Fig. 1

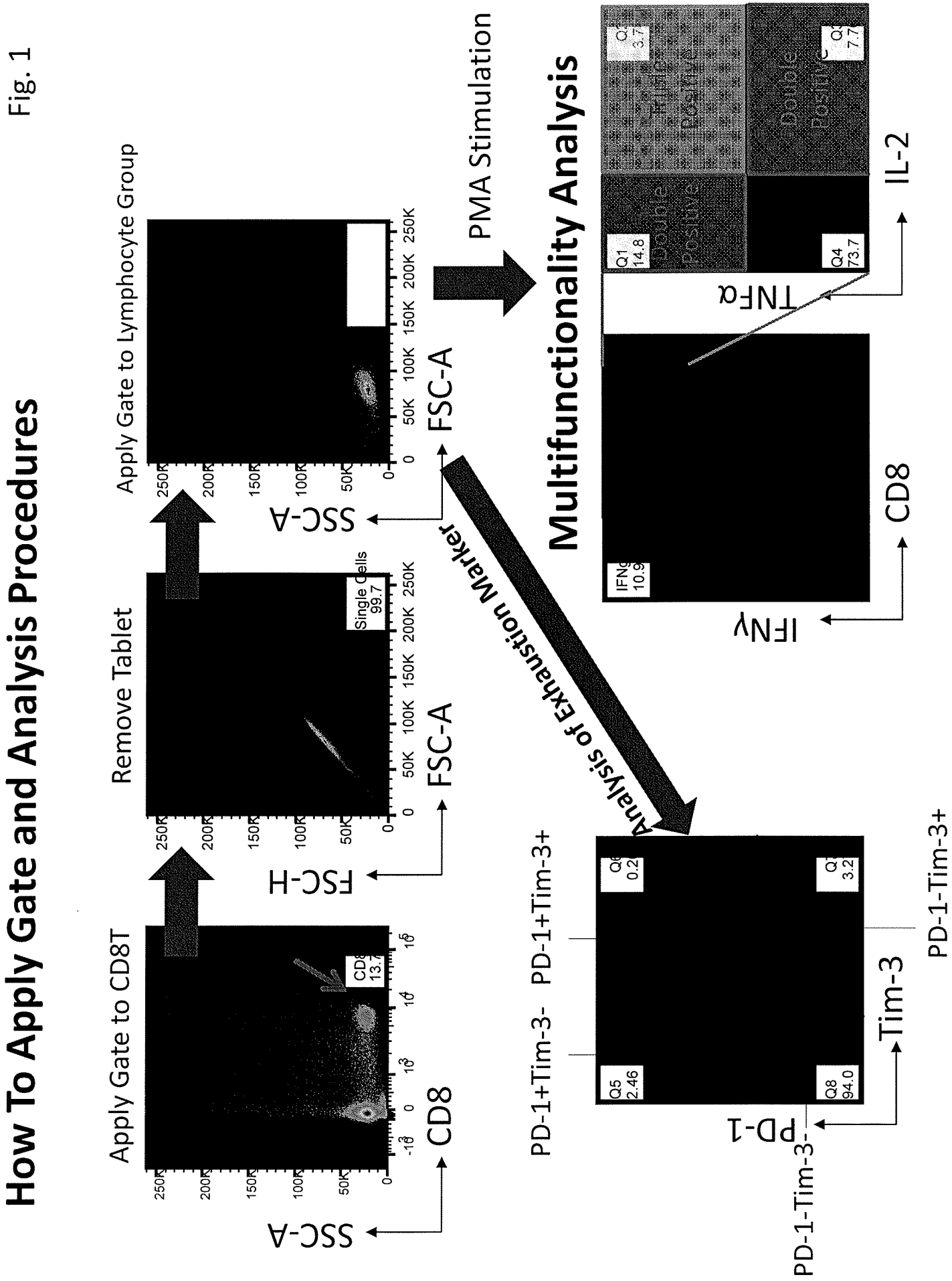
