Figure 1

Table 1.

Tumor Type		Fresh Product post-Harvest	Cryopreserved Product post-Thaw	Cryopreserved Product 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

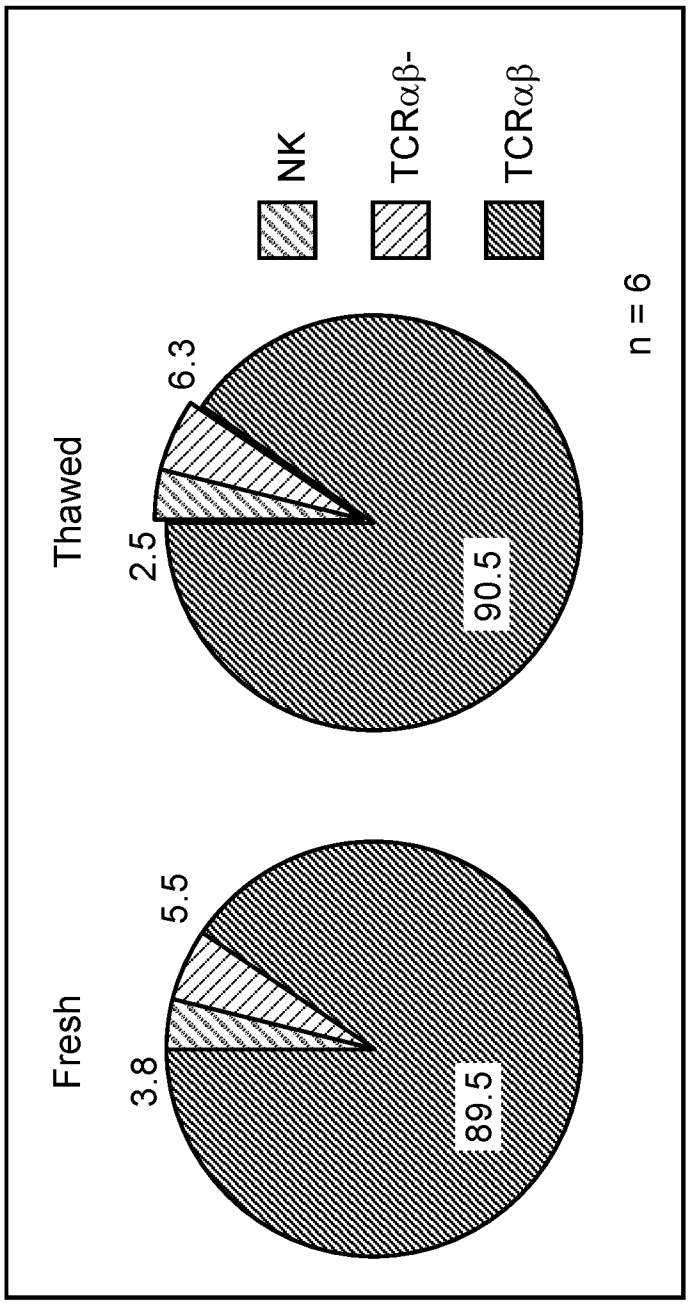


Figure 3

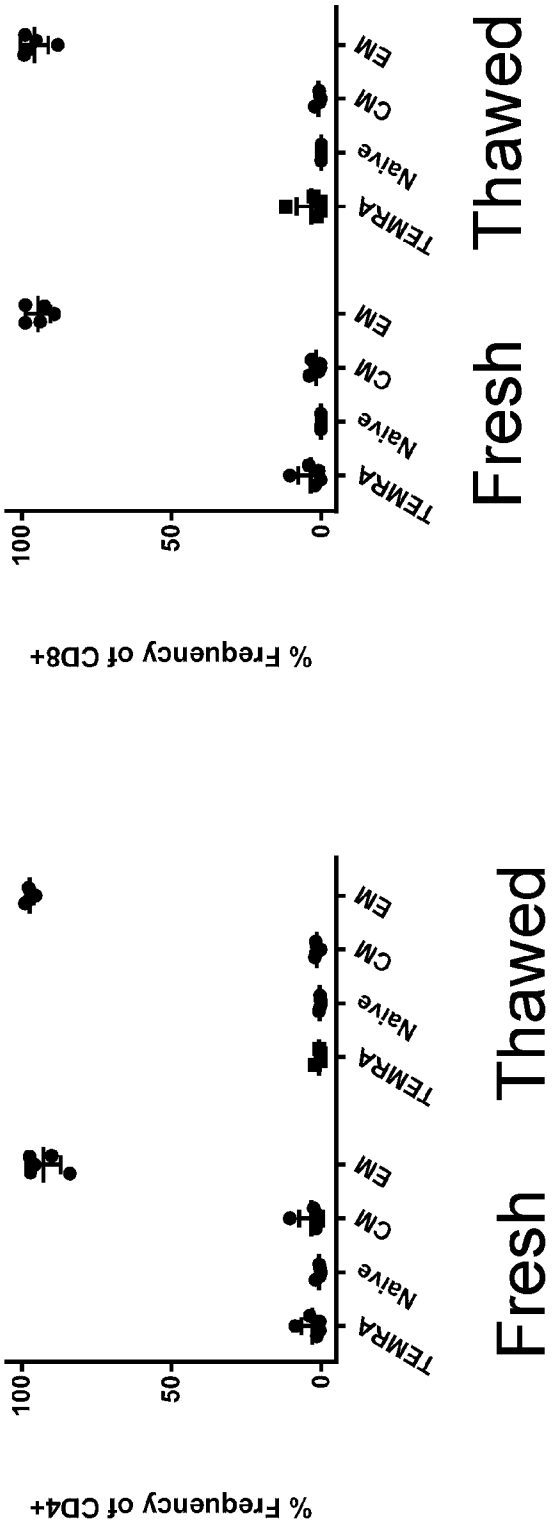
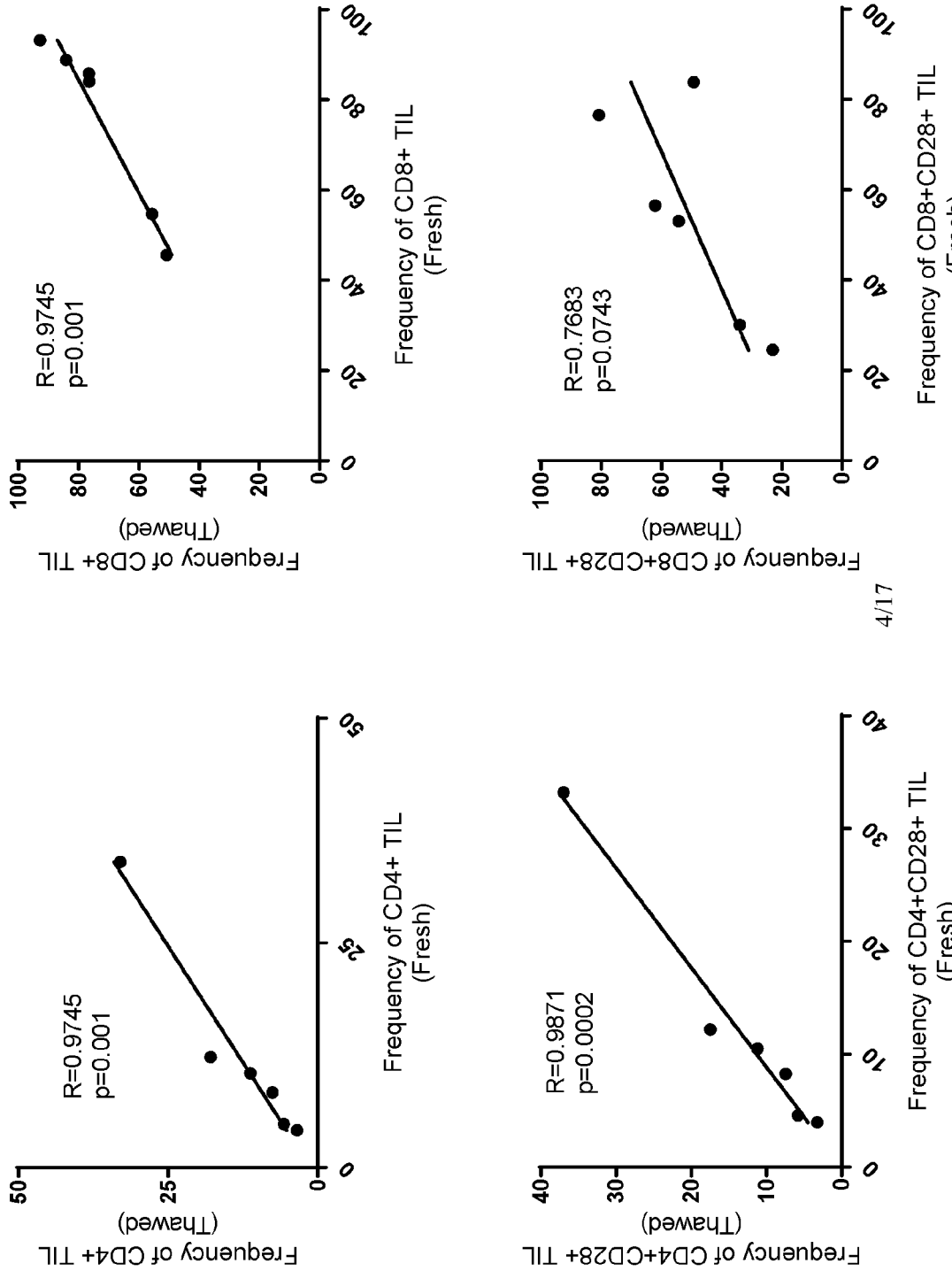

% Frequency of CD8+
100
50
0
TEMRA
Naive
CM
EM
Fresh
Thawed
% Frequency of CD4+
100
50
0
TEMRA
Naive
CM
EM
Fresh
Thawed

