frontiers

Supplementary Material

Glucose-restricted diet regulates the tumor immune microenvironment and prevents tumor growth in lung adenocarcinoma

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1. Supplementary Tables

Supplementary Table S1: Clinical data of the cohort of patients with NSCLC analyzed in this study

Patient Code	Histological classification	Grading	Age	Gender	Tumor Diameter (cm)	pT	pN	M	TNM-Stadium	BMI = Weight/ Hight²	IGF1R Expression TU	glucose (mg7dl)
3-MP	ADC	G3	79	Male	5	2b	0	0	IIA	27,44	-	-
9-MP	ADC	G2	84	Female	2,7	1c	2	0	IIIA	30,04	-	83
10-MP	ADC	G2	72	Male	1,4	1b	0	0	IA2	22,20	-	-
15-MP	ADC	G3	63	Male	2,5	1c	0	0	IA3	31,38	-	125
16-MP	ADC	G3	70	Female	1,8 and 2,8	3	0	0	IIB	27,68	-	107
17-MP	ADC	G2	74	Male	2,6	1c	0	0	IA3	30,42	-	110
19-MP	ADC	-	55	Female	7	3	0	0	IIB	23,67	-	
20-MP	ADC	G3	60	Male	2,7	1c	0	0	IA3	21,04	-	110
22-MP	ADC	G3	68	Male	7	3	1	0	IIIA	27,32	-	117
23-MP	ADC	G2	73	Male	4,5	2b	0	0	IIA	23,55	-	101
26-MP	ADC	G3	52	Female	1,3	1b	0	1b	IVA	28,52	-	104
27-MP	ADC	G3	70	Female	1,4	1b	0	0	IA2	26,40	-	93
28-MP	ADC	G3	76	Male	1,2	1b	0	0	IA2	28,40	-	100
32-MP	ADC	G3	60	Female	4,4	2b	2	0	IIIA	26,44	-	
34-MP	ADC	G2	51	Female	1,8	1b	0	0	IA2	20,20	-	84
35-MP	ADC	G3	72	Female	3	1c	0	0	IA3	35,56	-	102
39-MP	ADC	G3	65	Male	6	3	0	0	IIB	33,66	-	134
40-MP	ADC	G3	82	Male	1,8	1b	1	0	IIB	17,65	-	86
43-MP	ADC	G3	72	Female	4	2a	2	0	IIIA	30,85	-	96
44-MP	ADC	G1	53	Male	1,5	1b	0	0	IA2	34,32	-	108
45-MP	ADC	G1	78	Male	2,3	1c	0	0	IA3	35,43	-	94
51-MP	ADC	G3	61	Male	2,3 and 2,5	3	2	0	IVA	24,07	-	96
53-MP	ADC	G2	62	Male	1,4	1b	0	0	IA2	30,98	-	101
55-MP	ADC	G3	64	Female	1,8	1b	2	0	IIIA	29,30	-	100
56-MP	ADC	G2	68	Female	4	2a	0	0	IB	40,40	-	92
57-MP	ADC	G2	36	Female	3,8	2a	0	0	IB	49,25	-	112
59-MP	ADC	G2	71	Male	0,9 and 2,1	4	0	0	IIIA	21,47	-	100
62-MP	ADC	G2	80	Female	3,5	2a	0	0	IB	45,91	-	161

