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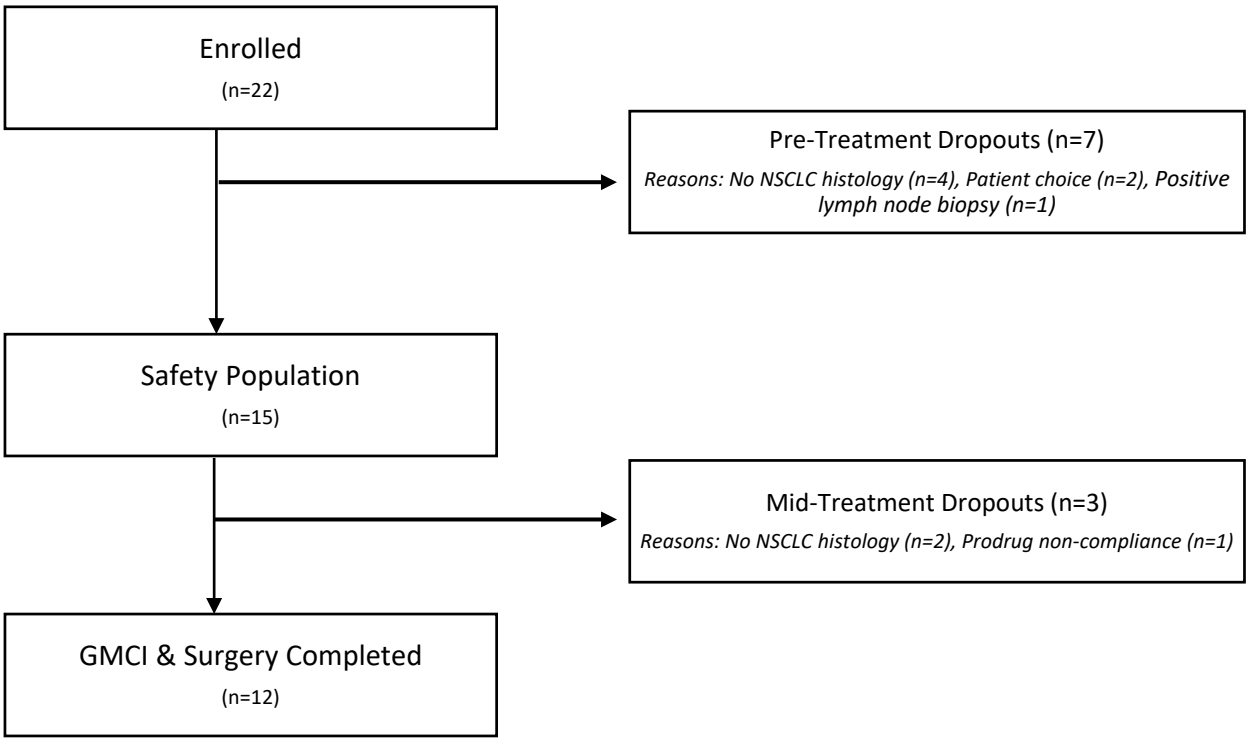
Supplemental Information

Neoadjuvant Gene-Mediated Cytotoxic Immunotherapy for Non-Small-Cell Lung Cancer: Safety and Immunologic Activity

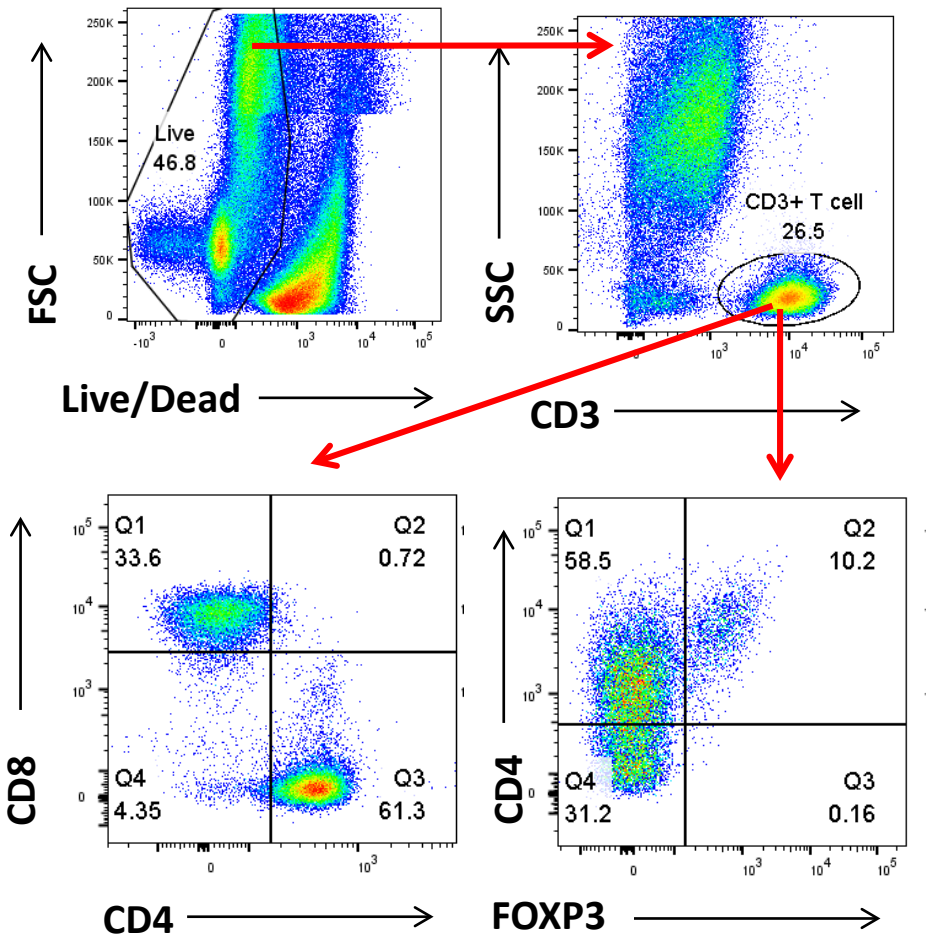
Jarrod D. Predina, Andrew R. Haas, Marina Martinez, Shaun O'Brien, Edmund K. Moon, Patrick Woodruff, Jason Stadanlick, Christopher Corbett, Lydia Frenzel-Sulyok, Mitchell G. Bryski, Evgeniy Eruslanov, Charuhas Deshpande, Corey Langer, Laura K. Aguilar, Brian W. Guzik, Andrea G. Manzanera, Estuardo Aguilar-Cordova, Sunil Singhal, and Steven M. Albelda

