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## Supplemental Information

# Overcoming effector T cell exhaustion in ovarian cancer ascites with a novel adenovirus encoding for a MUC1 bispecific antibody engager and IL-2 cytokine

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**Table S1. List of antibodies used in this study.**

| Experiment | Antibodies | Manufacturer |
|---|---|---|
| T cell activation assay (in vitro) and immunological analysis of tumor (in vivo) | FITC anti-human CD3 (clone OKT3) | Biolegend, California, USA |
| | AF-700 anti- human CD4 (clone A161A1) | |
| | BV-421 anti- human CD8 (clone SK1) | |
| | PE-Cy™7 anti- human CD69 (clone FN50) | |
| | PE-Cy™5 anti- human CD25 (clone M-A251) | |
| | PE-CF594 anti-human CD127 (clone HIL-7R-M21) | BD Sciences, New Jersey, USA |
| | APC anti-human Tbet (clone 4B10) | Biolegend, California, USA |
| | PE anti-human EOMES (clone X4-83) | BD Sciences, New Jersey, USA |
| Expression of cell surface MUC1 antigen | APC anti-human MUC1 (clone 16A) | Biolegend, California, USA |
| Profiling of NK and NK-T like cells | BV-605 anti-human CD56 (HCD56) | Biolegend, California, USA |
| Activation of γδ T cell | PE anti-human TCR-γδ (clone 11F2) | Miltenyi, Bergisch Gladbach, Germany |
| | FITC anti-human Vδ1 (clone REA173) | |
| | APC anti-human Vδ2 (clone REA771) | |
| | AF-700 Perforin (clone B-D48) | |
| | BV421 GranzymeB (clone GB11) | BD Sciences, New Jersey, USA |
| | BV650 Interferon gamma (clone 4S.B3) | Biolegend, California, USA |
| T cell exhaustion panel | FITC anti-human CD3 (clone OKT3) | BioLegend, California, USA |
| | AF-700 anti-human CD4 (clone A161A1) | |
| | BV-510 anti-human CD8 (clone SK1) | BD Sciences, New Jersey, USA |
| | PE anti-human TIM-3 (clone A18087E) | BioLegend, California, USA |
| | BV-605 anti-human TIGIT (clone A15153G) | |
| | PE anti-human TIM-3 (clone A18087E) | |
| | BV-605 anti-human TIGIT (clone A15153G) | |
| | PE-Cy™5 anti-human LAG-3 (clone 11C3C65) | |
| | PE-Cy7 anti-human BTLA (clone MIH26) | |
| | APC anti-human CTLA-4 (clone BNI3) | |
| | PE-CF594 anti-human PD-1 (clone NAT105) | |
| | PE anti-human TOX (clone TXRX10) | Invitrogen, Massachusetts, USA |
| | BV-421 anti-human TCF-1 (clone S33-966) | BD Sciences, New Jersey, USA |

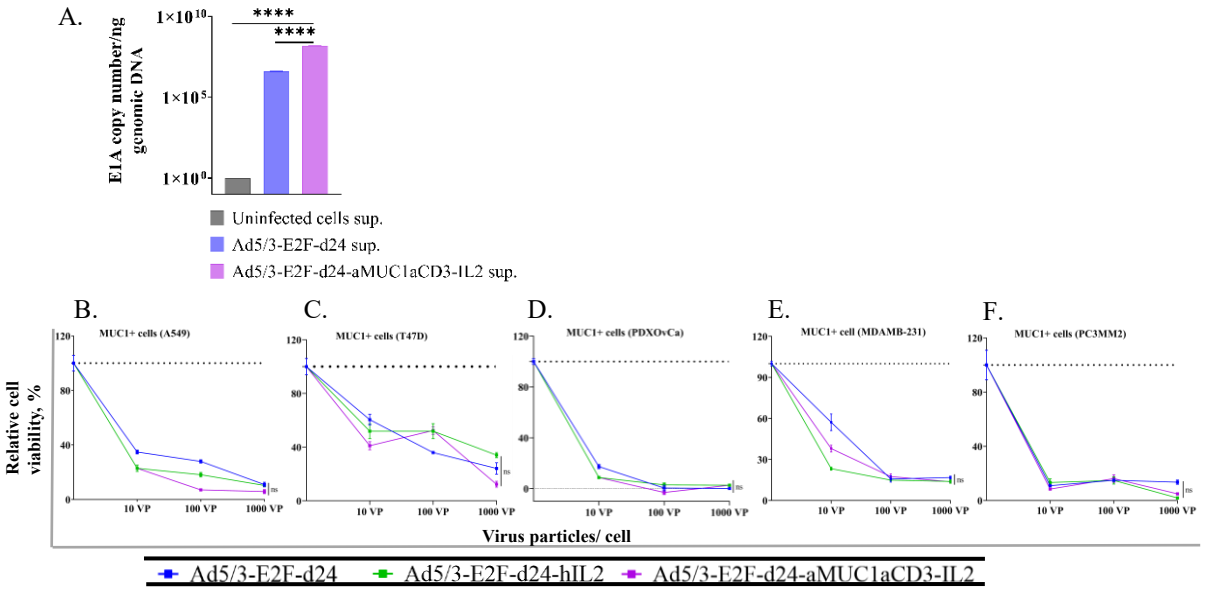



**Figure S1. Oncolytic activity of TILT-322 *in vitro*. (A)** Quantification of E1A gene copy number as a measure of virus replication in TILT-322 infected A549 cells supernatant. **(B-F)** Relative cell viability of human cancer cells followed by the infection of Ad5/3-E2F-d24, Ad5/3-E2F-d24-hIL2, and TILT-322 virus different concentration of virus ranging from 10, 100 or 1000 VP/ cell. Cells were incubated with the viruses for 3 days (A549, PDX-OvCa, and MDAMB-231), 5 days (PC3MM2), or 7 days (T47D) before determining cell viability. The untreated control group is indicated by the dashed line.