64-MP	ADC	G2	55	Male	3,5	2a	1	1a	IVA	24,93	-	89
68-MP	ADC	G3	42	Male	8,5	4	0	0	IIIA	26,47	4,026	94
69-MP	ADC	G2	76	Female	3,1	2a	0	0	IB	25,24	-	81
70-MP	ADC	G2	70	Female	2,7	1c	0	0	IA3	22,10	-	-
71-MP	ADC	G3	67	Male	3,7	2a	0	0	IB	30,42	-	109
72-MP	ADC	-	53	Male	3,5				IIIC	24,98	-	104
73-MP	ADC	G2	67	Female	4,8	2b	0	0	IIA	27,36	-	93
74-MP	ADC	G2	58	Female	3,2	2a	0	0	IB	30,73	-	-
77-MP	ADC	G2	64	Female	0,9	1a	0	0	IA1	24,09	-	259
78-MP	ADC	G3	80	Female	2,1	1c	2	0	IIIA	26,29	-	102
80-MP	ADC	G3	62	Male	5,4	3	0	0	IIB	34,87	-	106
81-MP	ADC	G3	61	Male	1,6	1b	0	0	IA2	26,09	-	98
83-MP	ADC	G3	60	Female	5,5	3	0	1a	IVA	23,42	-	98
86-MP	ADC	G2	54	Male	1,8	1b	1	1b	IVA	17,76	-	65
88-MP	ADC	G2	73	Male	2,1	1c	0	0	IA3	25,61	-	102
89-MP	ADC	-	61	Female	3,7	2a	2	0	IIIA	24,88	-	94
91-MP	ADC	G2	67	Male	3,6	2a	2	0	IIIA	24,06	0,44	101
93-MP	ADC	G3	66	Male	2,8	2a	1	0	IIB	29,07	-	104
94-MP	ADC	G2	77	Female	2,2	1c	0	0	IA3	23,57	-	108
97-MP	ADC	G2	73	Male	5	3	0	0	IIB	29,14	1,999	127
98-MP	ADC	G3	55	Female	2,9	1c	2	0	IIIA	17,22	0,127	102
99-MP	ADC	G3	62	Male	3,5 and 1,5	4	0	0	IIIA	18,93	1,034	-
102-MP	ADC	G2	66	Male	2,1	1c	1	0	IIB	20,90	0,281	101
103-MP	ADC	G3	70	Female	3,9	2a	0	0	IB	24,52	0,806	108
105-MP	ADC	G3	77	Male	1,8	1b	0	0	IA2	25,86	1,288	-
106-MP	ADC	G2	79	Male	1,2	1b	0	0	IA2	24,45	1,191	-
107-MP	ADC	G1	54	Female	1,7	1b	0	0	IA2	21,48	0,71	97
108-MP	ADC	G2	60	Female	3,6	2a	0	0	IB	20,03	1,015	116
110-MP	ADC	G3	77	Female	7,2	4	2	0	IIIB	25,64	2,371	-
112-MP	ADC	G2	74	Female	1,6	4	0	0	IIIA	29,36	2,577	101
113-MP	ADC	G3	68	Male	7,5	3	0	0	IIB	27,77	-	166
118-MP	ADC	G1	67	Female	1,5	1b	0	0	IA2	33,25	1,4	-
121-MP	ADC	G3	59	Male	12,5	4	0	0	IIIA	25,00	-	-
122-MP	ADC	G3	61	Female	2,8	1c	0	0	IA3	37,03	-	94
124-MP	ADC	G2	71	Male	1,2	1b	0	0	-	-	-	-

Abbreviations: MP= molecular pneumology, ADC=adenocarcinoma; BMI= body mass index

Histopathological grading: G1=well differentiated; G2=moderately differentiated; G3=poorly differentiated

T-primary tumor: 0: No evidence of primary tumor; 1a: Tumor 2 cm or less in greatest dimension; 1b: Tumor more than 2 cm but not more than 3 cm in greatest dimension; 2a: Tumor more than 3 cm but not more than 5 cm in greatest dimension; 2b: Tumor more than 5 cm but not more than 7 cm in greatest dimension; 3: Tumor more than 7 cm

N-regional lymph nodes: 0: No regional lymph node metastasis; 1: Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension; 2: Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)

M-distant metastasis: 0: No distant metastasis; 1: Distant metastasis.

Supplementary Table S2: Summary of relevant clinical data of the cohort of patients with NSCLC analyzed in this study

Number of Patients	63	
Male	33 (52,4%)	
Female	30 (47,6%)	
Av. Age	65,98412698	(σ=9,704)
G1	3	
G2	26	
G3	30	
tumor diameter	3,42	(σ=2,164) (n=63)
Av. BMI	27,36	(σ=6,157) (n=62)
Av. Glucose	106,7	(σ=27,753) (n=51)

Supplementary Table S3: Survival data of the cohort of patients with NSCLC analyzed in this study