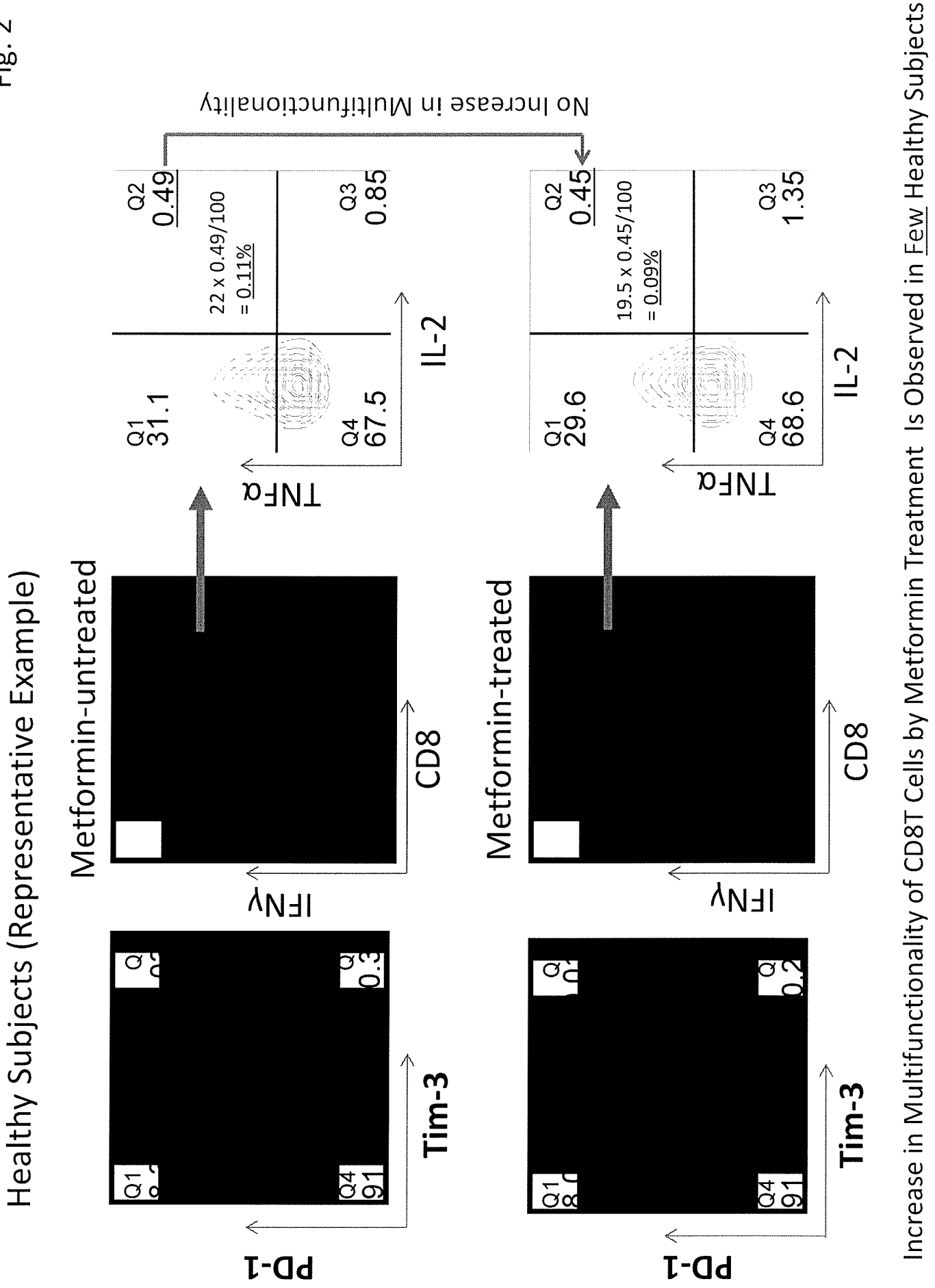
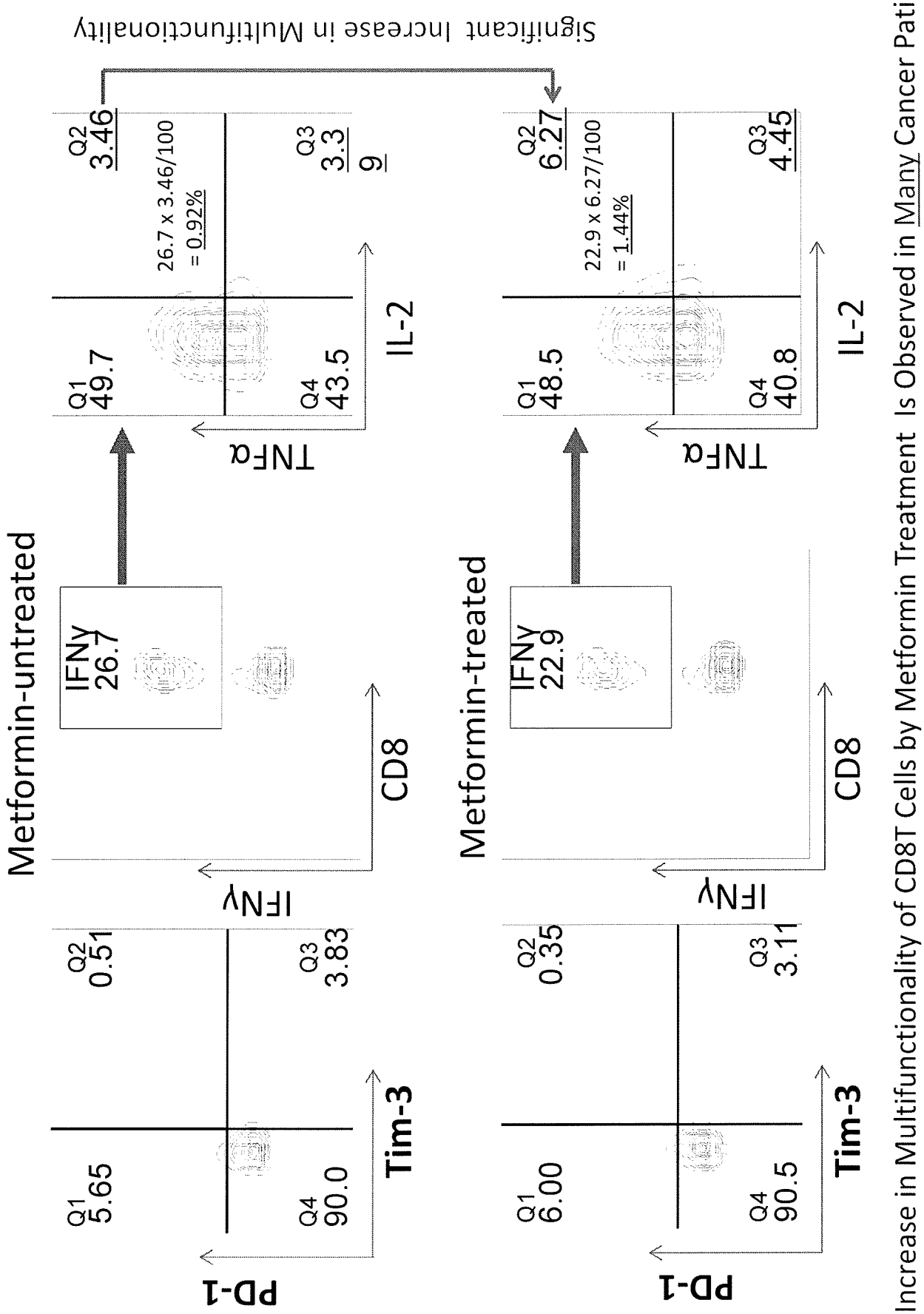