All experiments were run in quadruplet, and the resulting data are presented as mean ± SEM. One-way ANOVA with Tukey's post hoc test was used to compare more than two groups in the activation assay. Statistical significance is represented as *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ and ns = non-significant.

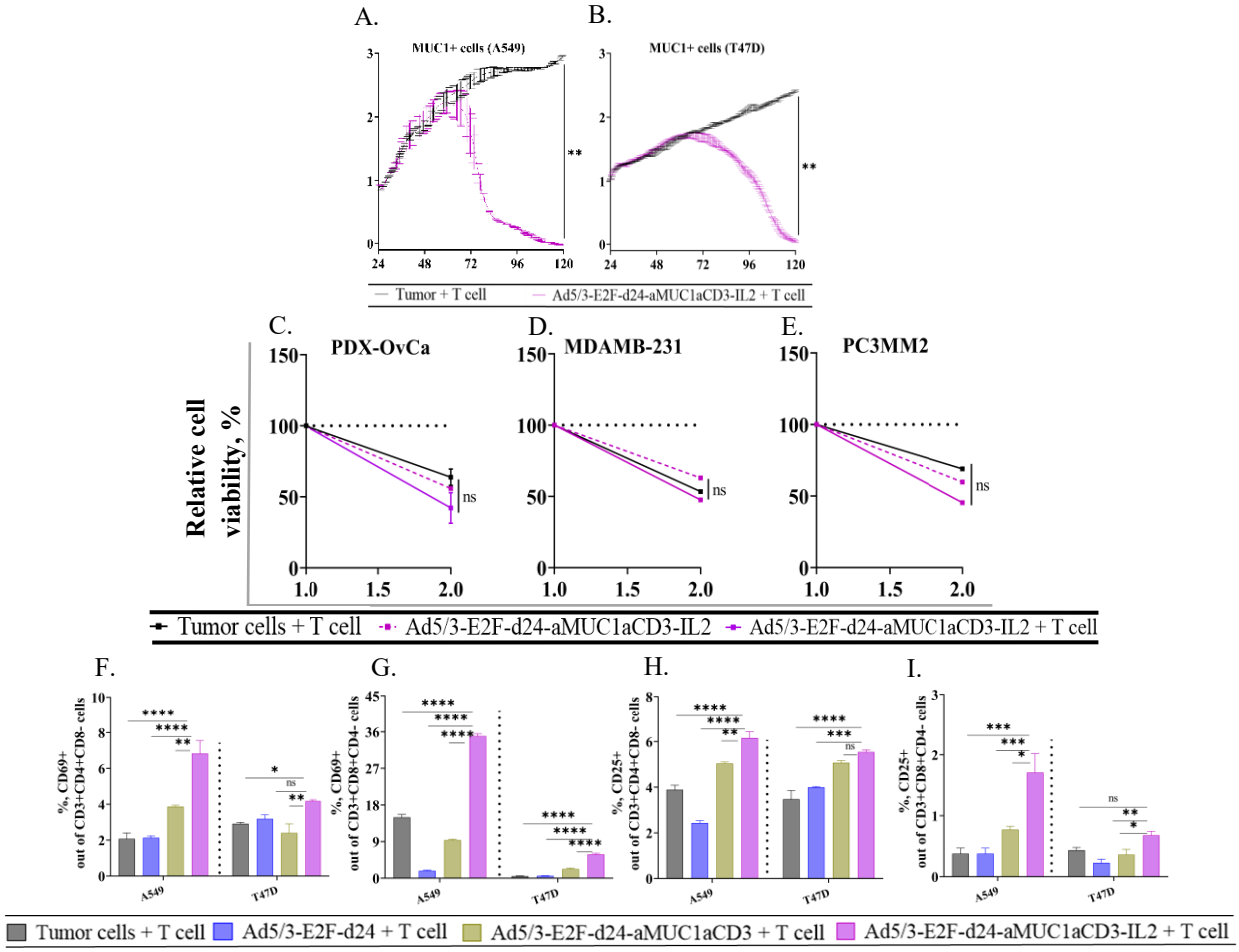

A.
MUC1+ cells (A549)
B.
MUC1+ cells (T47D)
Tumor + T cell
Ad5/3-E2F-d24-aMUC1aCD3-IL2 + T cell
C.
PDX-OvCa
D.
MDAMB-231
E.
PC3MM2
Relative cell viability, %
ns
Tumor cells + T cell
Ad5/3-E2F-d24-aMUC1aCD3-IL2
Ad5/3-E2F-d24-aMUC1aCD3-IL2 + T cell
F.
G.
H.
I.
%, CD69+ out of CD3+CD4+CD8- cells
%, CD69+ out of CD3+CD8+CD4- cells
%, CD25+ out of CD3+CD4+CD8- cells
%, CD25+ out of CD3+CD8+CD4- cells
A549
T47D
Tumor cells + T cell
Ad5/3-E2F-d24 + T cell
Ad5/3-E2F-d24-aMUC1aCD3 + T cell
Ad5/3-E2F-d24-aMUC1aCD3-IL2 + T cell

**Figure S2**. **TILT-322 mediated tumor cell killing and T cell activation *in vitro*.** Real-time iCELLigence-based cytotoxicity assay showing the killing of MUC1-positive **(A)** A549 and **(B)** T47D tumor cells in the presence of virus and T cells at E:T ratio of 1 for up to 120 hours. **(C-E)** Evident of faster tumor cell killing by TILT-322 in the presence of unstimulated human CD3+ cells at E:T ratio of 5 and 1 pfu/cell virus concentration 48 hours post-infection. **(F-G)** Percentage of activated CD69+ and **(H-I)** CD25+ lymphocytes in TILT-322 treated tumors. All experiments were run in quadruplet, and the resulting data are presented as mean ± SEM. Welch's t-test was used to determine statistical significance in the iCELLigence assay and one-way ANOVA with Tukey's post hoc test was used to compare more than two groups in the activation assay. Statistical significance is represented as *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ and ns = non-significant. Statistical significance in xCELLigence assay is represented as *$p = 0.0282$, and **$p = 0.0057$.