Patient Code	Survival after 3 months (CT)	Survival after 6 months (CT)	Survival after 9 months (CT)	Survival after 12 months (CT)	Survival after 3 years (CT)	Survival after 5 years (CT)
3-MP	No					
9-MP	yes	yes	yes	yes	yes	yes
10-MP	yes	yes	yes	yes	yes	
15-MP	yes					
16-MP	yes	yes	yes			
17-MP	yes	yes	yes	yes	yes	yes
19-MP						
20-MP	yes	yes				
22-MP	yes	yes	yes	yes	yes	
23-MP	yes	yes	yes	yes		
26-MP						
27-MP	yes	yes	yes	yes	yes	yes
28-MP						
32-MP	yes	yes	yes	yes		
34-MP	yes	yes	yes	yes	yes	yes
35-MP	yes	yes	yes	yes	yes	yes
39-MP	yes	yes	yes	yes		
40-MP	yes	yes	yes	yes		
43-MP						
44-MP	yes	yes	yes	yes		
45-MP						
51-MP	yes	yes	yes	yes	yes	
53-MP	yes	yes	yes	yes		
54-MP	yes	yes				
55-MP	yes	yes	yes	yes		
56-MP	yes	yes				
57-MP	yes	yes				
59-MP	yes	yes				
62-MP	yes	yes	yes	yes		
64-MP	yes	yes	yes	yes	yes	
68-MP	yes	yes	yes	yes		
69-MP	yes	yes	yes			
70-MP						
71-MP						
72-MP	yes	yes	yes	yes		
73-MP	yes	yes	yes			
74-MP	yes	yes	yes	yes		
77-MP						
78-MP	yes	yes	yes	yes		
80-MP	yes	yes	yes	yes		
81-MP	yes	yes	yes	yes		
83-MP						
86-MP	yes	yes	yes	yes		
88-MP						
89-MP	yes	yes	yes	yes		
91-MP						
93-MP	yes	yes	yes			
94-MP						
97-MP	yes	yes	yes	yes		
98-MP	yes	yes				
99-MP	no					
102-MP	yes	yes	yes	yes		
103-MP						
105-MP	yes	yes	yes	yes		
106-MP	yes	yes	yes	yes		
107-MP	yes	yes	yes	yes		
108-MP	yes	yes	yes			
110-MP	yes	yes	yes			
112-MP	yes	yes	yes			
113-MP						
118-MP	yes					
121-MP	yes					
122-MP	yes					
124-MP						

Supplementary Table S4: List of primers used for Quantitative Real-Time PCR.

GENE	PRIMER SEQUENCE
hIGF1R	fw: 5´- GGG GCT CCT GTT TCT CTC C -3´ rev: 5´- GCC TTG GAG ATG AGC AGG AT-3´
hAKT	fw: 5´- CAC ACC ACC TGA CCA AGA TGA -3´ rev: 5´- CCT CCA AGC TAT CGT CCA GC-3´
hSLUG	fw: 5´- GAA CTG GAC ACA CAT ACA GTG ATT-3´ rev: 5´- AGT GAT GGG GCT GTA TGC TC-3´
hmTOR	fw: 5 - AGC ATC GGA TGC TTA GGA GTG G – 3´ rev: 5´ - CAG CCA GTC ATC TTT GGA GAC C – 3´
hGAPDH	fw: 5´- AAA TCA AGT GGG GCG ATG CT – 3´ rev: 5´- CAA ATG AGC CCC AGC CTT CT – 3´
mPD-L1	fw: 5´- TCA CTT GCT ACG GGC GTT T – 3´ rev: 3´- CCC AGT ACA CCA CTA ACG CA – 5´
mGAPDH	fw: 5´- AAC TTT GGC ATT GTG GAA GG– 3´ rev: 3´- CAC ATT GGG GGT AGG AAC AC– 5´
mIGFR	fw: 5´- CTG CGG CGA TGA AGA GAA GAA AA – 3´ rev: 5´- TAC CGG TGC CAC GTT ATG ATG ATT – 3´

Supplementary Table S5: Cell culture media used for human and mouse experiments

MEDIUM	INGREDIENTS
RPMI 0 mg/dl glucose	500 ml Gibco™ RPMI 1640 Medium, Thermo Fisher Scientific, no glucose (cat# 11879020); 50 ml heat-inactivated fetal bovine serum (FCS), Sigma-Aldrich (cat# S0615); 2mM L-Glutamine, anprotec (cat# AC-AS-0001); 5 ml Penicillin-Streptomycin (Pen/Strep), anprotec (cat# AC-AB-0024)
RPMI 100 mg/dl glucose	For RPMI 100 mg/dl final medium for 0 mg/dl and 200 mg/dl were mixed 1:1
RPMI 200 mg/dl glucose	500 ml Gibco™ RPMI 1640 Medium, Thermo Fisher Scientific (cat# 21875091); 50 ml heat-inactivated fetal bovine serum (FCS), Sigma-Aldrich (cat# S0615); 2mM L-Glutamine, anprotec (cat# AC-AS-0001); 5 ml Penicillin-Streptomycin (Pen/Strep), anprotec (cat# AC-AB-0024)
DMEM medium	500 ml Gibco™ DMEM, high glucose, Thermo Fisher Scientific (cat# 11965092); 50 ml heat-inactivated fetal bovine serum (FCS), Sigma-Aldrich (cat# S0615); 2mM L-Glutamine, anprotec (cat# AC-AS-0001); 5 ml Penicillin-Streptomycin (Pen/Strep), anprotec (cat# AC-AB-0024)
DMEM medium without additions	500 ml Gibco™ DMEM, high glucose, Thermo Fisher Scientific (cat# 11965092)