Figure 4


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

4/17

Figure 5

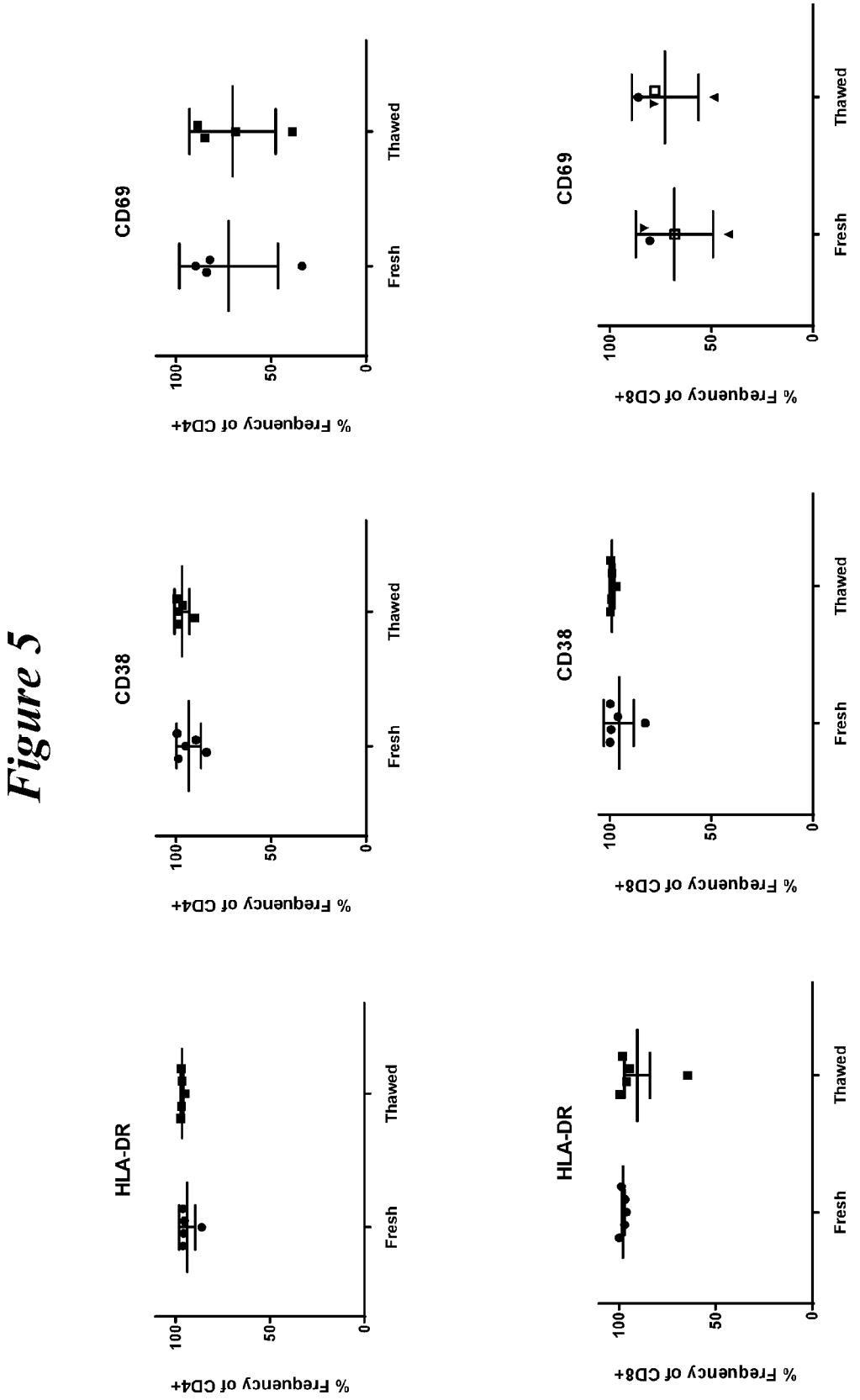

CD69
CD38
HLA-DR