Figure S1. CONSORT Diagram

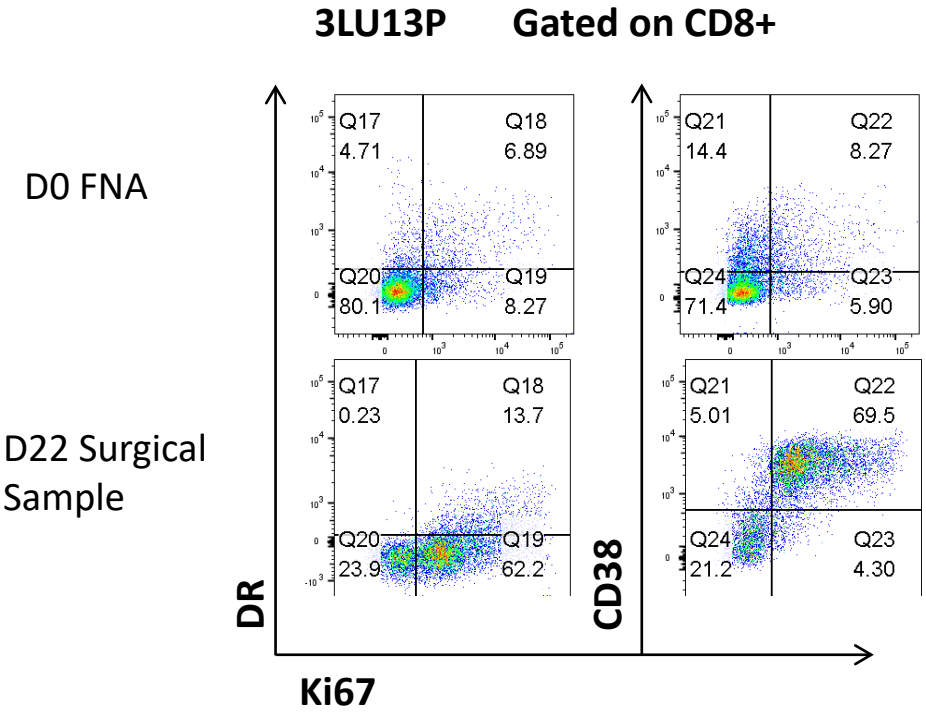


Supplementary Figure 2
Example of Cell Markers on Tumor Cells

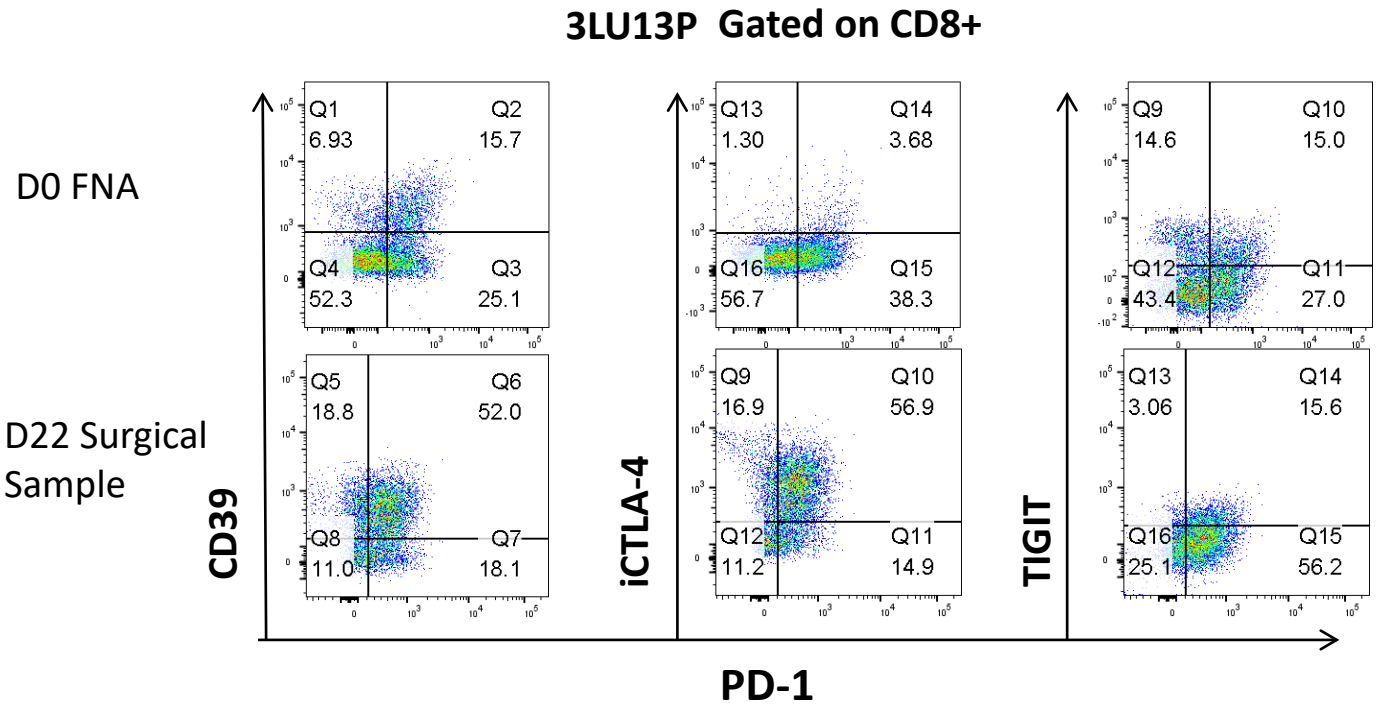
3LU13P



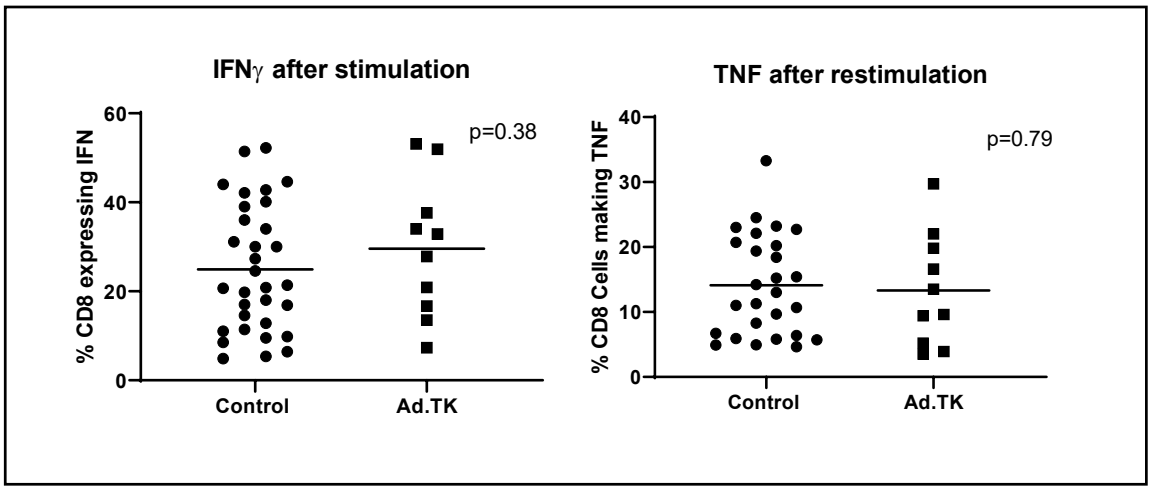
Supplementary Figure 3.