Supplementary Table S6: List of antibodies used for human flow-cytometry

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse monoclonal anti-human-CD221, BV421	BD Biosciences	Cat#562593
Mouse monoclonal anti-human-EGFR, AlexaFlour488	BioLegend	Cat#352907
Annexin V, APC	BD Biosciences	Cat#550474
Propidium Iodide Staining Solution	BD Biosciences	Cat#51-66211E
Mouse Anti-Ki-67- FITC	BD Biosciences	Cat#556026
Human BD Fc Block	BD Biosciences	Cat#564220

Supplementary Table S7: List of cell culture antibodies and recombinant proteins

Chemicals, peptides, cell culture antibodies and recombinant proteins		
Human IGF1	R&D Systems	Cat#291-G1
Murine aPD1	InVivoMab	Cat#BE0146
Murine IgG2a	InVivoMab	Cat#BE0089
Murine aCD3	BD Biosciences	Cat#555329
Murine aCD28	BD Biosciences	Cat#555725
Murine aCD16/CD32	BD Biosciences	Cat#553142

Supplementary Table S8: List of antibodies used for murine flow-cytometry

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Monoclonal anti-murin-CD3, FITC	eBioscience	Cat#11-0031-82
Hamster monoclonal anti-murin-CD3, PE-Cy7	eBioscience	Cat#552774
Rat monoclonal anti-murin-CD4, BB700	BD Biosciences	Cat#566408
Rat monoclonal anti-murin-CD4, PE-Cy5.5	BD Biosciences	Cat#550954
Rat monoclonal anti-murin-CD8, BV510	BD Biosciences	Cat#563068
Monoclonal anti-murin-CD8, PerCP	BioLegend	Cat#100732
Rat monoclonal anti-murin-CD25, BV421	BioLegend	Cat#102033
Hamster monoclonal anti-murin-PD1, PE	BD Biosciences	Cat#561788
Rat monoclonal anti-murin-CD62L, APC-Cy7	BD Biosciences	Cat#560514
Rat monoclonal anti-murin-CCR7, AlexaFlour488	BD Biosciences	Cat#560682
Monoclonal anti-murin-CD103, PE	eBioscience	Cat#12-1031-83
Mouse BD Fc Block	BD Biosciences	Cat# 553142

Supplementary Table S9: Enzyme-linked immunosorbent assay (ELISA)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ELISA		
Murine IFN-γ	eBioscience	Cat#555138
Murine IL-2	eBioscience	Cat#555148
Murine IL-10	BD Bioscience	Cat#555252
Murine TGF-β	R&D system	Cat#DY9679