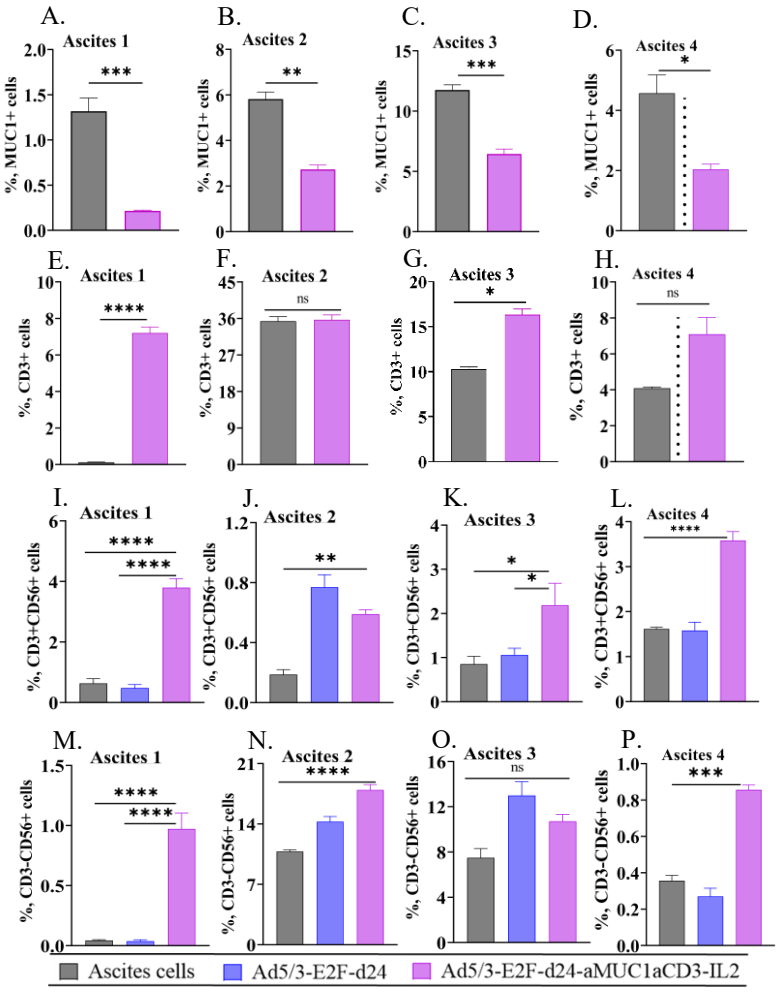



**Figure S3. Detection of MUC1, T cells, and other cells followed by TILT-322 infection in ascites samples. (A-D)** Detection of reduced proportion of MUC1 antigen in ascites cells and **(E-H)** Total percentage of CD3+ T cell detected in ascites samples **(I-L)** Total percentage of CD3+CD56+ cells and **(M-P)** CD3-CD56+ NK cells followed detected in four ascites samples infected with TILT-322 virus. The resulting data are presented as mean ± SEM of quadruplet. One-way ANOVA with Tukey's post hoc test was used to run the statistical significance and the result is represented as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

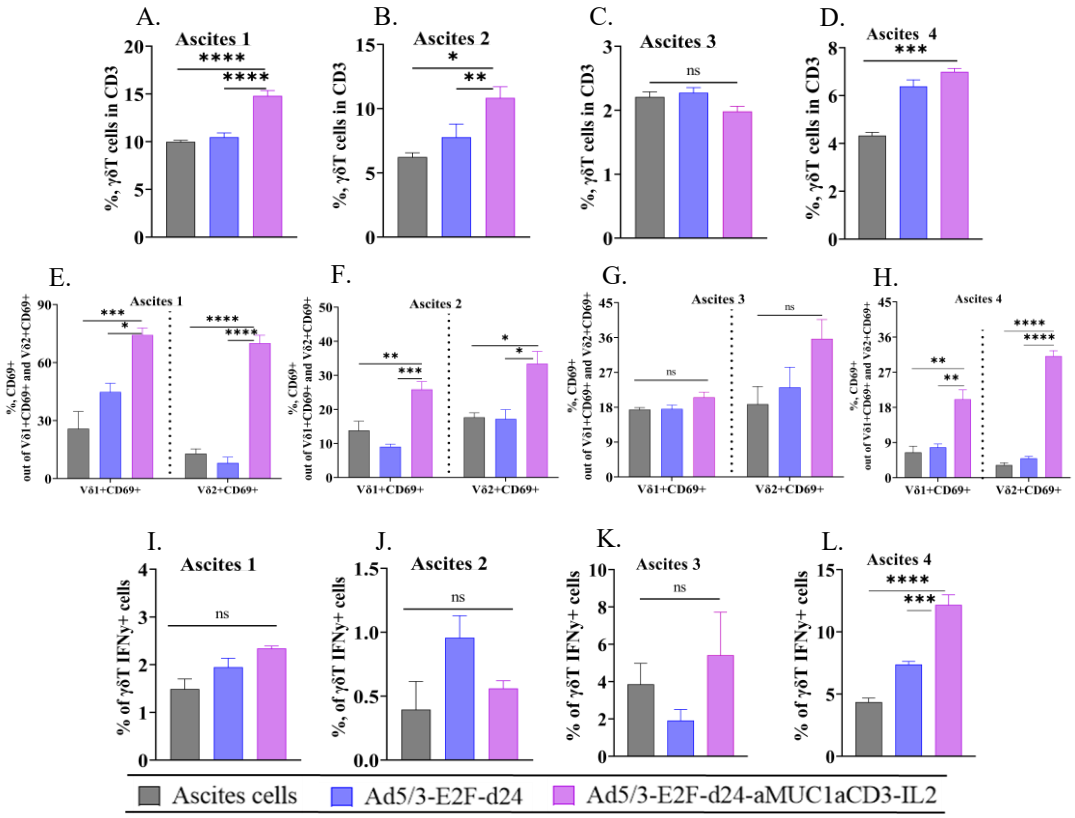



**Figure S4. TILT-322 mediated activation of γδT cell.**

The graph shows the percentage of **(A-D)** γδ T cells and **(E-H)** Percentage of activated CD69+ cells among the Vδ1+ and Vδ2+ subsets. **(I-L)** Interferon-gamma positive γδ T cells in all three tested ascites samples infected by TILT-322. The resulting data are presented as mean ± SEM of quadruplet. One-way ANOVA with Tukey's post hoc test was used to run the statistical significance is represented as ***p < 0.001, ****p < 0.0001 and, ns = non-significant.