Example of Activation Receptors on CD8 TILs



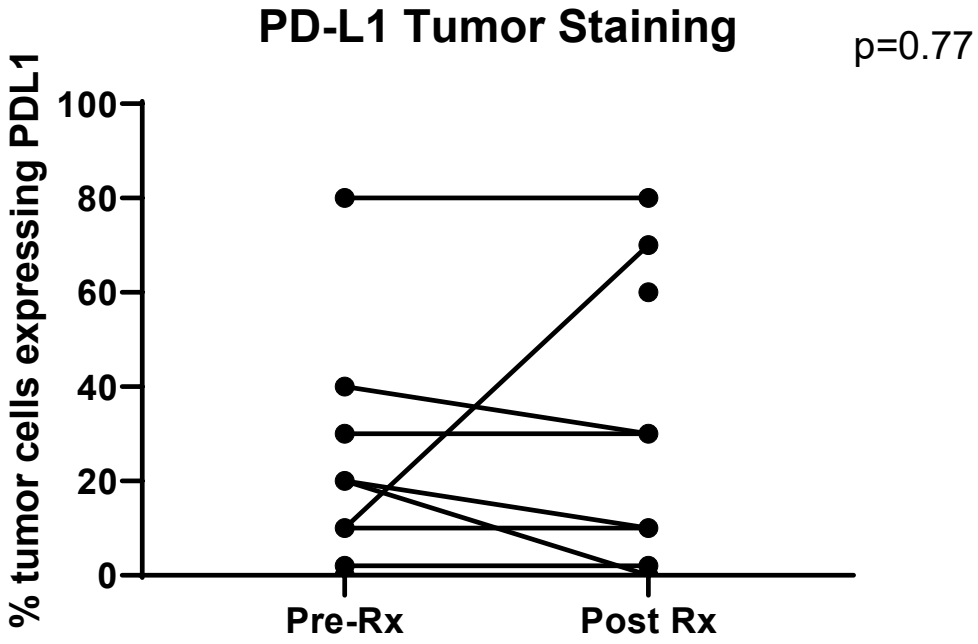
Supplementary Figure 4.
Example of Inhibitory Receptors on TILs



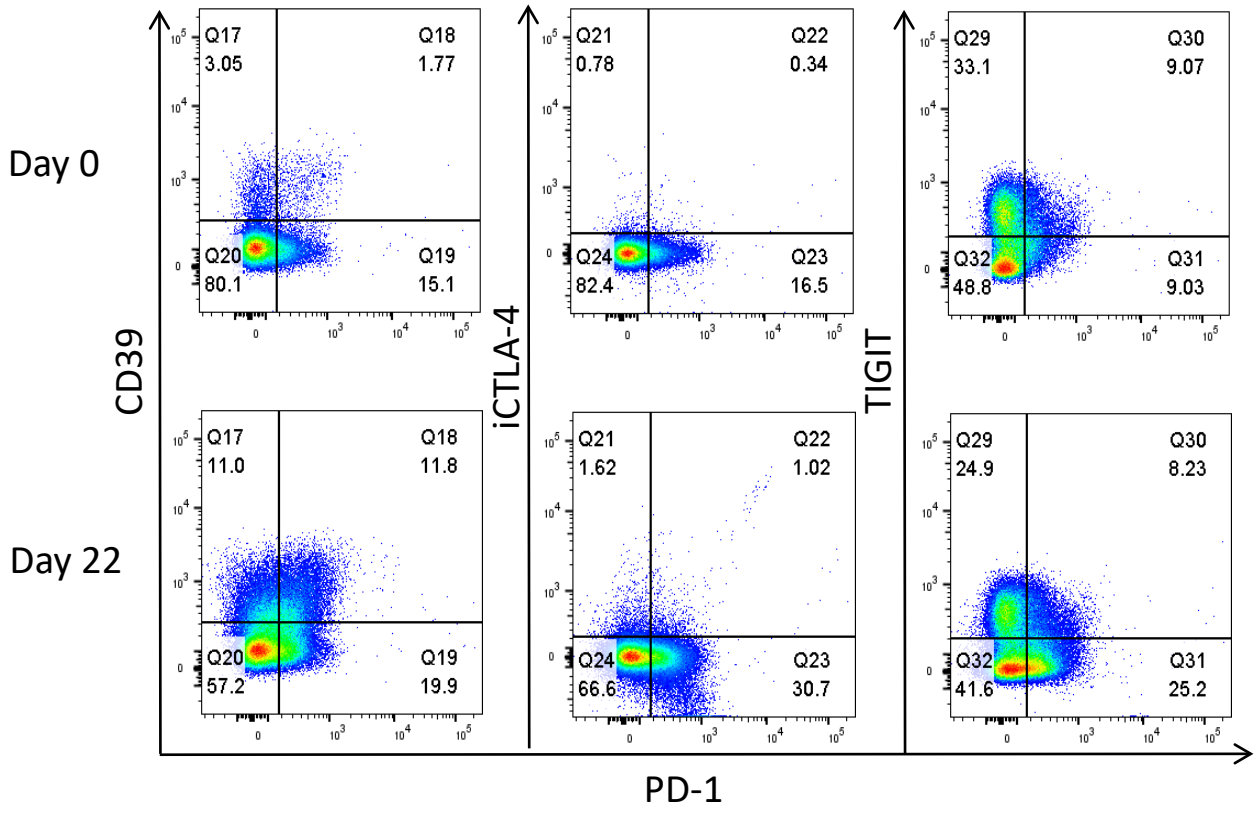
Supplemental Figure 5. TIL cytokine production after stimulation