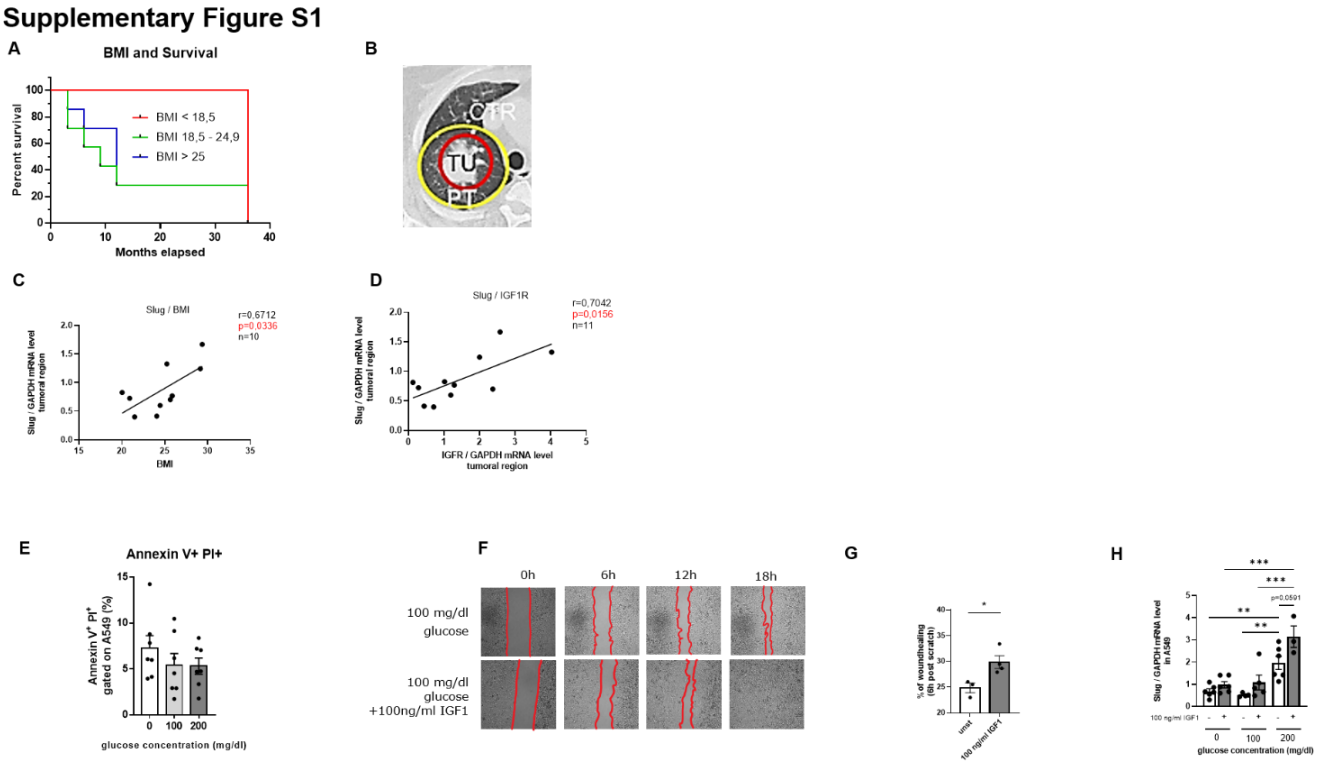
Supplementary Table S10: Micro nutrition of the mice diet

Diet	**High carbohydrate diet**	**Low carbohydrate diet**	**Breeding diet**
Trace elements	**[mg/kg]**	**[mg/kg]**	**[mg/kg]**
Aluminium	3,71	16,03	82,46
Chlorine	3.630,00	3.630,00	3.369,44
Iron	178,58	179,3	189,98

Flourine	4,17	4,17	3,1
Iodine	0,51	0,51	1,47
Cobalt	0,15	0,14	0,48
Copper	5,75	5,65	14,52
Manganese	100,89	101,52	72,88
Molybdenum	0,2	0,2	1,67
Sulfur	2.791,54	2.712,65	918,24
Selenium	0,33	0,31	0,26
Zinc	29,3	30,04	83,65
Added vitamins per kg			
Biotin	201 µg	201 µg	290 µg
Choline chloride	1.012 mg	1.012 mg	717 mg
Folic acid	10 mg	10 mg	2 mg
Nicotinic acid	50 mg	50 mg	36 mg
Pantothenic acid	50 mg	50 mg	21 mg
Vitamine A	15.000 IU	15.000 IU	15.000 IU
Vitamine B1	20 mg	20 mg	18 mg
Vitamine B2	20 mg	20 mg	12 mg
Vitamine B6	15 mg	15 mg	9 mg
Vitamine B12	41 µg	41 µg	24 µg
Vitamine C	20 mg	20 mg	36 mg
Vitamine D3	500 IU	500 IU	600 IU
Vitamine E	180 mg	192 mg	80 mg
Vitamine K3	10 mg	10 mg	3 mg
Fatty acids	**[mg/kg]**	**[mg/kg]**	**[mg/kg]**
Arachidic acid C-20:0	50	70	340
Eicosanoic acid C-20:1	150	210	425
α-Linolenic acid C-18:3	150	210	4.491
Linolenic acid C-18:2	28.500	39.900	32.681
Palmitic acid C-16:0	2.500	3.500	8.010