% Frequency of CD4+
% Frequency of CD8+
100
50
0
Fresh
Thawed

Figure 6A

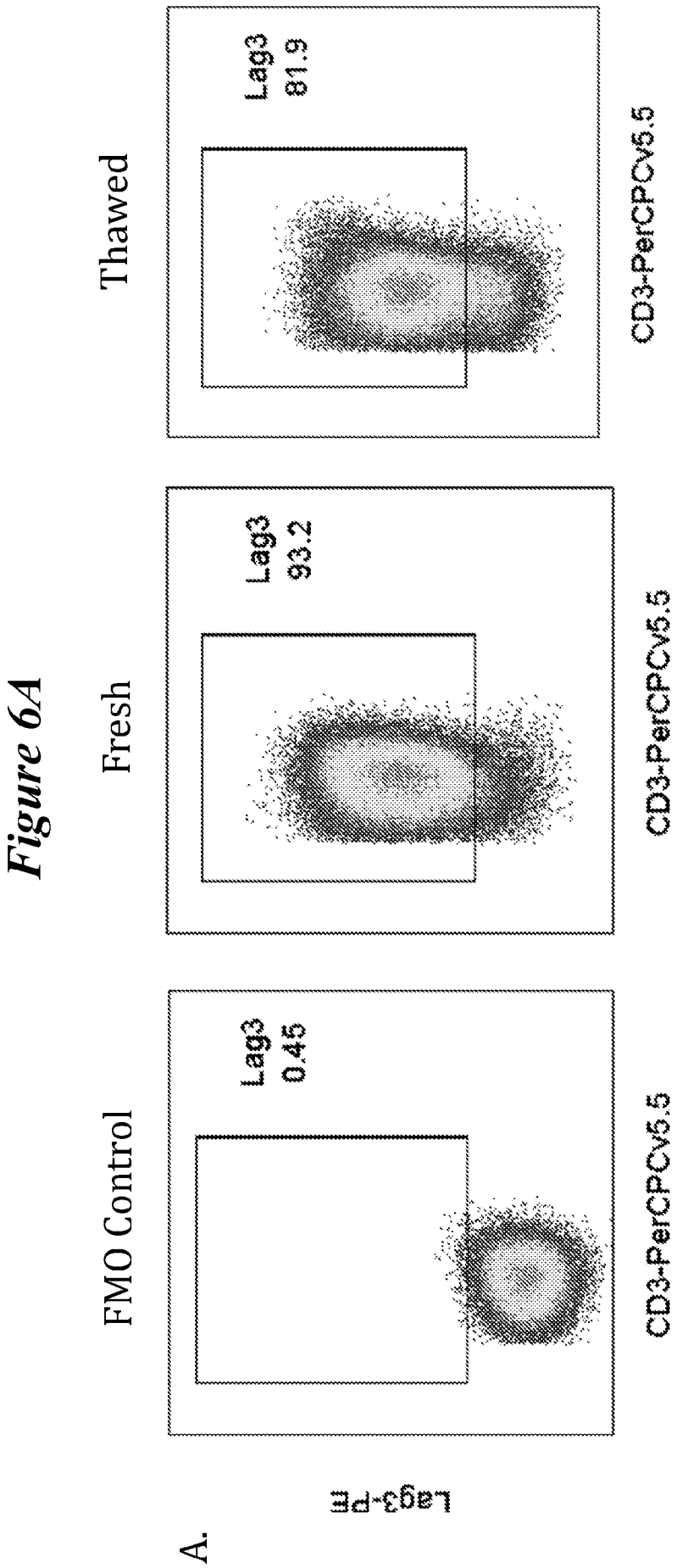
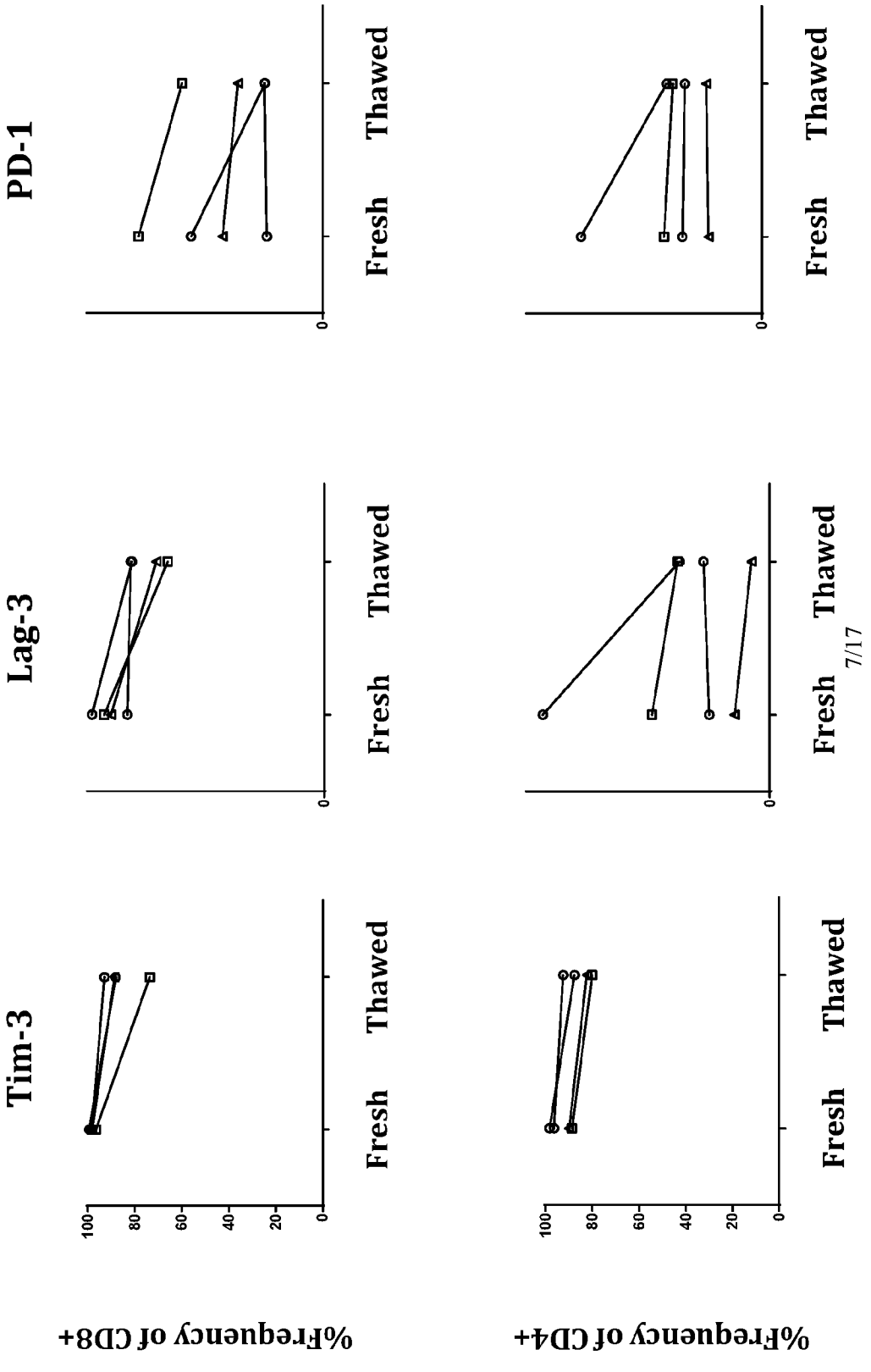