How To Apply Gate and Analysis Procedures
Apply Gate to CD8T
CD8
SSC-A
Remove Tablet
FSC-H
FSC-A
Single Cells
99.7
Apply Gate to Lymphocyte Group
SSC-A
FSC-A
PMA Stimulation
Analysis of Exhaustion Marker
Multifunctionality Analysis
PD-1+Tim-3-
PD-1+Tim-3+
PD-1-Tim-3-
PD-1-Tim-3+
PD-1
Tim-3
Q5 2.46
Q8 94.0
Q1 3.2
IFNγ
CD8
IFNγ 10.9
TNFα
IL-2
Q1 14.8
Q4 73.7
Double Positive
Triple Positive
Double Positive
Q1 7.7

Fig. 2



Increase in Multifunctionality of CD8T Cells by Metformin Treatment Is Observed in Few Healthy Subjects

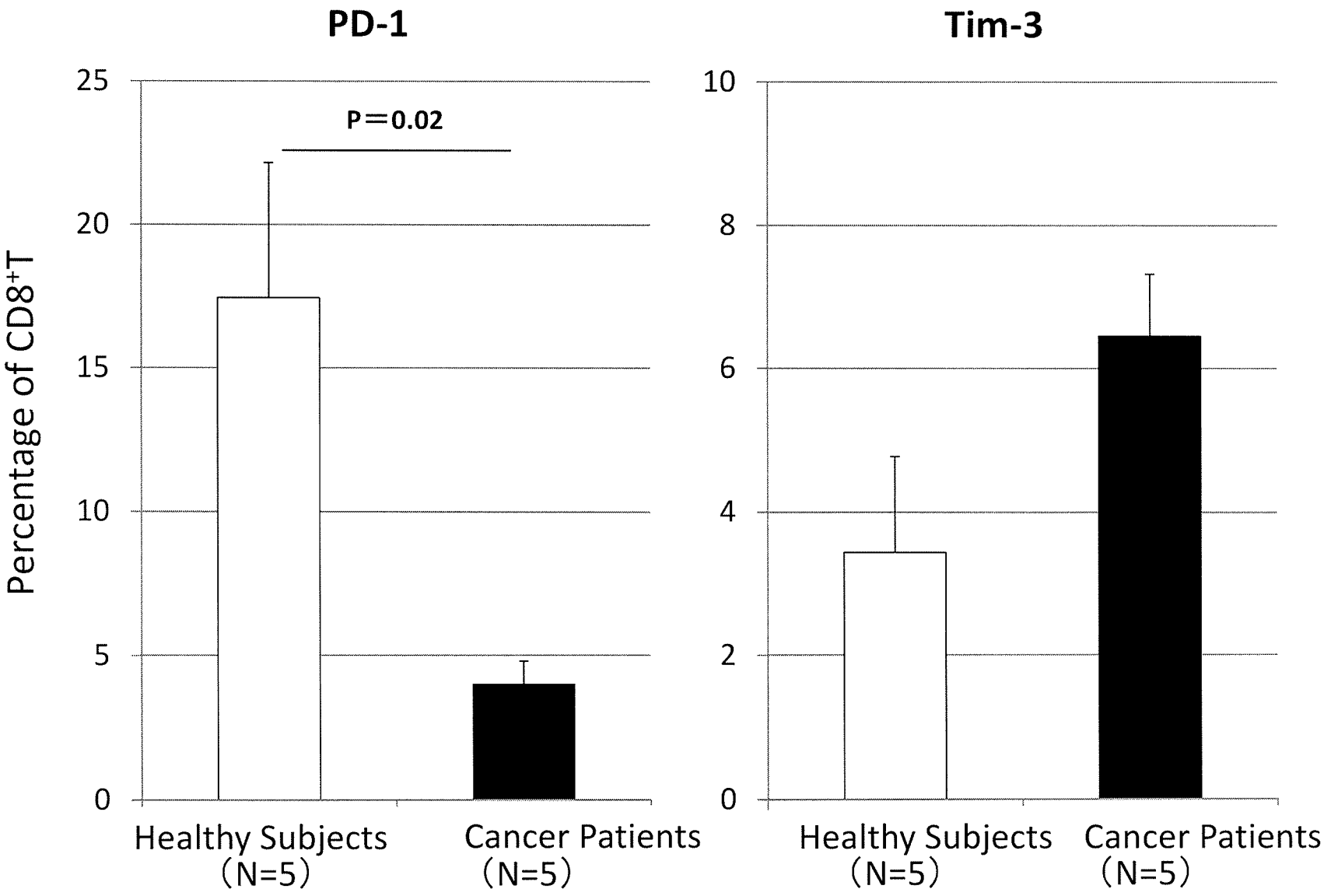
Fig. 3



Increase in Multifunctionality of CD8T Cells by Metformin Treatment Is Observed in Many Cancer Patients

Fig. 4

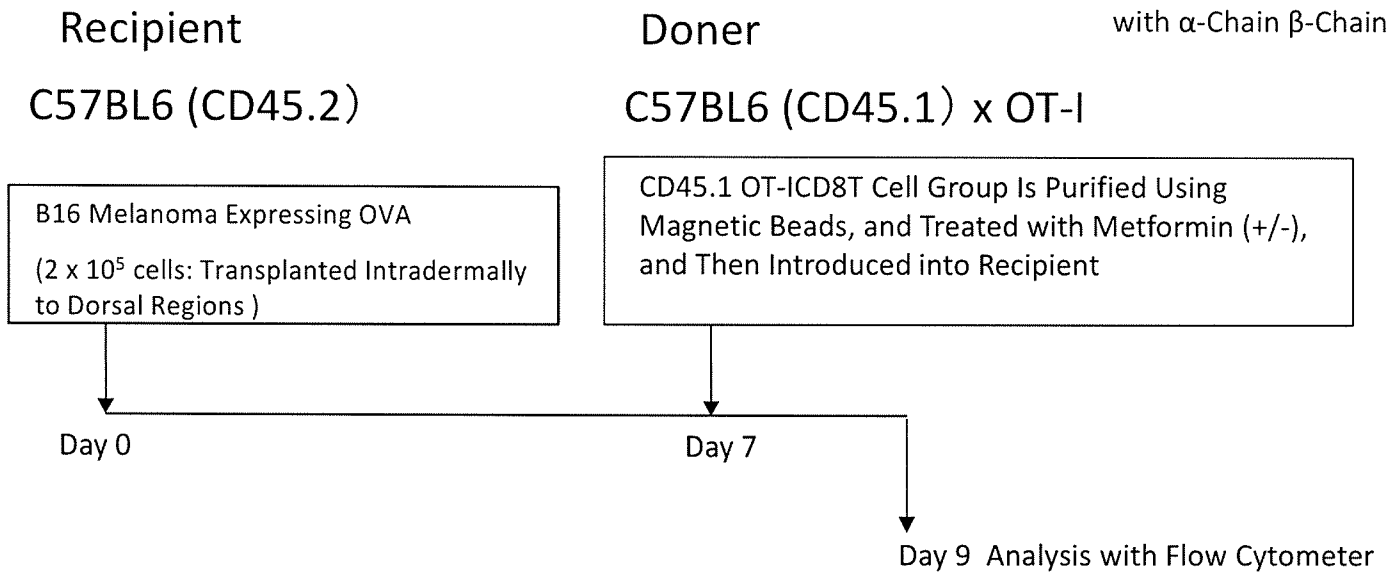
Comparison of Expressions of Exhaustion Molecules PD-1 and Tim-3 in CD8T Cells (Healthy Subjects vs. Cancer Patients)



Expression Frequency of PD-1 is Significantly Lower in Cancer Patients. In Contrast, Expression Frequency of Tim-3 Tends to be High in Cancer Patients.