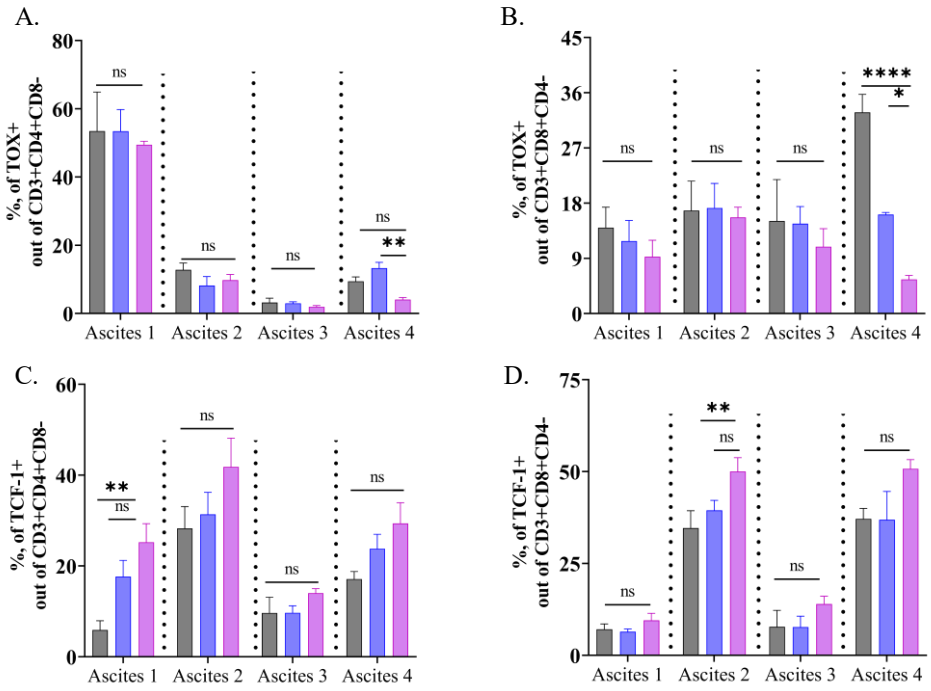



**Figure S5. T cell exhaustion at the transcriptional level followed by the TILT-322 treatment.** The graph shows the percentage of **(A-B)** TOX+ cells and **(C-D)** TCF-1+ cells on CD4+ and CD8+ T cells followed by TILT-322 treatment in four different ascites samples. The resulting data are presented as mean ± SEM of quadruplet. One-way ANOVA with Tukey's post hoc test was used to run the statistical significance is represented as ***$p < 0.001$, ****$p < 0.0001$ and, ns = non-significant.

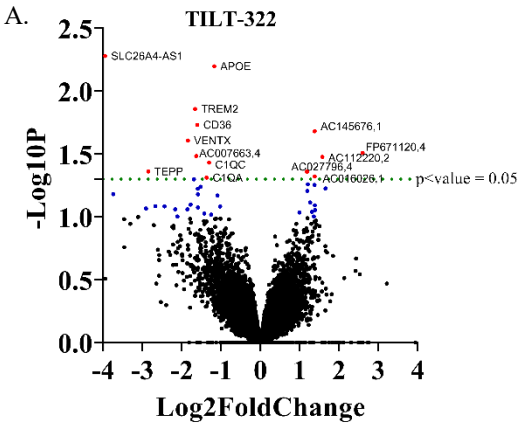
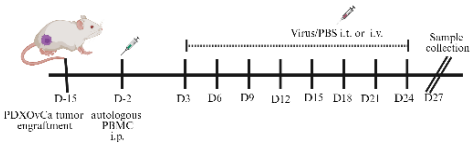



**Figure S6. Differentially expressed genes in TILT-322 infected ascites cells.** TILT-322 infection of ovarian ascites samples led to downregulation of 14 and upregulation of 5 differentially expressed genes in ascites samples.

A.




| Group | Dose | Route |
|---|---|---|
| PBMC | 1 x $10^{7}$ | Intraperitoneal |
| Ad5/3-E2F-d24 | 1 x $10^{9}$ | Intratumoral |
| Ad5/3-E2F-d24 aMUC1aCD3 | 1 x $10^{9}$ | Intratumoral |
| Ad5/3-E2F-d24 aMUC1aCD3-IL2 | 1 x $10^{9}$ | Intratumoral |
| Ad5/3-E2F-d24 aMUC1aCD3-IL2 | 1 x $10^{10}$ | Intravenous |