A.
Lag3-PE
FMO Control
Lag3
0.45
CD3-PerCPCv5.5
Fresh
Lag3
93.2
CD3-PerCPCv5.5
Thawed
Lag3
81.9
CD3-PerCPCv5.5

Figure 6B



7/17

Figure 7

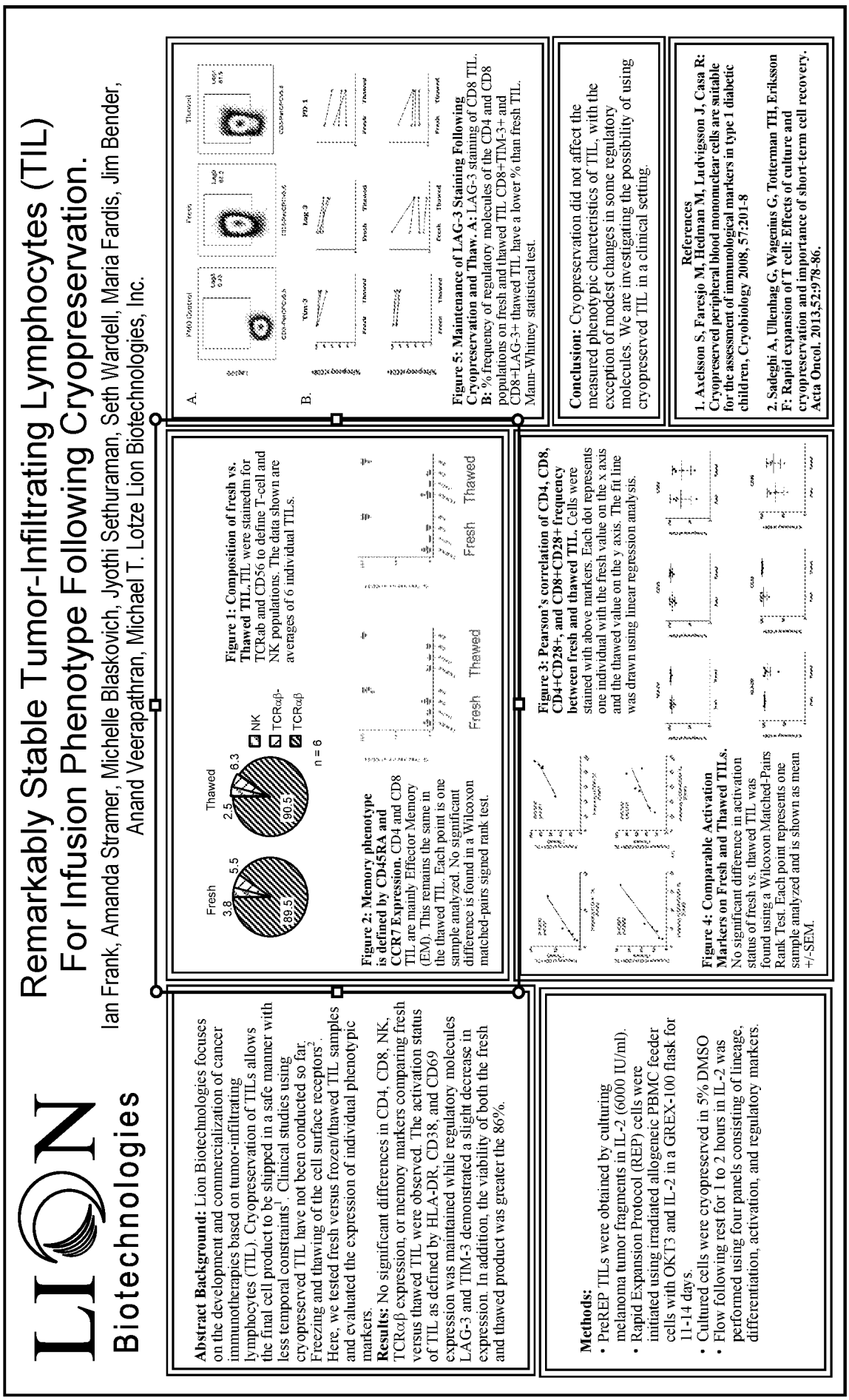

LION
Biotechnologies
Remarkably Stable Tumor-Infiltrating Lymphocytes (TIL) For Infusion Phenotype Following Cryopreservation.
Ian Frank, Amanda Stramer, Michelle Blaskovich, Jyothi Sethuraman, Seth Wardell, Maria Fardis, Jim Bender, Anand Veerapathran, Michael T. Lotze Lion Biotechnologies, Inc.
Abstract 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.
Fresh
Thawed
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.
PMD Control
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, Faresjo M, Hedman M, Ludvigsson J, Casa 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