Fig. 5

OT-I Mice Are Transgenic Mice Having OVA-Antigen-Recognizing $CD8^+$T Cells with α-Chain β-Chain T Cell Receptors



CD45.1 OT-1

→ Collect Spleen Cells
→ Purification of CD8 T Cells Using Magnetic Beads
→ Expose Cells to Metformin for 6 Hours
→ Adoptive Transfer of Treated OT-I CD8 T Cells
→ After 2 Days, Remove Tumor and Collect Cells Infiltrated in Tumor
→ Donor Cells are Specified by CD8, CD45.1, Vα2, and Multifunctionality is Analyzed Based on IL-2,TNFα,IFNγ

Fig. 6

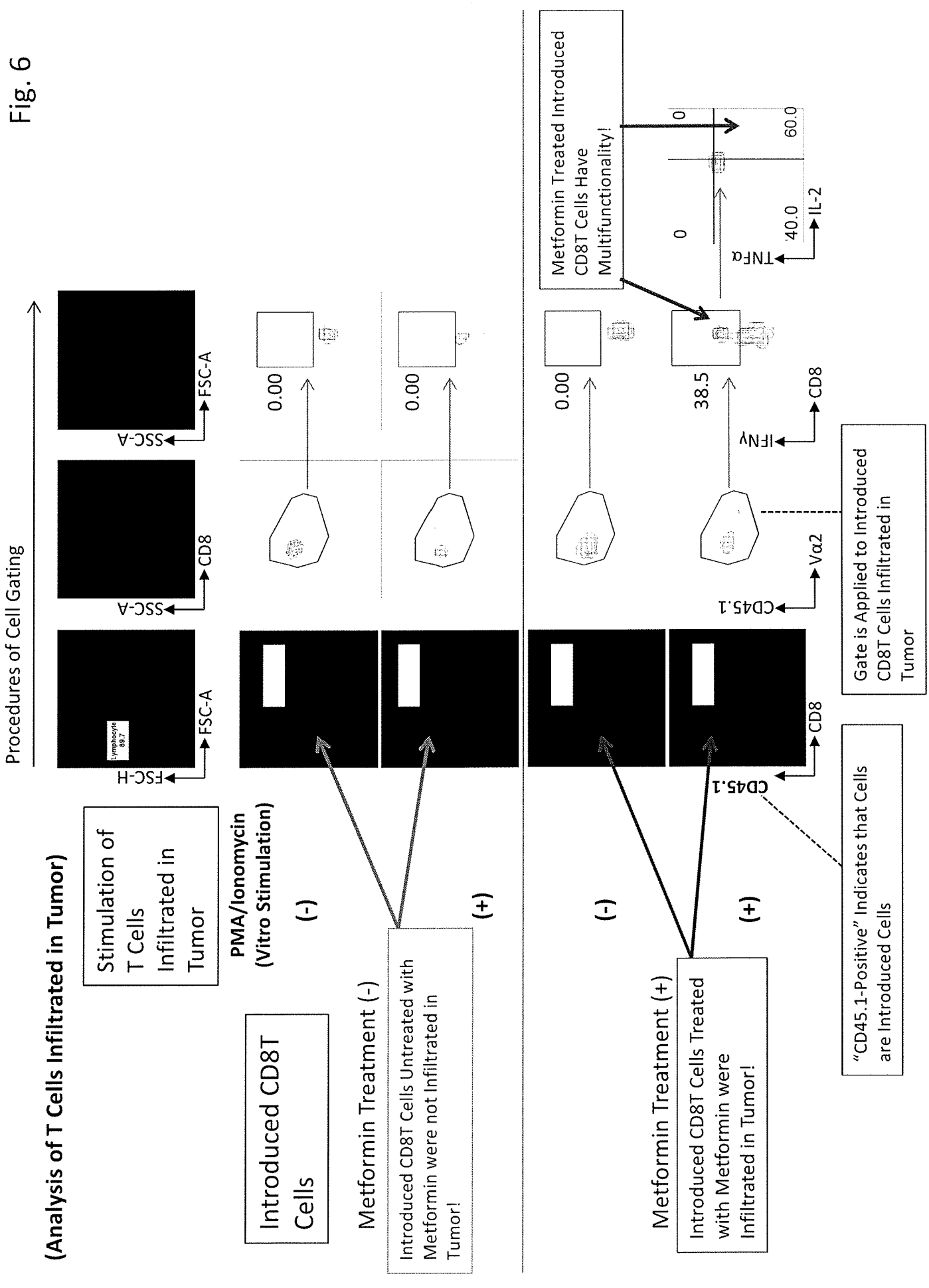

(Analysis of T Cells Infiltrated in Tumor)
Procedures of Cell Gating
Stimulation of T Cells Infiltrated in Tumor
FSC-H
FSC-A
SSC-A
CD8
PMA/Ionomycin (Vitro Stimulation)
Introduced CD8T Cells
Metformin Treatment (-)
Introduced CD8T Cells Untreated with Metformin were not Infiltrated in Tumor!
(-)
(+)
0.00
0.00
CD45.1
CD8
Vα2
IFNγ
TNFα
IL-2
Metformin Treatment (+)
Introduced CD8T Cells Treated with Metformin were Infiltrated in Tumor!
"CD45.1-Positive" Indicates that Cells are Introduced Cells
Gate is Applied to Introduced CD8T Cells Infiltrated in Tumor
Metformin Treated Introduced CD8T Cells Have Multifunctionality!
0.00
38.5
0
0
40.0
60.0