Stearic acid C-18:0	1.350	1.890	2.399
Oleic acid C-18:1	13.500	18.900	13.795
Amino acids	**[mg/kg]**	**[mg/kg]**	**[mg/kg]**
Alanine	2.528	2.316	10.395
Arginine	9.829	9.700	15.977
Aspartic acid	3.583	3.404	23.673
Cystine	3.196	3.140	3.285
Glutaminc acid	23.675	23.172	46.578
Glycine	3.136	3.030	10.055
Histidine	5.276	5.200	5.800
Isoleucine	7.223	7.120	10.406
Leucine	14.763	14.440	17.772
Lysine	17.401	17.322	12.372
Methionine	7.223	7.170	3.530
Phenylalanine	7.172	7.040	11.293
Proline	12.763	12.516	14.511
Serine	5.268	5.130	12.016
Threonine	7.154	7.054	8.758
Tryptophan	1.977	1.960	3.192
Tyrosine	9.285	9.170	7.953
Valine	3.296	3.170	11.405

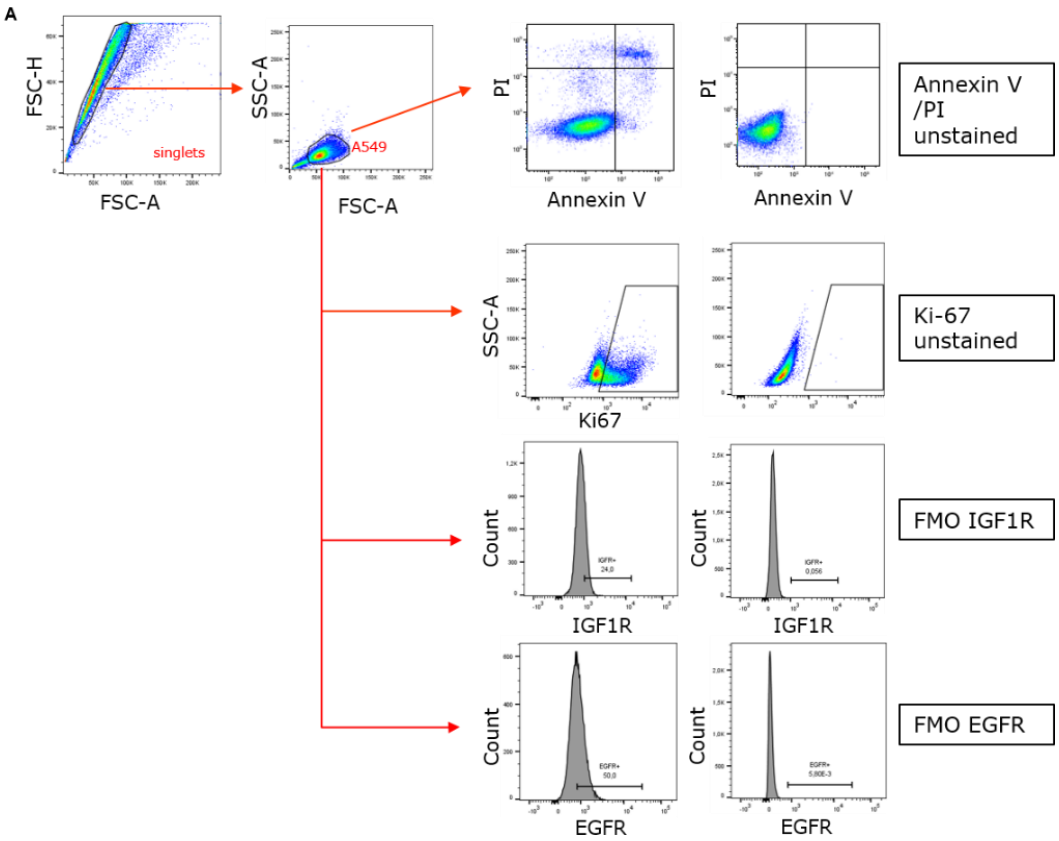
2. Supplementary Figures



Supplementary Figure S1. Human cohort of NSCLC patients. A) Kaplan-Meier curve depending on patients BMI (BMI < 18,5 n=1, BMI 18,5-24,9 n=7 BMI >25 n=7). **B)** CT illustration of the areas for lung tissue resected after surgery of NSCLC patients. **C)** qPCR analysis of relative SLUG/GAPDH mRNA expression level correlated with BMI of patients (n=10), p= 0,0336, r=0,6712, **(B-H** p= 0,05). **D)** qPCR analysis of relative Slug/GAPDH mRNA expression level correlated with relative IGF1R/GAPDH mRNA expression level of patients (n=12); p=0,0156, r= 0,7042 (B-H p= 0,025). **E)** Annexin V/PI FACS analysis of untreated A549 cell culture, cultured in 0, 100 or 200mg/dl glucose; late apoptotic (AnnexinV+ PI+) A549 cells (n=7). **F)** Scratched A549 cell mono layer. Photos were taken 0 h, 6 h, 12 h, 18 h after the scratch to detect cellular migration. Upper panel shows untreated cells with 100 mg/dl glucose in cell culture medium. In the lower panel, A549 cells were treated with 100 ng/ml IGF1 cultured with the same glucose concentration. The scratch is highlighted in red. **G)** Relative migration in percent of scratched A549 cells simulating wound healing capability after 6h; p (unst vs. 100 ng/ml IGF1)=0.0285 (n=4). **H)** qPCR analysis of relative SLUG/GAPDH mRNA expression level in A549 cells cultured as described in figure 3h; p (0 mg/dl glucose without IGF1 vs. 200 mg/dl glucose without IGF1) =0,0063, p (100 mg/dl glucose without IGF1 vs. 200 mg/dl glucose without IGF1)=0,0056, p (100 mg/dl glucose with IGF1 vs. 200 mg/dl glucose with IGF1) =0,0004, p (0 mg/dl glucose with IGF1 vs. 200 mg/dl glucose with IGF1) =0,0001

Correlations are shown using simple linear regression. The two-tailed Pearson correlation analysis was performed to get the r and p value for figures B and C. One-way ANOVA test was used for figure H and unpaired T-Test for G.