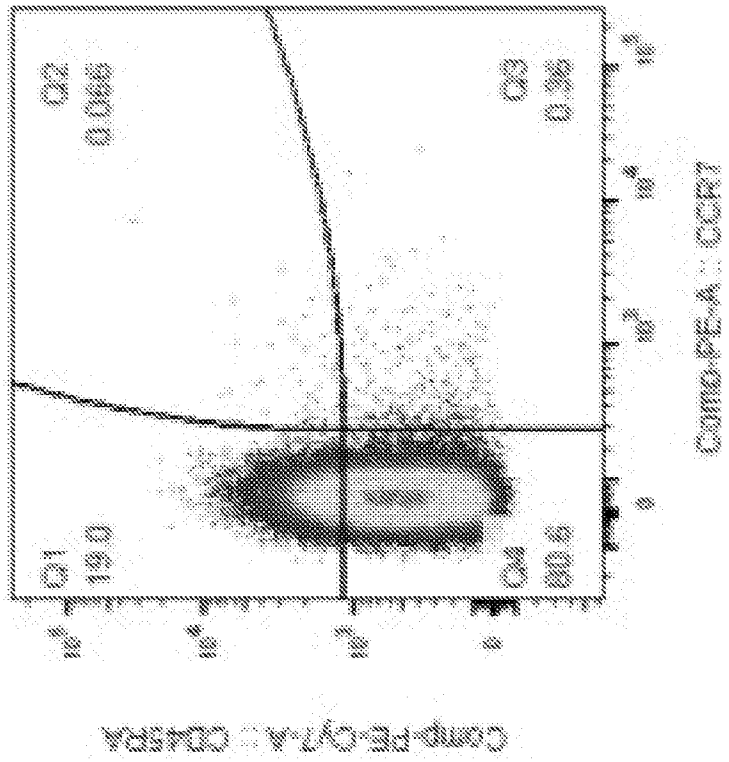


Figure 9

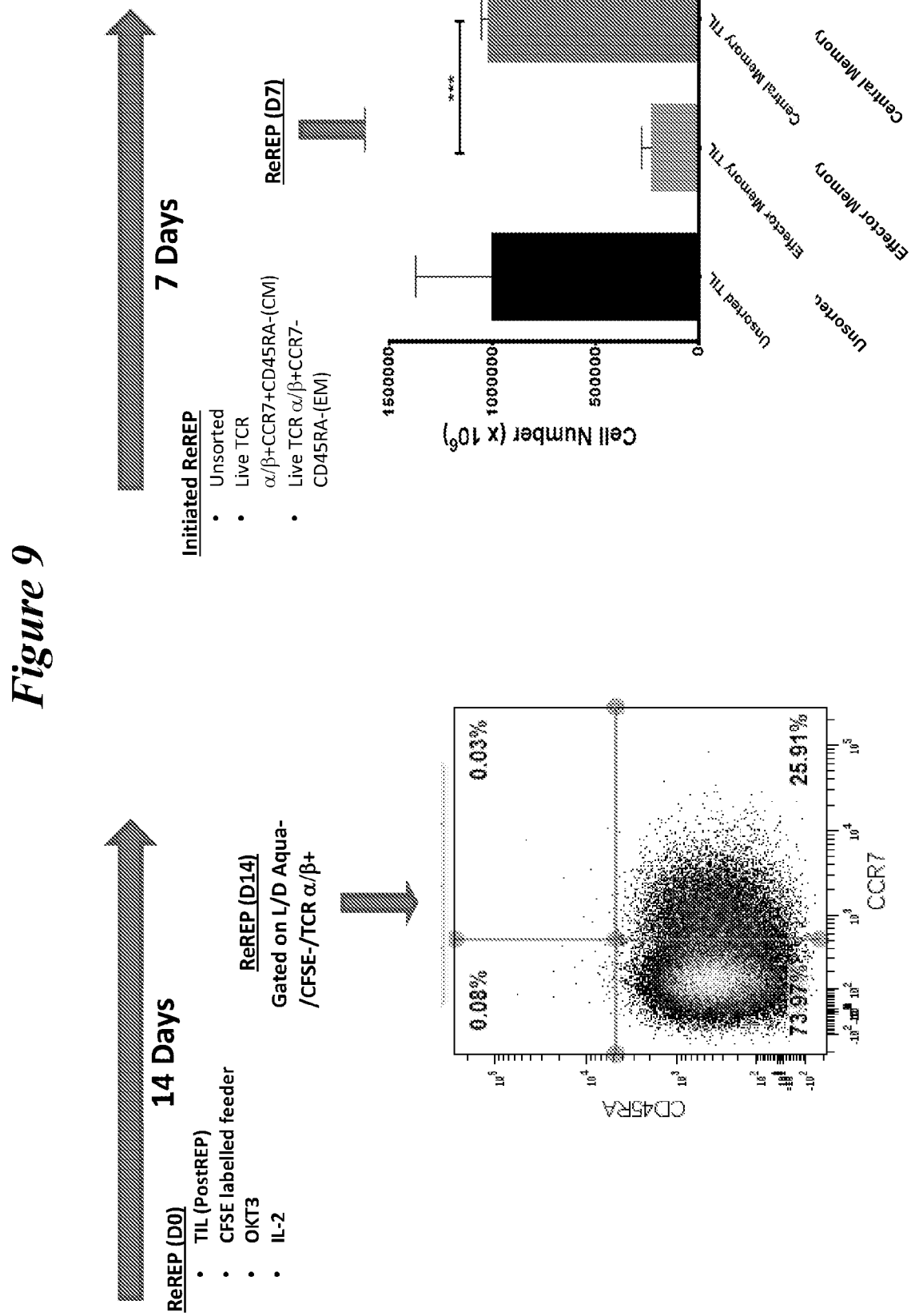

14 Days
ReREP (D0)
TIL (PostREP)
CFSE labelled feeder
OKT3
IL-2
ReREP (D14)
Gated on L/D Aqua-/CFSE-/TCR α/β+
0.08%
0.03%
73.97%
25.91%
CD45RA
CCR7
7 Days
Initiated ReREP
Unsorted
Live TCR α/β+CCR7+CD45RA-(CM)
Live TCR α/β+CCR7-CD45RA-(EM)
ReREP (D7)
Cell Number (x 10^6)
1500000
1000000
500000
0
***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