Fig. 7

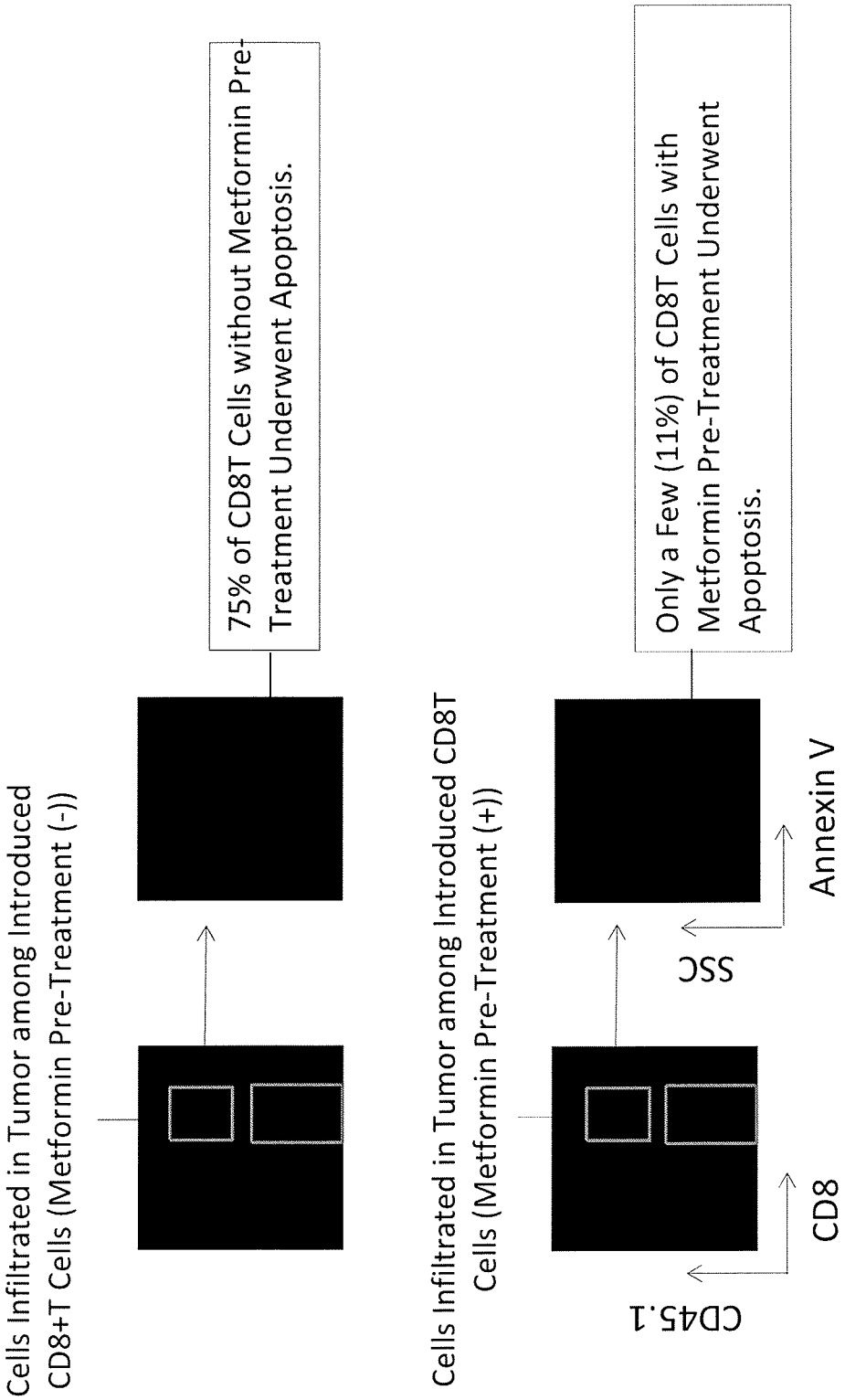

Cells Infiltrated in Tumor among Introduced CD8+T Cells (Metformin Pre-Treatment (-))
75% of CD8T Cells without Metformin Pre-Treatment Underwent Apoptosis.
Cells Infiltrated in Tumor among Introduced CD8T Cells (Metformin Pre-Treatment (+))
Only a Few (11%) of CD8T Cells with Metformin Pre-Treatment Underwent Apoptosis.
CD45.1
CD8
SSC
Annexin V