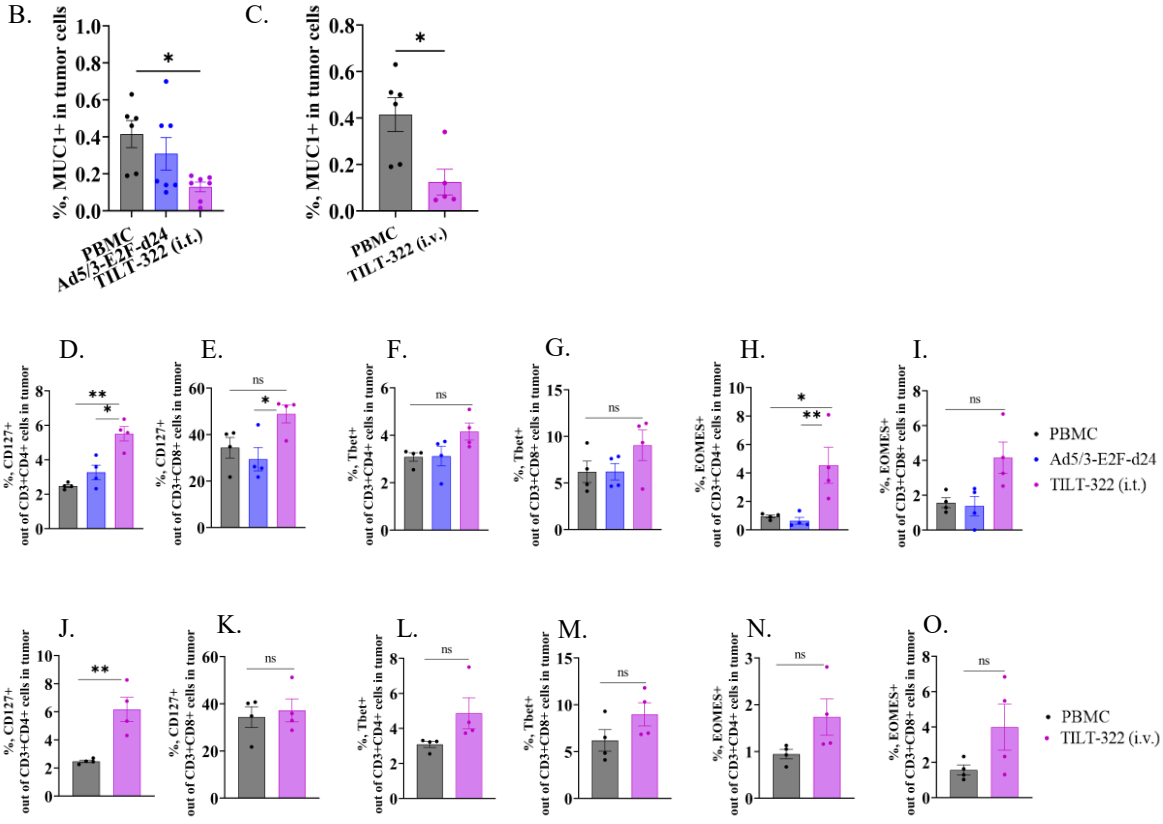


**Figure S7. Schedule for the treatment of TILT-322 *in vivo* experiment and immunological analysis of animal tumor samples. (A)** Schematic representation of TILT-322 animal experiment with dosage information. **(B)** Total percentage of MUC-1 positive cells detected in TILT-322 virus-treated tumors with intratumoral and **(C)** intravenous delivery. Total percentage of **(D-E)** CD127+ cells, **(F-G)** T-bet+ cells, **(H-I)** EOMES+ cells on CD4+ or CD8+ T cells in *in vivo* tumor samples followed by the local delivery of TILT-322. When the TILT-322 was given systemically, the total percentage of (**J-K)** CD127+ cells, **(L-M)** T-bet+ cells, **(N-O)** EOMES+ cells on CD4+ or CD8+ T cells in *in vivo* tumor samples is shown. The resulting data are presented as mean ± SEM of quadruplet. One-way ANOVA with Tukey's post hoc test was used to run the statistical significance is represented as ***$p < 0.001$, ****$p < 0.0001$, and, ns = non-significant.

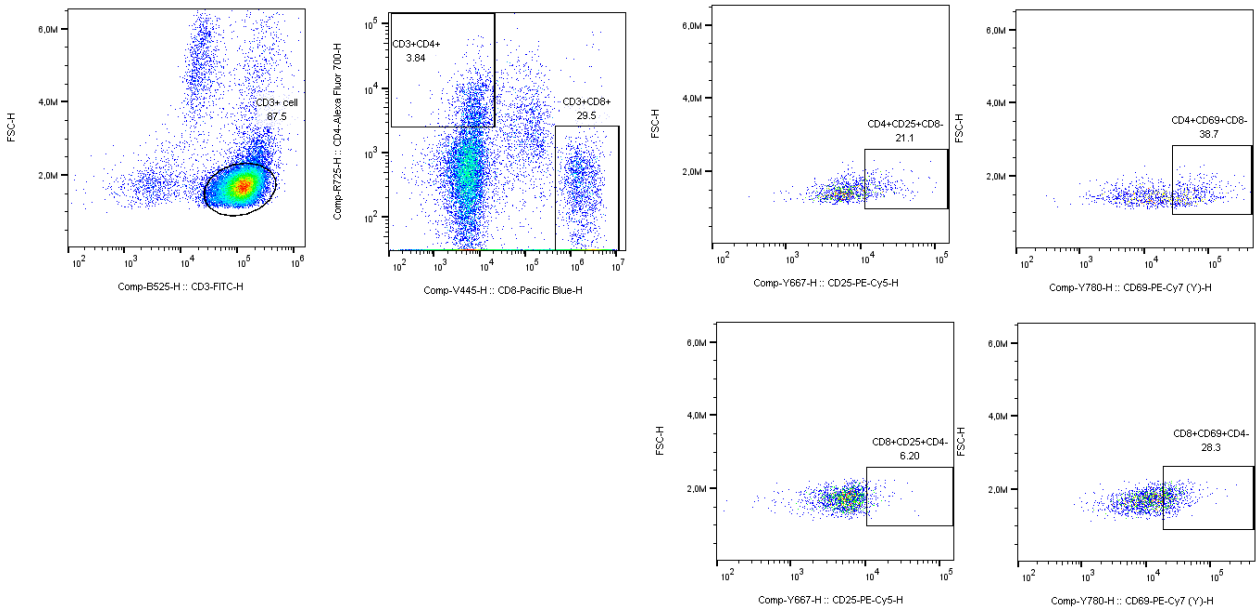



**Figure S8. Flow gating strategy used to access activated CD69 and CD25 T cells *in vitro*.** Gating is done from lymphocytes → CD3 → CD4 → CD69+/CD25+ or CD3 → CD8 → CD69+/CD25+.