Figure 10A

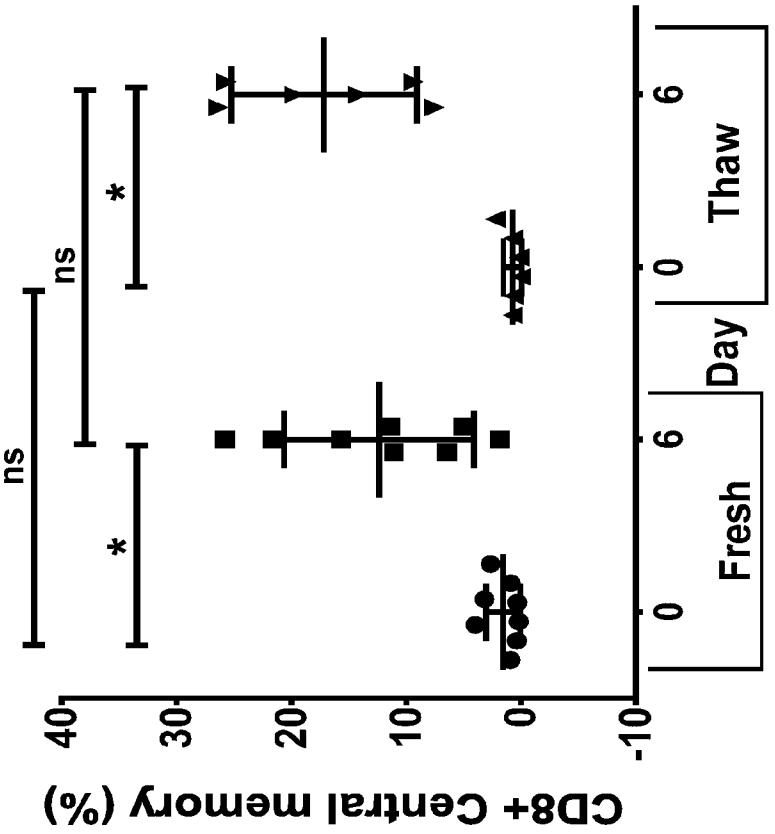


Figure 10B

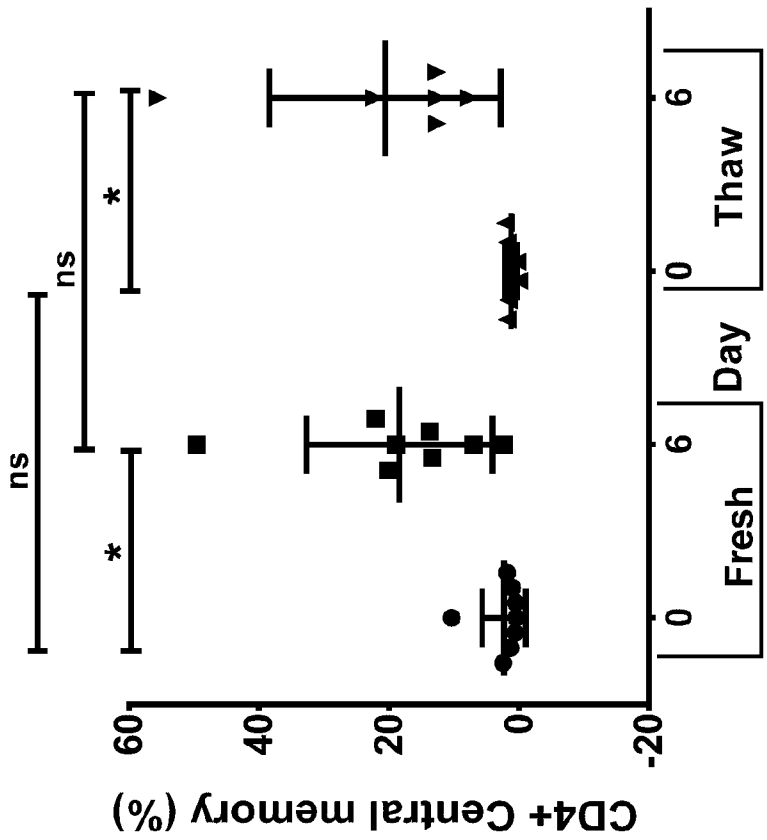


Figure 11

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

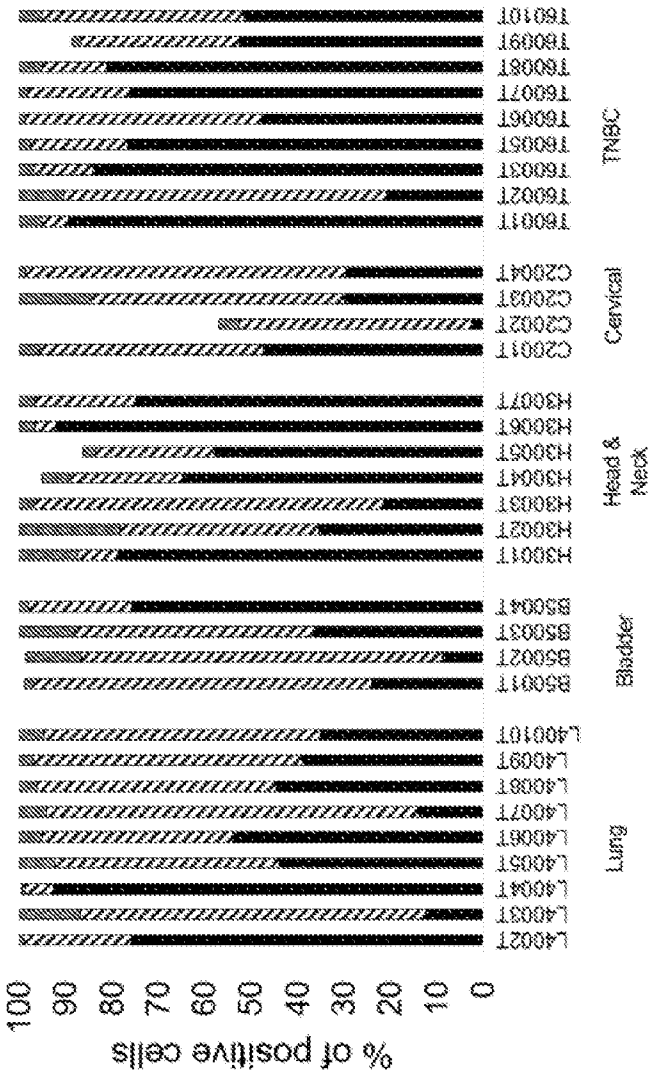

CD4+
CD8+
CD56+
% of positive cells
100
90
80
70
60
50
40
30
20
10
0
L40027T
L40037T
L40047T
L40057T
L40067T
L40077T
L40087T
L40097T
L40107T
Lung
B50011T
B50021T