Fig. 8

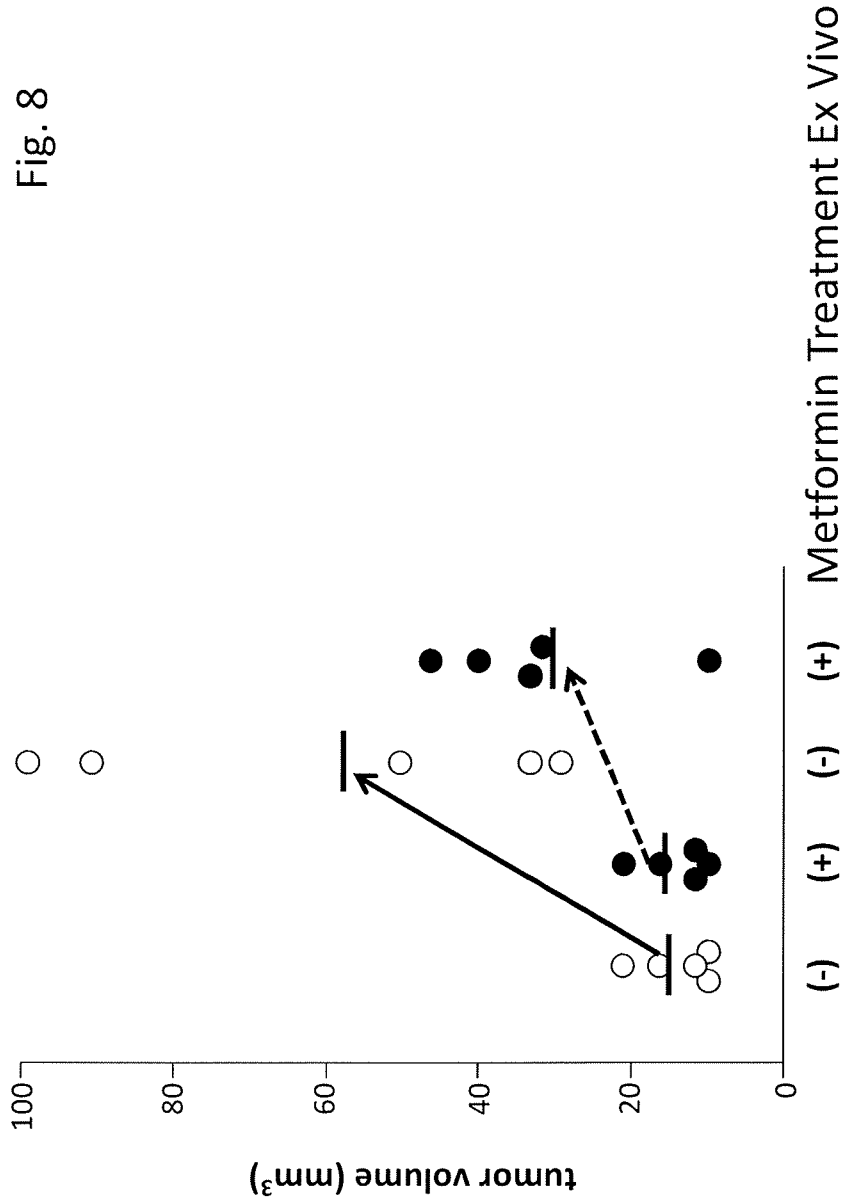

tumor volume (mm³)
100
80
60
40
20
0
(-)
(+)
(-)
(+)
Metformin Treatment Ex Vivo
Day 7
Day 9

Fig. 9

Cellular Immune Treatment Using Metformin

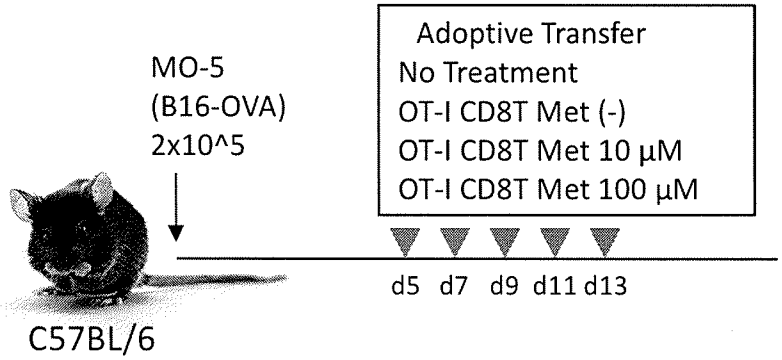
OT-ICD8T Cells Are Cultured for 6 Hours with 0, 10, 100 μM Metformin to Prepare PJ Cells