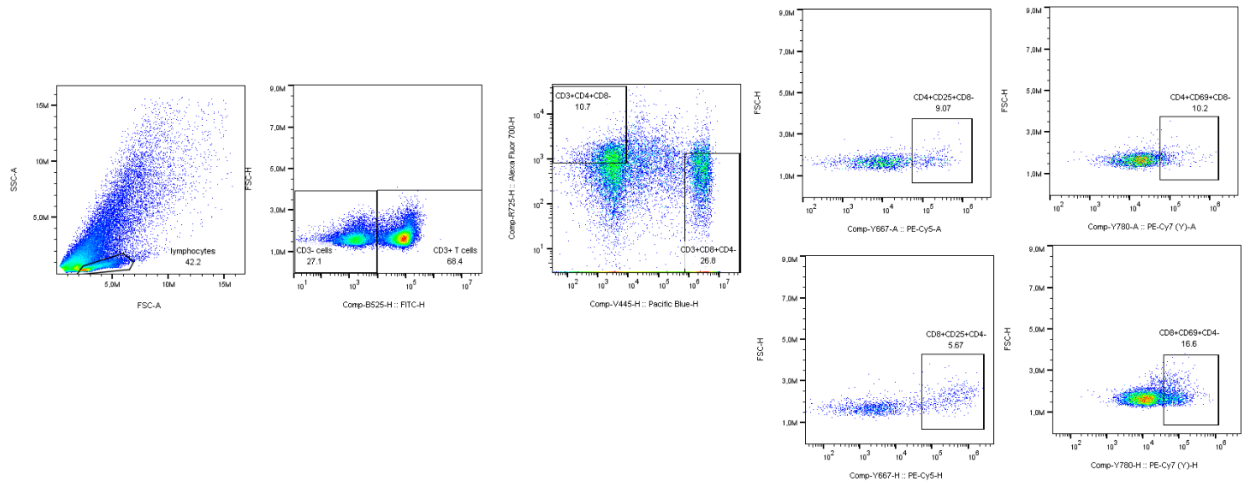



**Figure S9. Flow gating strategy used to access activated CD69 and CD25 T cell *ex vivo*.** Gating is done from lymphocytes→CD3+→CD4+→CD69+/CD25+ or →CD3+→CD8+→CD69+/CD25+.

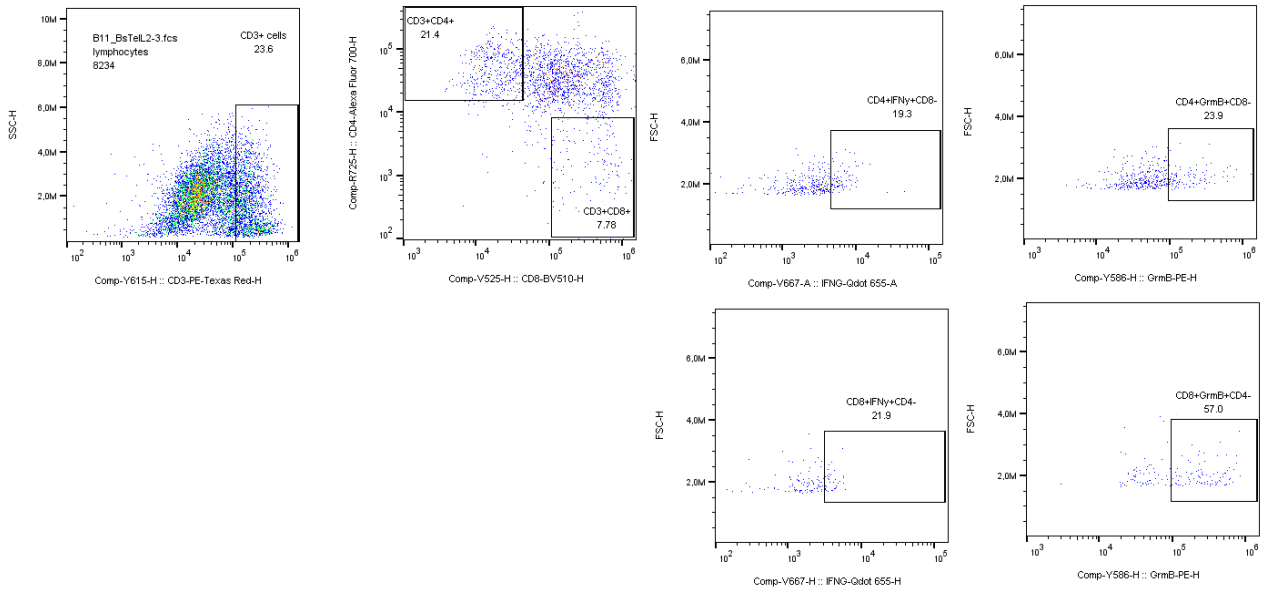



**Figure S10. Flow Gating Strategy used to access granzymeB and interferon-gamma positive cells in TILT-322 infected ascites samples.** Gating is done from lymphocytes→CD3+→CD4+→GranzymeB+/Interferon-y+ or →CD3+→CD8+→ GranzymeB+/Interferon-y+.