B50037T
B50047T
Bladder
H30011T
H30021T
H30031T
H30047T
H30057T
H30067T
H30077T
Head & Neck
C20011T
C20027T
C20037T
C20047T
Cervical
T60011T
T60027T
T60037T
T60047T
T60057T
T60067T
T60077T
T60087T
T60097T
T60107T
TNBC

Figure 13

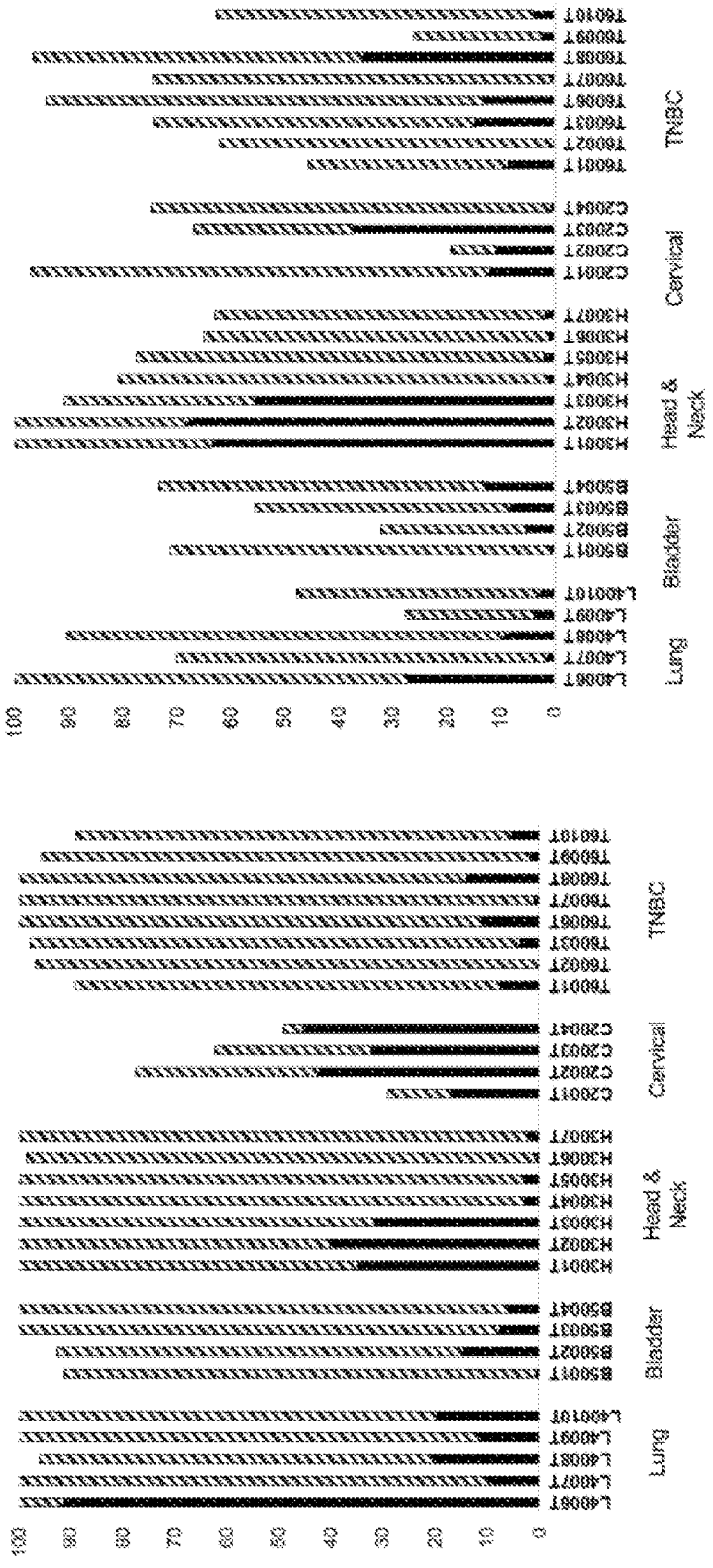

% CD8/CD28+
CD8/CD27+
CD4/CD28+
CD4/CD27+
Lung
Bladder
Head & Neck
Cervical
TNBC
100
90
80
70
60
50
40
30
20
10
0