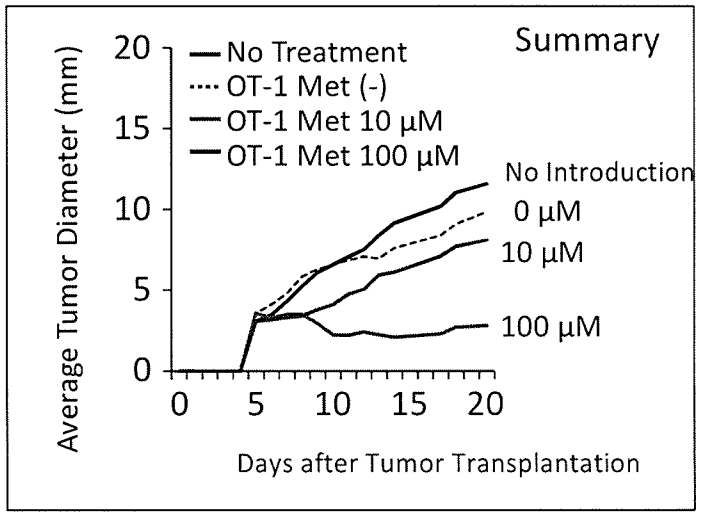
↓

Adoptive Transfer of PJ Cells to MO5 Cancer-bearing Mice

↓

Monitoring of Tumor Growth





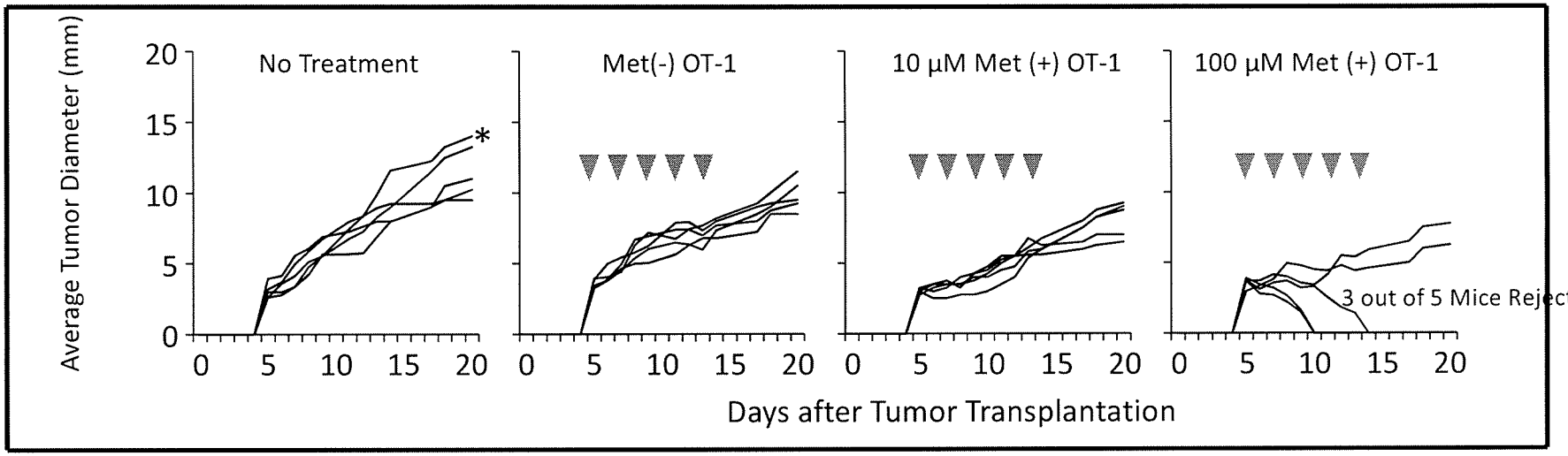


Fig. 10

Effects of Combined Use of Metformin and Cancer Vaccine

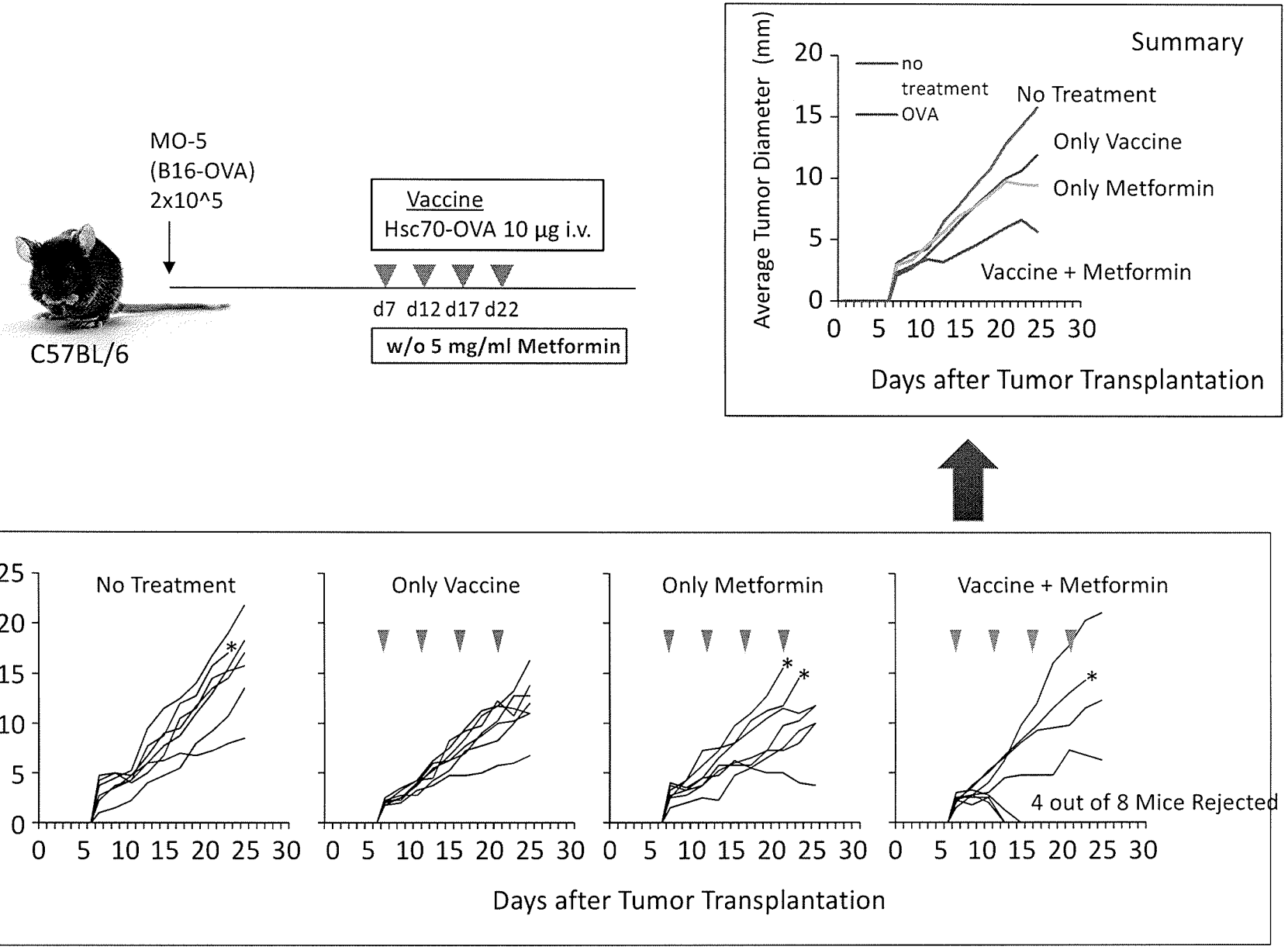


Fig. 11

Effects of Combined Use of PD-1 Antibody and Metformin-1

Test Condition	
Control	n=10
Metformin	n=10
αPD-1	n=10
Met + αPD-1	n=10
Treatment Started on Day 5	