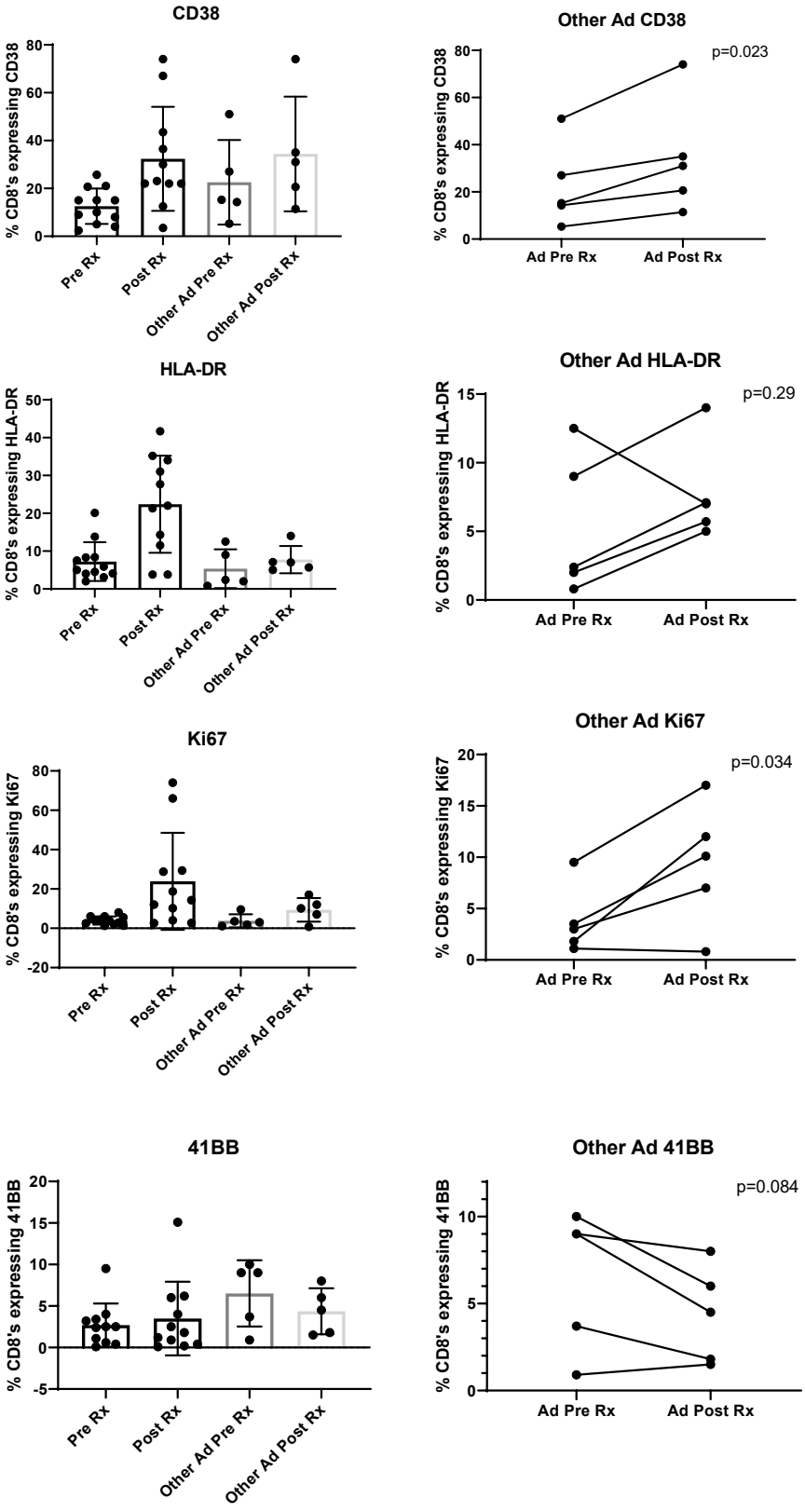
Supplemental Figure 6: Expression Level of PDL1 on tumors as assessed by IHC



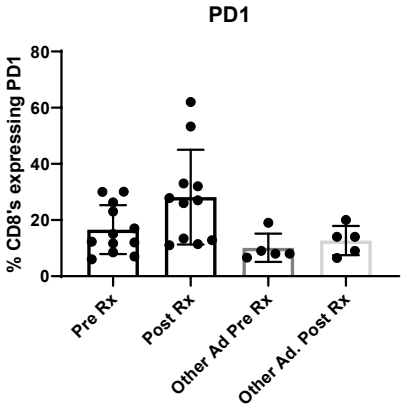
Supplemental Figure 7. Inhibitory Receptor Expression on CD8+ cells from Patient 3LU13P PBMC