Figure 14

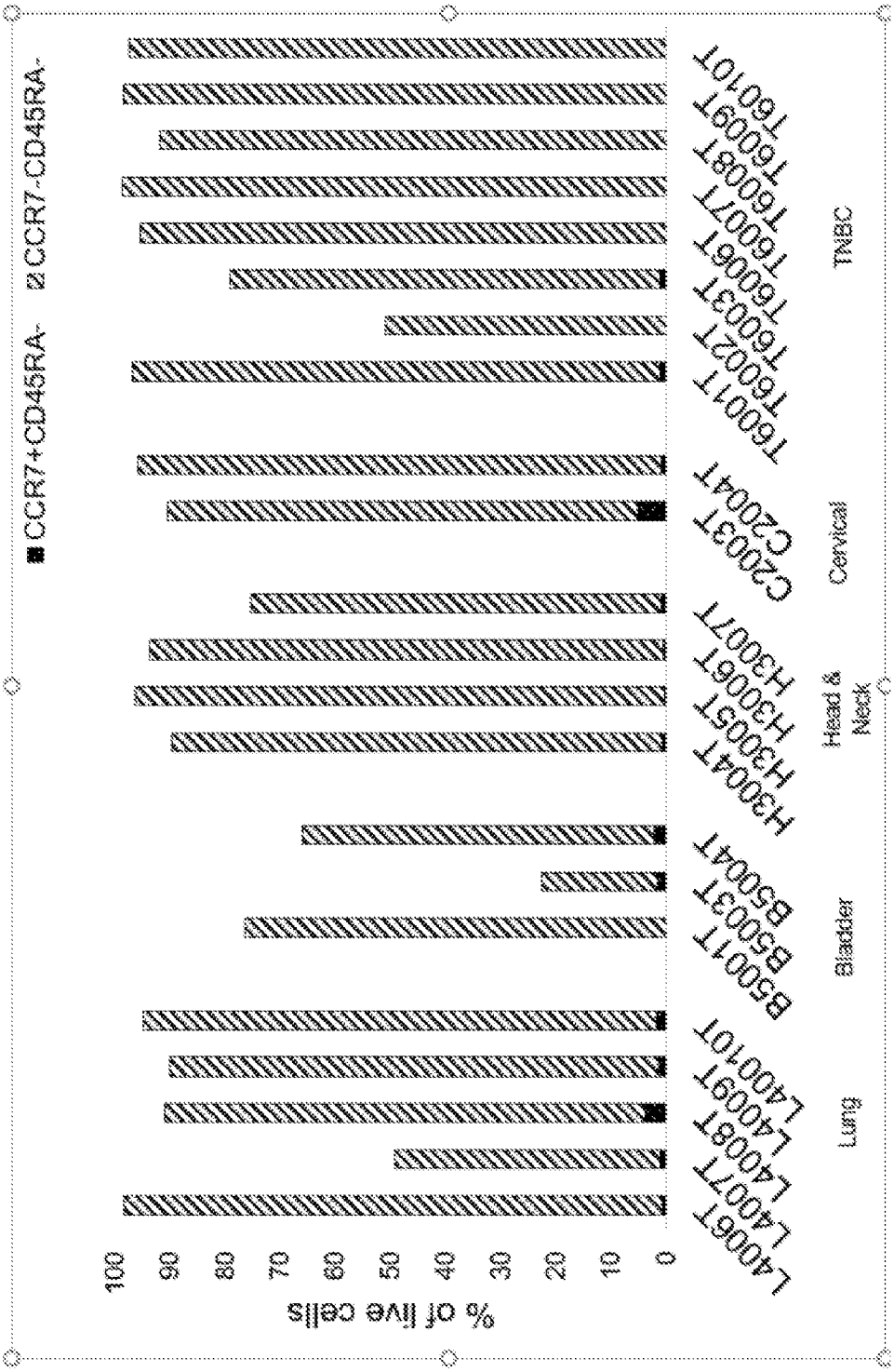

CCR7+CD45RA-
CCR7-CD45RA-
% of live cells
100
90
80
70
60
50
40
30
20
10
0
L40061
L40071
L40081
L40091
L40101
B50011
B50031
B50041
H30041
H30051
H30061
H30071
C20031
C20041
T60011
T60021
T60031
T60061
T60071
T60081
T60091
T60101
Lung
Bladder
Head & Neck
Cervical
TNBC

Figure 15

TUBE NAME	SSRC	S/ Live	S/ Live	S/ Live/ CD8	S/ Live/ CD8	S/ Live/ CD8	S/ Live/ CD8	S/ Live/ CD8	S/ Live/ CD8	S/ Live/ CD8
		CD4 Freq. of Parent	CD8 Freq. of Parent	Q1: CCR7-, CD45RA+ Freq. of Parent	Q2: CCR7+, CD45RA+ Freq. of Parent	Q3: CCR7+, CD45RA- Freq. of Parent	Q4: CCR7-, CD45RA- Freq. of Parent	Q5: CD28+, CD57+ Freq. of Parent	Q6: CD28+, CD57+ Freq. of Parent	Q7: CD28+, CD57- Freq. of Parent	Q8: CD28-, CD57- Freq. of Parent
1.00E+08	Not Rested	3.01	95	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	56.7	42.2	2.51	0.32	8.19	89
8.00E+08	Not Rested	2.85	95.6	0.34	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	55.1	43	1.05	0.32	12.8	85.9
4.00E+08	Rested	5.74	90	0.86	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	60.5	37	1.18	0.32	17.1	81.4