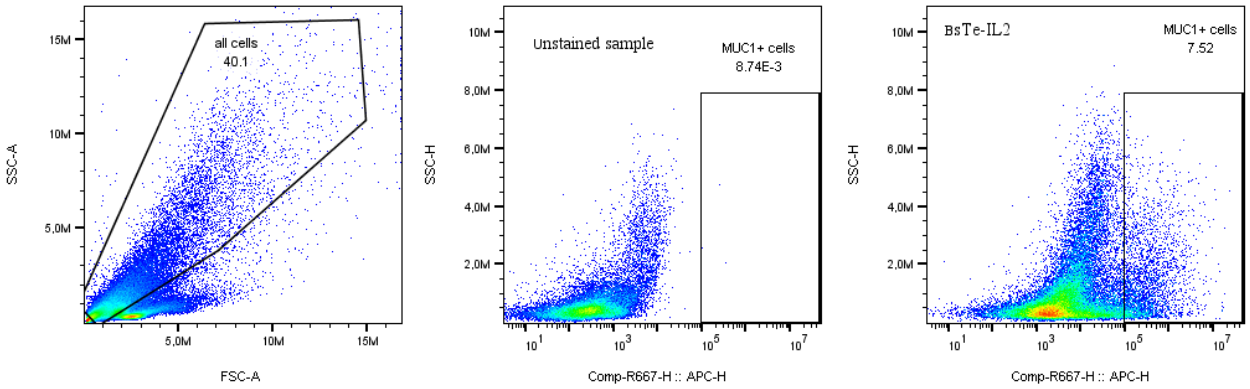



**Figure S11. Flow Gating Strategy used to access MUC1+ cells out of all ascites cells.** Gating is done from all cells → MUC1. MUC1 Fluorescence Minus One Control and unstained sample control were used to set the gating thresholds.

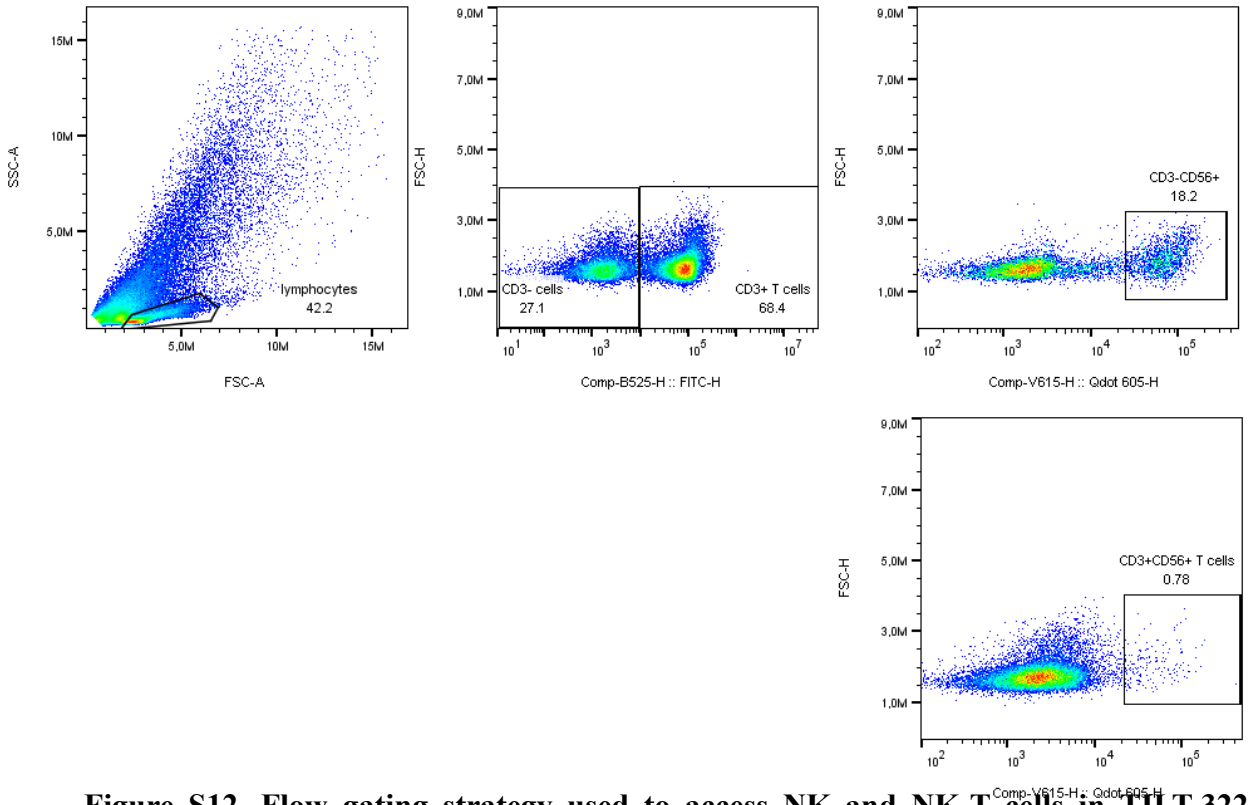



**Figure S12. Flow gating strategy used to access NK and NK-T cells in TILT-322 infected ascites samples.** Gating is done from lymphocytes → CD3+ cells → CD3+CD56+ cells or lymphocytes→ CD3- cells → CD3-CD56+ cells. All required Fluorescence Minus One Control and unstained sample control was used to set the gating thresholds.

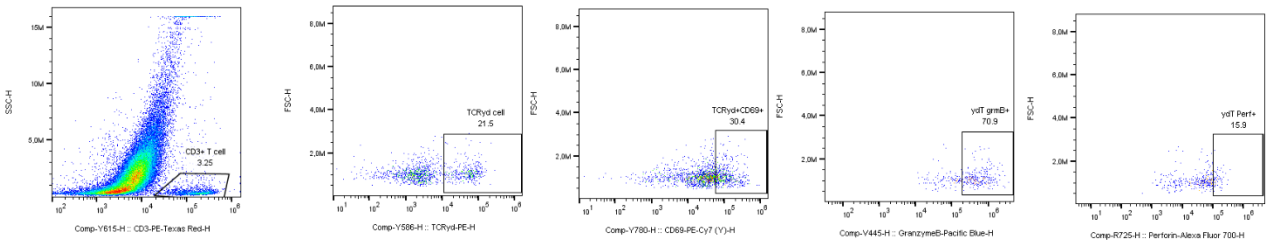


**Figure S13. Flow gating strategy used to access TCR gamma delta T cell activation in TILT-322 treated ascites samples *ex vivo*.** Gating is done from lymphocytes → CD3+ cells → TCRyd+ cells → CD69+ cells/ granzymeB+/ Perforin+ cells. All required Fluorescence Minus One Control and unstained sample control was used to set the gating thresholds.