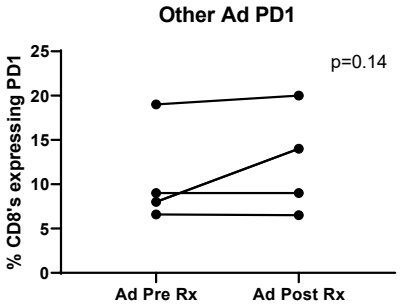


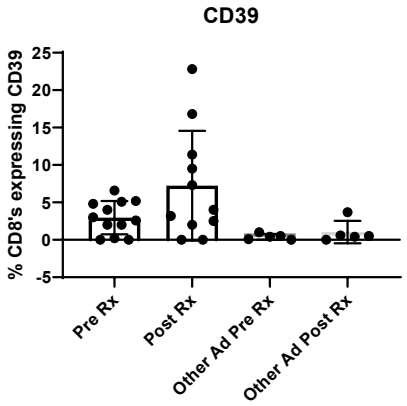
Supplemental Figure 8. Comparison of PBMC Activation Markers with other Ad Trials

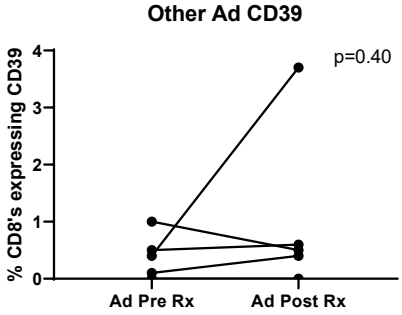


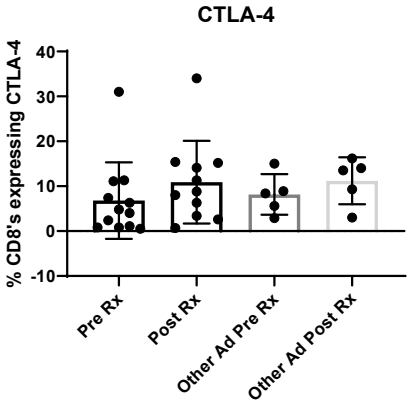
Supplemental Figure 9. Comparison of PBMC Inhibitory Receptors with other Ad Trials