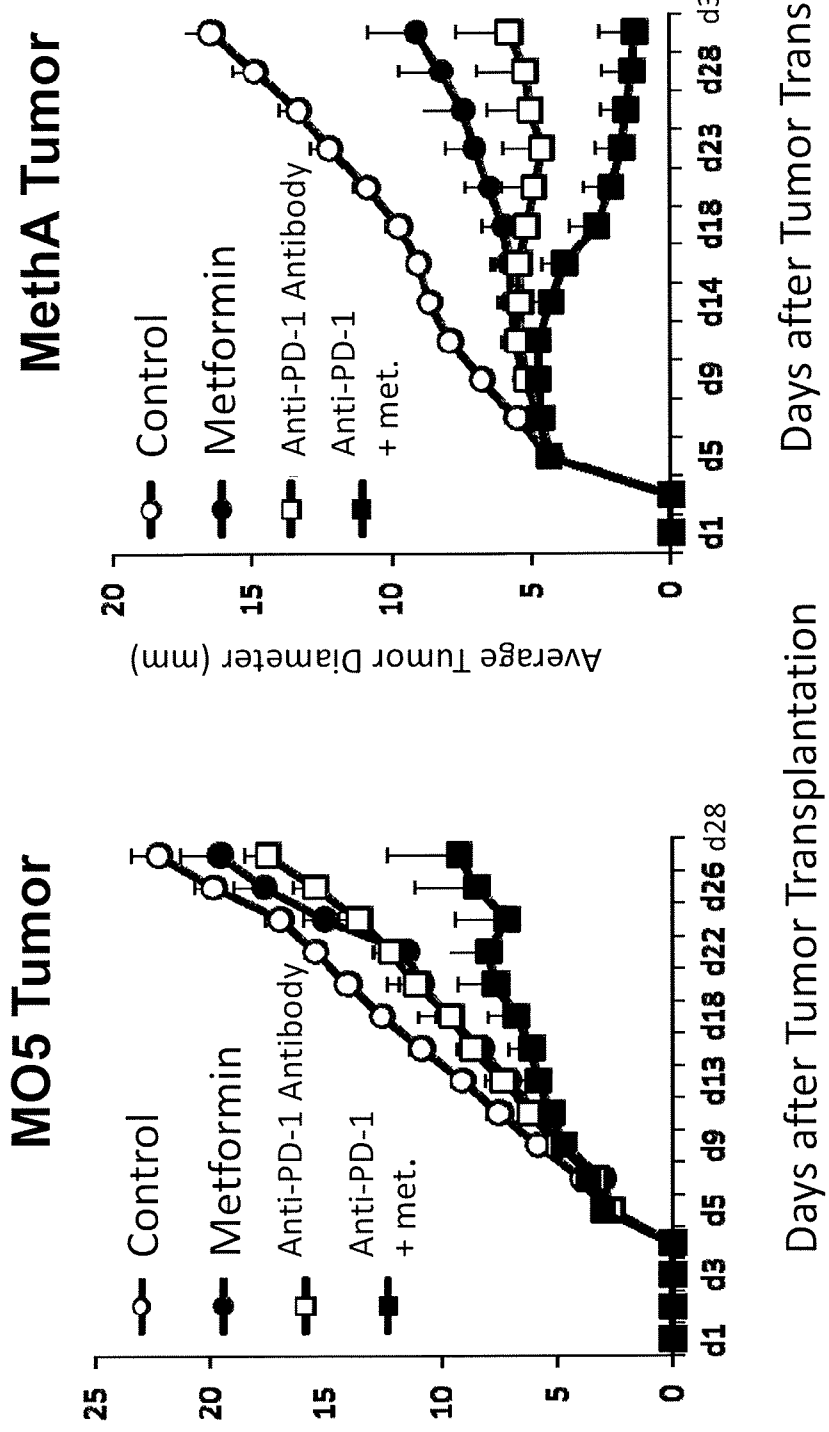


Fig. 12 **Effects of Combined Use of PD-1 Antibody and Metformin-2** MO5 (Tumor Curves of Individuals)

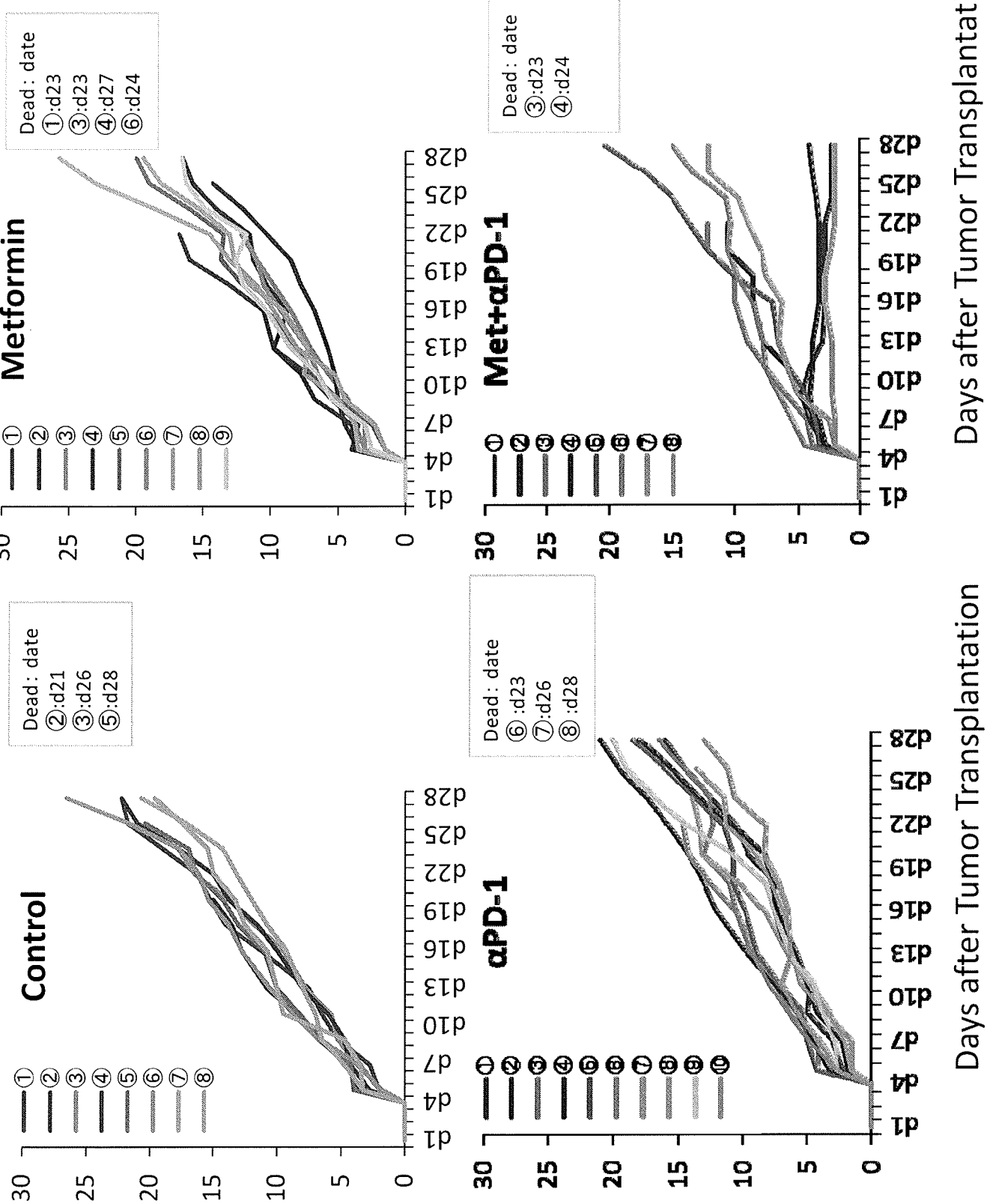


Fig. 13 **Effects of Combined Use of PD-1 Antibody and Metformin-3**