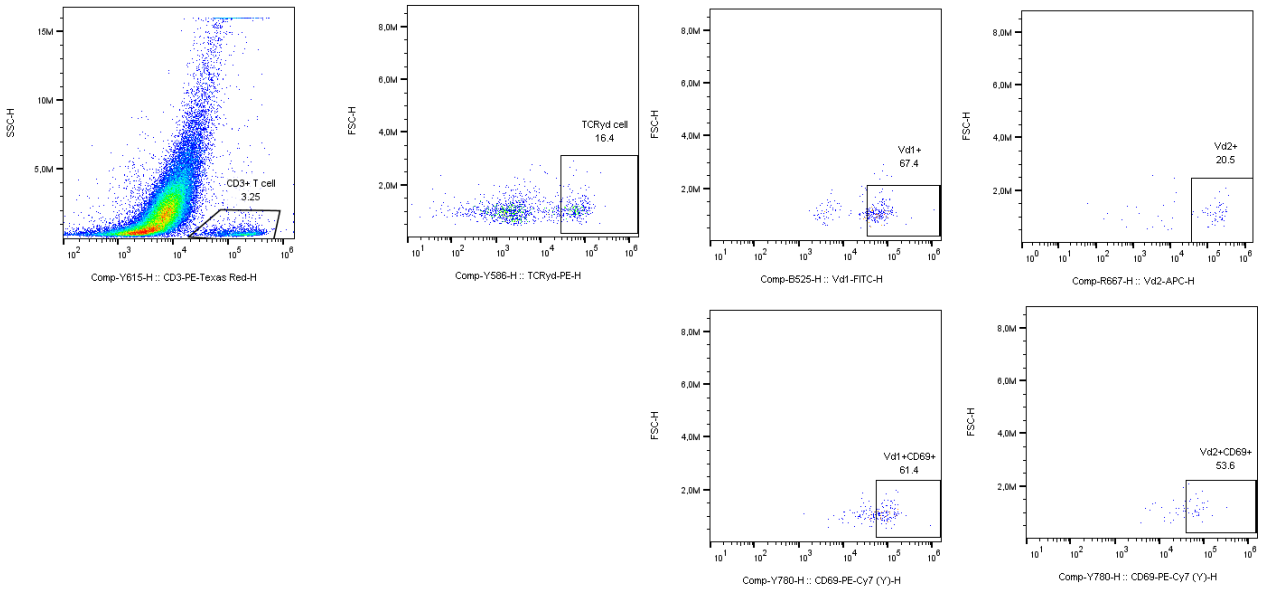



**Figure S14. Flow gating strategy used to access Vd1/Vd2 gamma delta T cell subset activation.** Gating is done from lymphocytes → CD3+ cells → TCRyd+ cells → Vd1+cells → CD69+ or lymphocytes → CD3+ cells → TCRyd+ cells → Vd2+cells → CD69+ cells. All required Fluorescence Minus One Control and unstained sample control was used to set the gating thresholds.

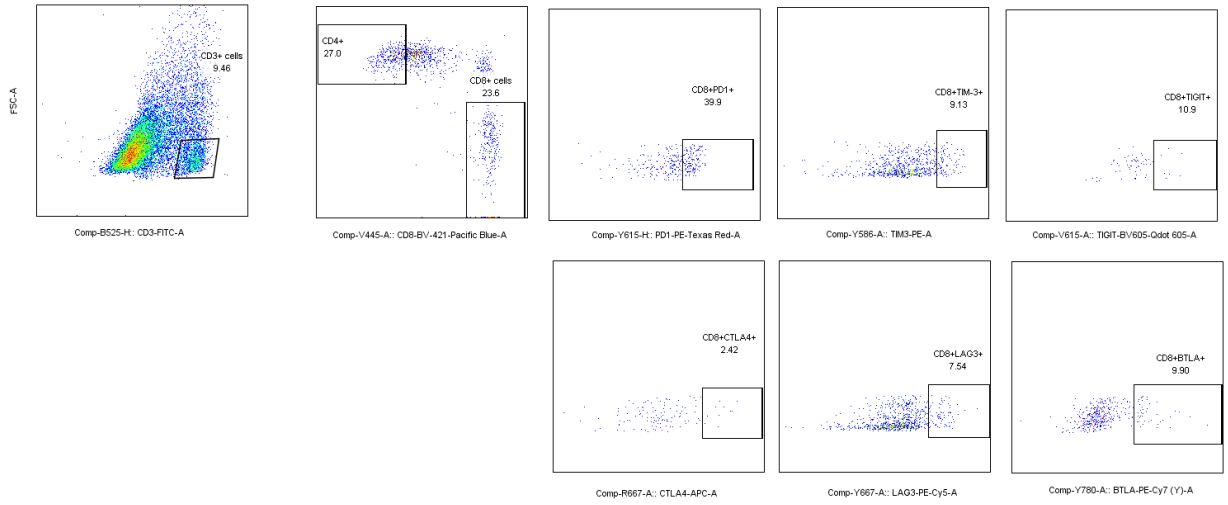



**Figure S15. Flow gating strategy used to examine T cell exhaustion in ovarian ascites followed by the TILT-322 treatment.** Gating is done from CD3+ cells → CD4+ → BTLA/CTLA-4/LAG-3/PD-1/TIGIT/TIM-3. A similar gating is done to access the expression on CD8+ T cells (CD3+ cells → CD8+ → BTLA/CTLA-4/LAG-3/PD-1/TIGIT/TIM-3). All required Fluorescence Minus One Control and unstained sample control was used to set the gating thresholds.