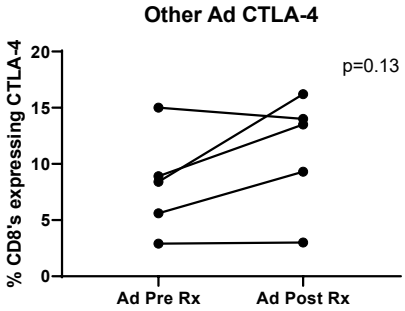


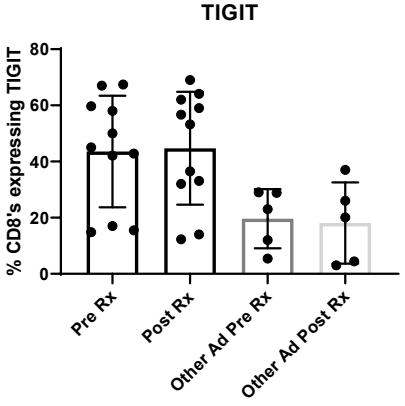


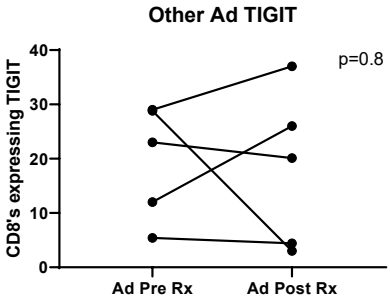




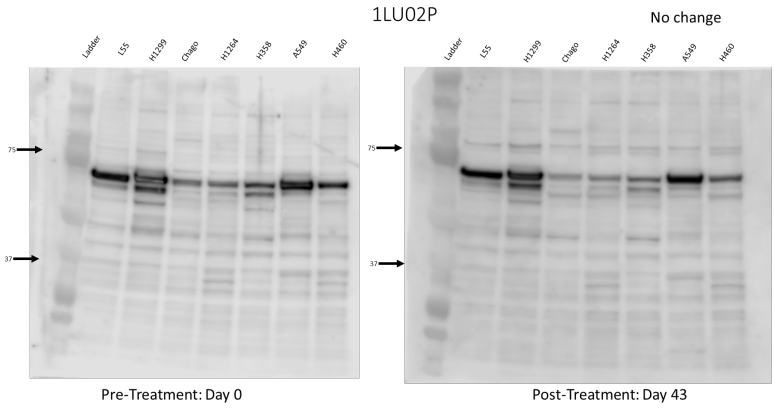


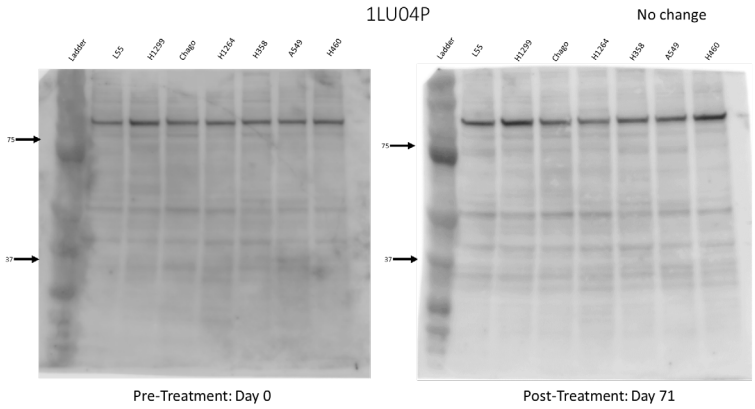


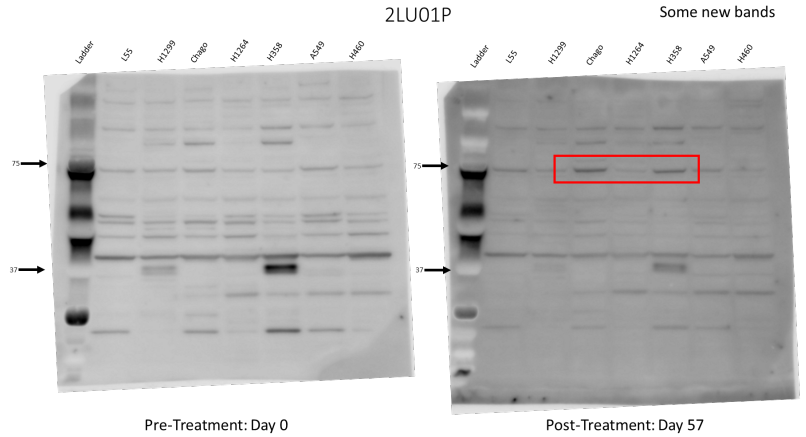


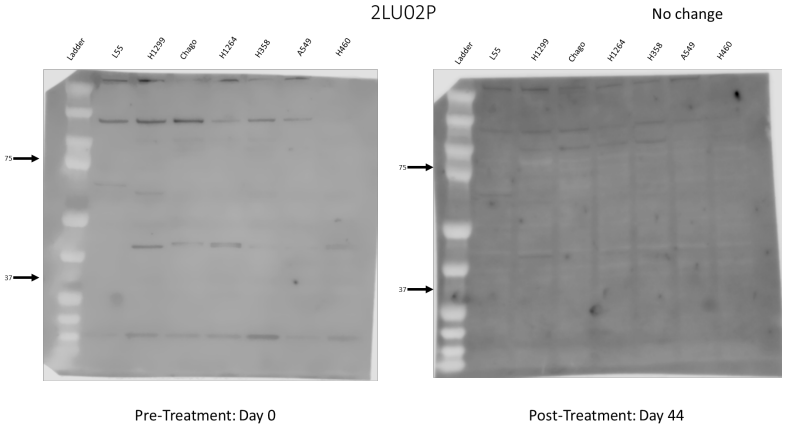


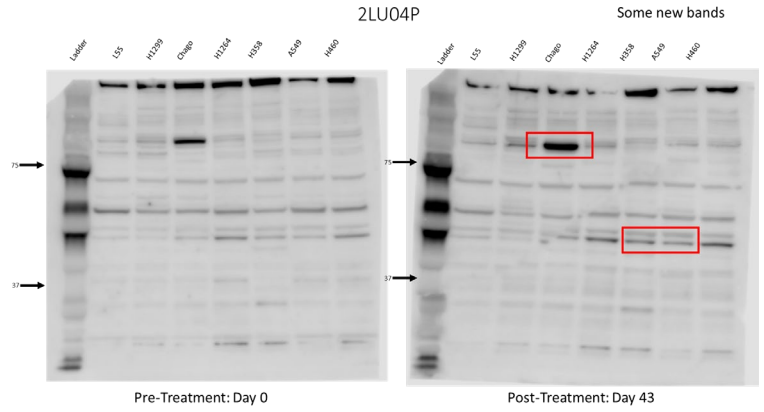
Supplemental Figure 10. Immunoblots before and after Ad.TK

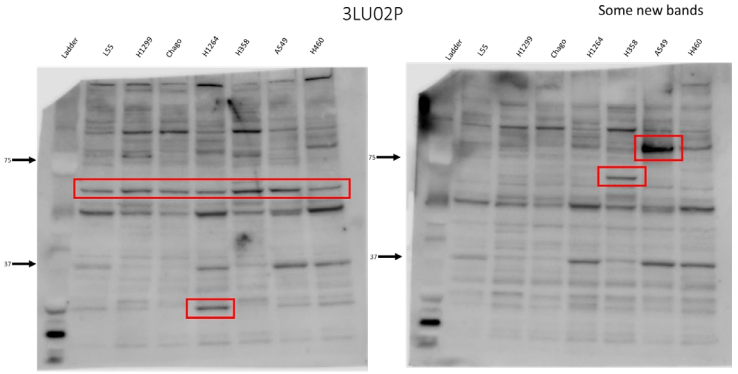


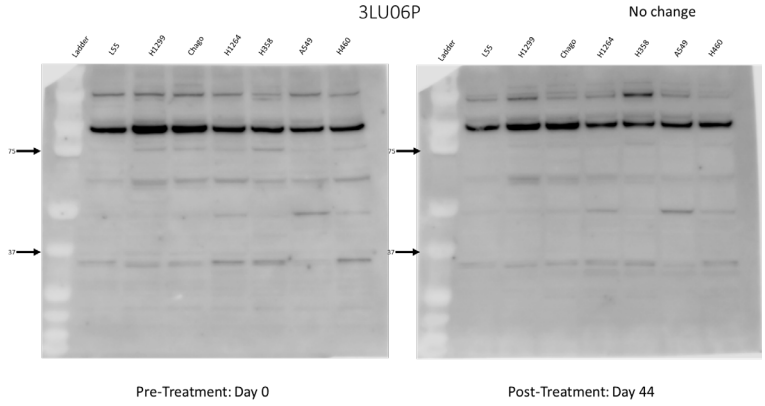


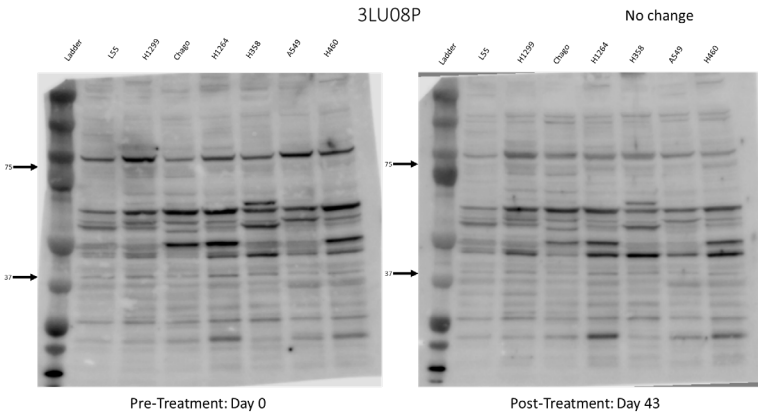












Supplemental Figure Legends

Supplemental Figure 1. Consort Diagram of the Phase 1 Trial
Twenty-two subjects met inclusion criteria and were enrolled in the Phase I trial. Seven patients were excluded before vector instillation (see- Pre-treatment Dropout box for reasons) and did not receive AdV-tk. Three patients were excluded after receiving AdV-tk (see Mid-treatment Dropout box for reasons). Twelve patients completed the trial. Three subjects received $2.5x10^{11}$ vp of AdV-tk (Cohort 1), 3 subjects received $5.0x10^{11}$ vp (Cohort 2), and 6 received $1.0x10^{12}$ vp (Cohort 3).

