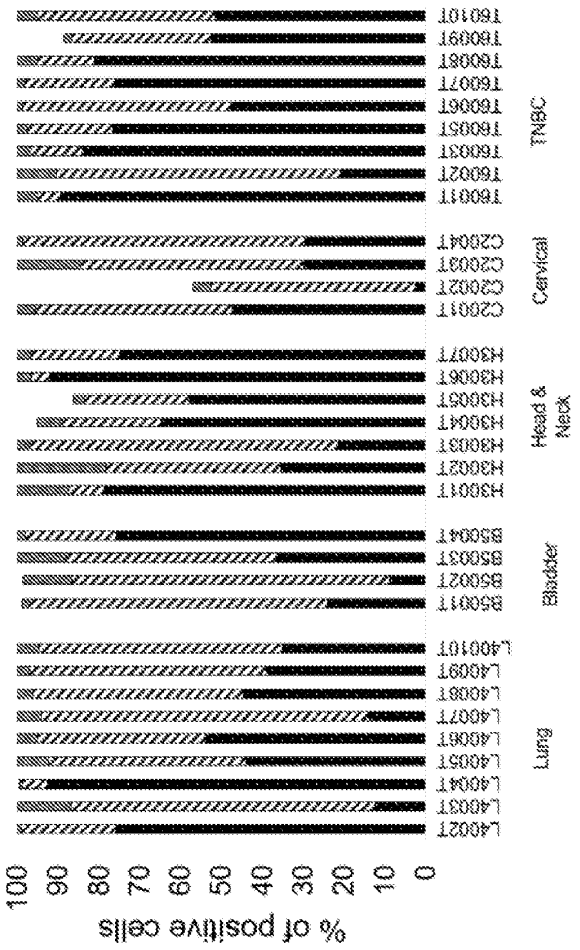

CD4+
CD8+
CD56+
% of positive cells
100
90
80
70
60
50
40
30
20
10
0
Lung
Bladder
Head & Neck
Cervical
TNBC

Figure 13

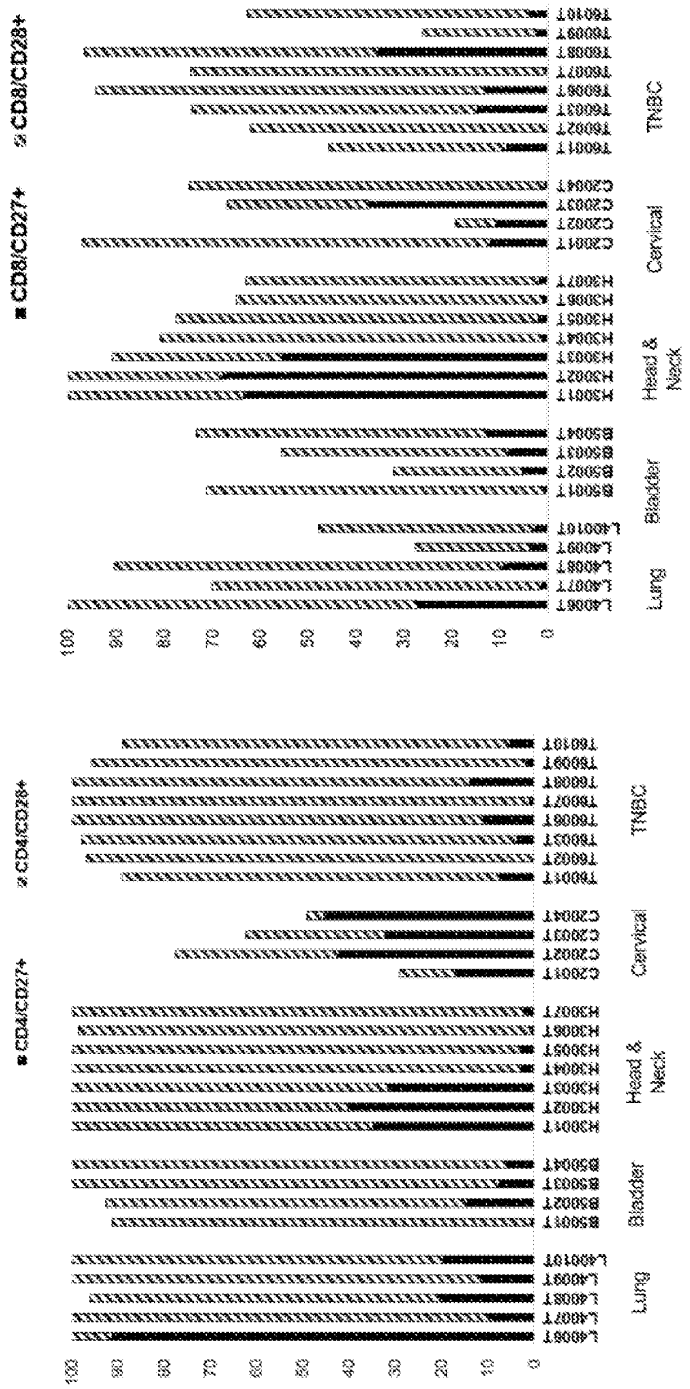

CD8/CD28+
CD8/CD27+
CD4/CD28+
CD4/CD27+
Lung
Bladder
Head & Neck
Cervical
TNBC
100
90
80
70
60
50
40
30
20
10
0