Supplemental Figure 2. Flow Cytometry Tracings Showing the Gating Strategy for Lymphocyte Analysis in tumors and blood. Tumor digest from Patient 3LU13P was examined for forward scatter (FSC) versus a live/dead stain (upper left tracing). Live cells were gated and examined for side scatter (SSC) versus expression of CD3 to identify T cells (circle in upper right tracing). CD3+ cells were gated and stained for CD8 and CD4 cells (lower left tracing). CD3 cells were gated and stained for CD4 and FOXP3 to identify T-regulatoy cells (upper right quandrant).

Supplemental Figure 3. Flow Cytometry Tracings showing Activation Markers on CD8+ TILs from Patient 3LU13P. Examples of flow tracings from the CD8+ T cells of pre-treatment (Day 0, upper 3 tracings) and post-treatment (D21, lower 3 tracings) from one patient (3LU13P) are shown. Expression of HLA-DR (DR), Ki67, and CD38 are shown as marked.

Supplemental Figure 4. Flow Cytometry Tracings showing Inhibitory Markers on CD8+ TILs from Patient 3LU13P. Examples of flow tracings from the CD8+ T cells of pre-treatment (Day 0, upper 3 tracings) and post-treatment (D21, lower 3 tracings) from one patient (3LU13P) are shown. Expression of PD1, CD39, CTLA4, and TIGIT are shown as marked.

Supplemental Figure 5. TIL activation

T cells isolated from each surgical sample were stimulated overnight with brefeldin/monensin and plate-bound anti-CD3 antibody and subjected to flow cytometry to detect intracellular cytokine production. The percent of CD8+ T cells expressing cytokine is plotted. No differences in the ability of the CD8+ TILs to produce intracellular IFN-γ or TNF-α were noted in the samples from this trial (AdV-tk) when compared to the CD8+ T cells from the early stage lung cancer patients (student t-test) (see Figure 2 population called Control).

Supplemental Figure 6: Expression Level of PDL1 on tumors as assessed by IHC. To determine if GCMI would upregulate the expression of PD-L1, a pre-treatment biopsy (when enough cells were present) and the surgical specimen after the treatment were stained for PD-L1 and the extent of tumor cells expressing membranous staining for PD-L1 was assessed by a pathologist (C.D.). We were able to assess staining in 8 pairs of samples. Paired t-tests were applied to define the statistical significance (p value) of the change.

Supplemental Figure 7. Flow Cytometry Tracings showing Inhibitory Markers on CD8+ Cells in PBMC from Patient 3LU13P. Examples of flow tracings from the CD8+ T cells of pre-treatment (Day 0, upper 3 tracings) and post-treatment (D21, lower 3 tracings) from the PBMCs from one patient (3LU13P) are shown. Expression of PD1, CD39, CTLA4, and TIGIT are shown as marked.

Supplemental Figure 8. Comparison of PBMC Activation Markers with other Adenoviral Vector Trials

CD8+ T cell activation changes in this trial were compared to 5 samples from two previous clinical trials in which the same AdV-tk vector followed by valacyclovir was injected intrapleurally in patients with malignant pleural effusions (3 patients) and a trial in which a similar replication-deficient type 5 Adenovirus encoding interferon alpha (IFN-α) was injected intrapleurally into patients with malignant mesothelioma (2 patients). Thawed PBMCs from the 5 patients before the delivery of the vector and then again 14 days later were analyzed using the same flow cytometry protocol as used for the this AdV-tk trial. The percentage of CD8+ T cells expressing each activation marker is plotted. In the right hand panels, "Pre Rx" = values from current neoadjuvant AdV-TK trial before vector instillation; "Post Rx" = values obtained at the time of surgery in the current trial; "Other Ad Pre Rx" = values from previous AdV-TK trials before vector instillation; "Other Ad Post Rx" = values from previous AdV-TK trials 2 weeks after vector instillation. In the right hand panels, the pre- and post-Rx values from the previous trials are compared. Paired t-tests were applied to define the statistical significance (p value) of the change. The following activation markers were assessed: CD38, HLA-DR, Ki67, and 41BB (CD137).

Supplemental Figure 9. Comparison of PBMC Inhibitory Receptors with other Ad Trials

Left hand panels: The percent of PBMC-derived CD8 T cells expressing PD1, CD39, CTLA-4, and TIGIT from pre-treatment and post-treatment (day of surgery) data from the current trial samples (first two bars) are compared with the pre- and post-vector PBMC CD8+ T cell data from two previous clinical trials in which we injected intrapleurally the same AdV-tk vector followed by valacyclovir (3 patients) into patients with malignant pleural effusions and a trial in which a similar replication-deficient type 5 Adenovirus encoding interferon alpha (IFN-α) was injected intrapleurally into patients with malignant mesothelioma (2 patients) (3rd and 4th bars).

Right hand panels: The changes in the percent of PBMC-derived CD8+ T cells expressing PD1, CD39, CTLA-4, and TIGIT from the from two previous clinical trials for each patient are plotted. None of these changes were significant by paired t-tests.

Supplemental Figure 10. Immunoblots before and after AdV-tk. We used pre-AdV-tk injection serum and 4-6 weeks post-resection serum to perform immunoblots on gels containing 7 different human lung cancer cells lines. Samples were available from 8 subjects. New or increased bands are shown in red boxes (in 3 of the 8 subjects).

Supplemental Information

Suppl. Table 1. Single cell surface and intracellular markers used in the study

Antibody	Clone	Source
CD103	Ber-Act8	Biolegend
CD137 (41BB)	4B4-1	Biolegend
CD3	UCHT1, SK7	Biolegend
CD38	HIT2	Biolegend
CD39	A1	Biolegend
CD4	OKT4	Biolegend
CD8	RPA-T8	BD Biosciences
CTLA-4	BNI3	BD Biosciences
FoxP3	206D	Biolegend
HLA-DR	G46-6	BD Biosciences
IFN-γ	4S.B3	Biolegend
Ki67	20Raj1	eBioscience
PD-1	EHI2-H7	Biolegend
PD-L1	M1H1	Biolegend
TIGIT	MBAS43	eBioscience
TIM-3	F38-232	Biolegend
TNF-α	Mab11	Biolegend

Supplemental Table 2. Unrelated Adverse Events

Time period 1 corresponds to day 0-1 relative to AdV-tk administration, Time period 2 is day 2 to surgery (approximately day 21). Time period 3 corresponds to the day of surgery to 4 weeks post-surgery. CTC= Common Terminology Criteria for Adverse Events.

Adverse Event	Time period 1			Time period 2			Time period 3		
	CTC 1	CTC 2	CTC 3	CTC 1	CTC 2	CTC 3	CTC 1	CTC 2	CTC 3
Blood and lymphatic system									
Splenomegaly							1		
Cardiac disorders									
Atrial fibrillation								1	
Heart failure									1
Sinus bradycardia								1	
Sinus tachycardia		1							
Ear and labyrinth disorders									
Hearing impaired						1			
Tinnitus							1		
Gastrointestinal disorders									
Abdominal distension	1								
Abdominal pain							1		
Constipation				1			2	1	
Diarrhea								1	
Dyspepsia								1	
Dysphagia								1	
Hepatic steatosis / Fatty liver	1								
Indigestion								1	
Nausea							1	1	
General disorders									
Diaphoresis							1		
Edema limbs							1		
Fatigue	1			1	1		3		
Fever	1						1		
Malaise					1		1		
Non-cardiac chest pain				1					
Pain	1						5	2	1
Infections and infestations									
Skin infection									1
Wound infection								1	
Lung infection (pneumonia)					1				
Injury and procedural									
Air leak							2		
Fracture								1	
Wound complication								1	

Adverse Event	Time period 1			Time period 2			Time period 3		
	CTC 1	CTC 2	CTC 3	CTC 1	CTC 2	CTC 3	CTC 1	CTC 2	CTC 3
Metabolism and nutrition									
Anorexia				1			2	1	
Diabetes Mellitus Exacerbation									1
Hyperglycemia									1
Severe protein-calorie malnutrition									1
Musculoskeletal									
Back pain					1				
Crepitus							1		
Muscle spasms							1		
Nervous system disorders									
Dysgeusia				1					
Headache			1						1
Paresthesia				1					
Tremor								1	
Psychiatric disorders									
Agitation							1		
Anxiety				1			2		
Insomnia				1					
Renal and urinary disorders									
Urinary retention		1						2	
Respiratory, thoracic, mediastinal disorders									
Bronchopleural fistula									1
Cough				4			1		
Crackles	1								
Dyspnea				3			2	1	
Hemoptysis				1					
Hypoxia								2	
Nasal congestion				1					
Pneumothorax							2		
Subcutaneous emphysema							2		
Wheezing				1					
Surgical and medical procedures									
Surgical pain							1		
Vascular disorders									
Hypotension		1					1	1	

Supplemental Table 3. Laboratory Abnormalities

Lab Abnormality	Post-Injection		Post-Surgery	
	CTC 1	CTC 3	CTC 1	CTC 2
Elevated AST/ALT	1			
Elevated Bilirubin	1			
Elevated Creatinine	1		1	
Elevated Potassium	1			
Elevated Sodium	1			
Low Albumin	2		1	
Low Calcium	1		9	
Low Hemoglobin	2		7	2
Low Leukocytes (WBC)			1	
Low Lymphocytes	2	2	2	1
Low Platelets	3		1	
Low Potassium			1	
Low Sodium	2		3	

Supplemental Table 4. All SAEs reported.

Time period 3 corresponds to the day of surgery to 4 weeks post-surgery.

PCN	Category	AE Name	Time Period	Time since injection of SAE (weeks)	CTC Grade	Relation
1LU04P	Infections and infestations	Skin infection	3	3.1	3	Unrelated
2LU01P	Cardiac disorders	Heart failure	3	5.3	3	Unrelated
3LU01P	Respiratory, thoracic and mediastinal disorders	Bronchopleural fistula	3	6.3	3	Unrelated
3LU05P	Metabolism and nutrition disorders	Diabetes Mellitus Exacerbation	3	3.4	3	Unrelated

Supplemental Table 5. Patient data related to Recurrence

Patient ID	Histology	Final TNM	Surgical Staging	Adjuvant Treatment	Recurrence	Time to recurrence (mos)	Date of surgery	Last Time of Contact	Median time of follow-up (mos)
1LU02	SCC	T3N2	IIIB	Yes	Yes	26	6/19/2017	1/9/2020	32
1LU04	SSC	T2aN0	IB	Yes	NED		11/14/2017	10/2/2019	23
1LU05	SSC	T2bN1	IIB	Yes	NED		12/12/2017	1/9/2020	25
2LU01	SCC	T4N0	IIIA	Yes	NED		3/8/2018	5/19/2020	14
2LU02	Adeno	T2aN0	IB	Unknown	Yes	24	5/4/2018	6/17/2020	26
2LU04	SSC	T2aN0	IB	No	NED		6/5/2018	5/20/2020	24
3LU01	SCC	T3N2	IIIB	Yes	Yes	6	8/3/2018	12/30/2019	17.7
3LU02	Sarco	T4N0	IIIA	Yes	NED		9/21/2018	2/29/2020	17
3LU06	Adeno	T3N2, T1N2	IIIB	Yes	NED		2/5/2019	5/21/2020	15
3LU08	SCC	T3N0	IIB	No	NED		4/5/2019	1/29/2020	10
3LU12	Adeno	T1N0	IA	Unknown	NED		10/29/2019	5/20/2020	7.5
3LU13	Adeno	T3N0	IIB	Yes	NED		10/29/2019	5/13/2020	7.5