Figure 14

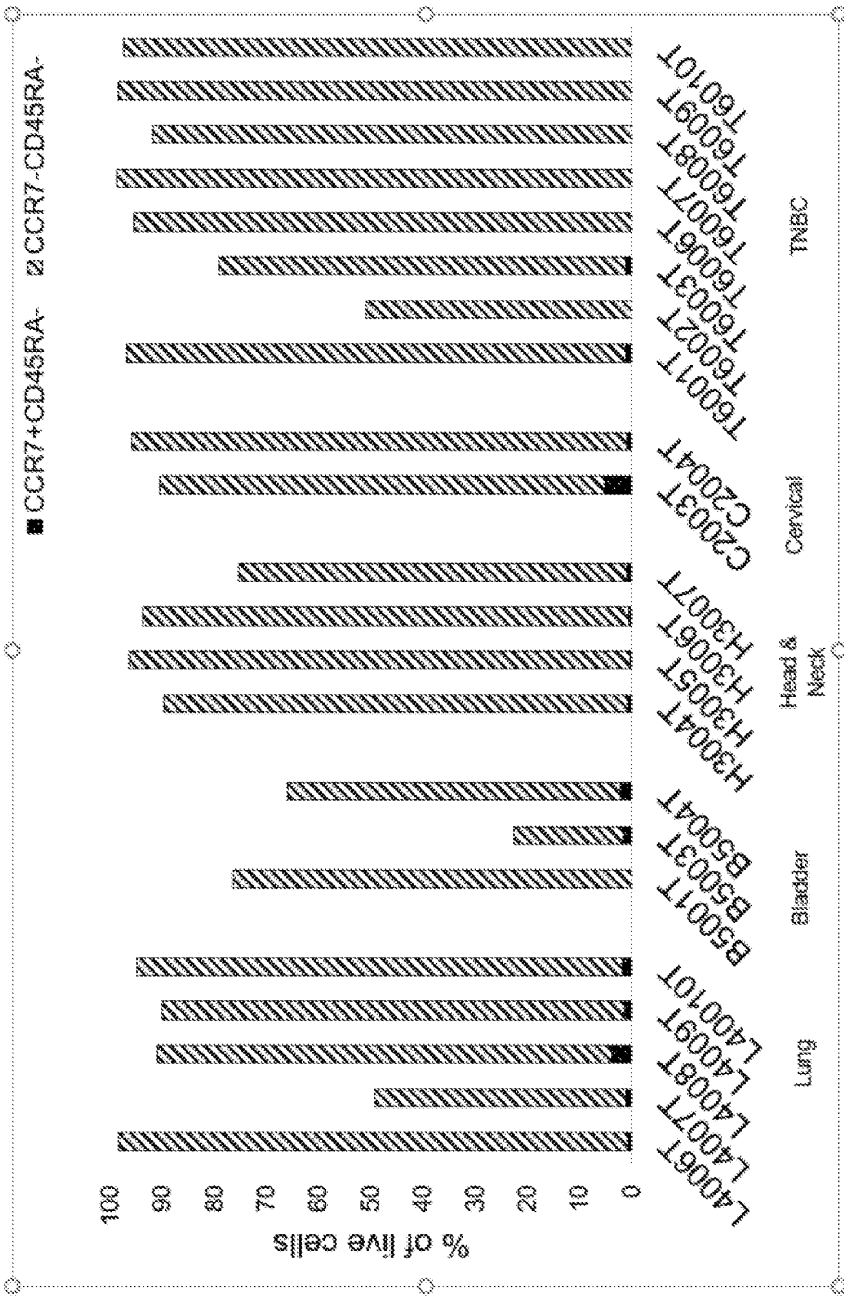

CCR7+CD45RA-
CCR7-CD45RA-
% of live cells
100
90
80
70
60
50
40
30
20
10
0
L4006T
L4007T
L4008T
L4009T
L4001O1T
B5001T
B5003T
B5004T
H3004T
H3005T
H3006T
H3007T
C2003T
C2004T
T6001T
T6002T
T6003T
T6006T
T6007T
T6008T
T6009T
T6010T
Lung
Bladder
Head & Neck
Cervical
TNBC

Figure 15

TUBE NAME	SSRC	CD4 Freq of Parent	S Live CD8	Q1: CCR7-, CD45RA+ Freq. of Parent	S Live CD8	Q2: CCR7+, CD45RA+ Freq. of Parent	S Live CD8	Q3: CCR7+, CD45RA- Freq. of Parent	S Live CD8	Q4: CCR7-, CD45RA- Freq. of Parent	S Live CD8	Q5: CD28+, CD57+ Freq. of Parent	S Live CD8	Q6: CD28+, CD57+ Freq. of Parent	S Live CD8	Q7: CD28+, CD57- Freq. of Parent	S Live CD8	Q8: CD28-, CD57- Freq. of Parent
1.00E+08	Not Rested	3.01	95	0.53	0.53	0.53	52	46.9	2.16	0.23	7.87	89.8						
4.00E+08	Not Rested	2.83	95	0.47	0.69	0.69	56.7	42.2	2.51	0.32	8.19	89						
8.00E+08	Not Rested	2.85	95.6	0.34	0.58	0.58	55.4	43.6	2.26	0.29	8.37	89.1						
1.00E+08	Rested	5.78	90.4	1.1	0.78	0.78	55.1	43	1.05	0.32	12.8	85.9						
4.00E+08	Rested	5.74	90	0.86	0.82	0.82	61.1	37.2	1.11	0.32	13.6	85						
8.00E+08	Rested	7.64	87.5	1.42	1.07	1.07	60.5	37	1.18	0.32	17.1	81.4						