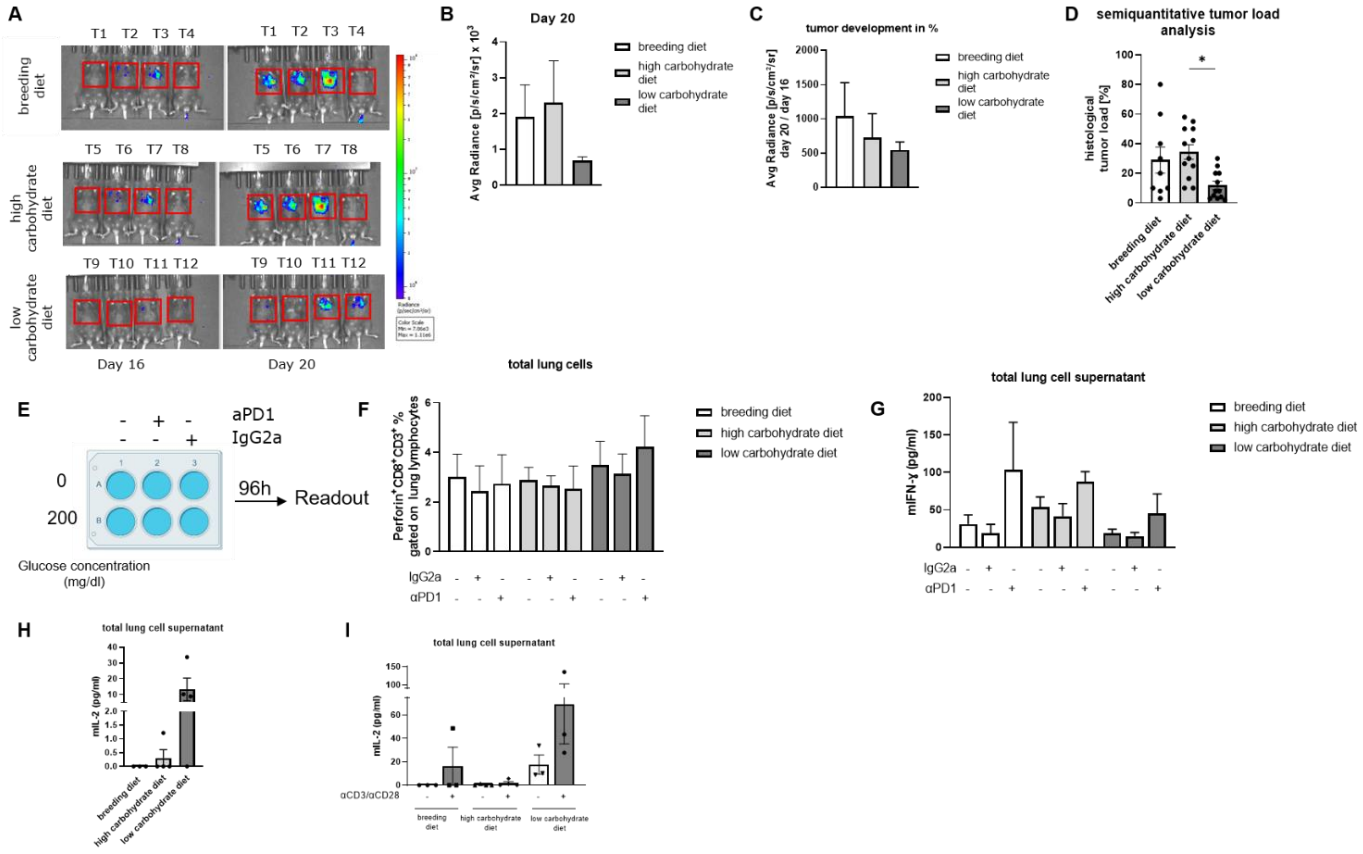
Supplementary Figure S2



Supplementary Figure S2. Gating strategy for A549 analysis.

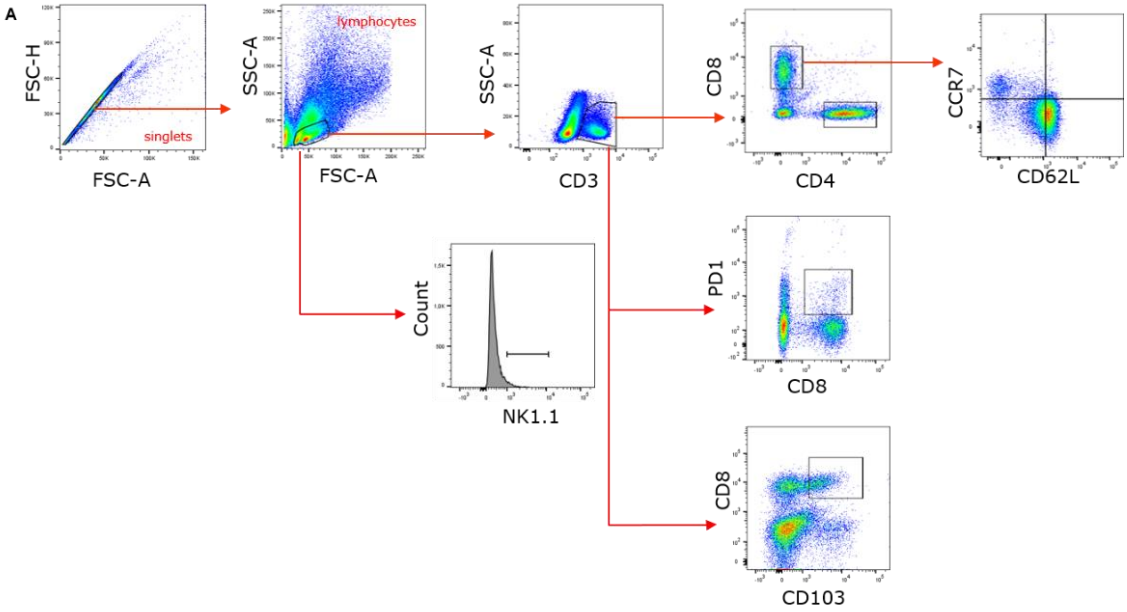
A) Representative flow cytometry staining of A549 sample. For cell preparation, cells were collected and washed. Next, cells were stained with fluorochrome labeled antibodies for the identification of the different expression levels of proteins. Cells that are shown were gated to identify the Ki67, AnnexinV, Pi, IGF1R and EGFR. Therefore, doublets were excluded gating FSC-A against FSC-H. Afterwards, the A549 cells were gated in FSC-A against SSC-A. Next, different expression levels were identified gating SSC-A against the protein of interest or Annexin V against Pi to differentiate early and late apoptosis as well as necrotic cells. For identification of EGFR and IGFR1 plots are shown as histograms and respective FMOs are shown on the right.

Supplementary Figure S3



Supplementary Figure S3. Carbohydrate deprivation diet in a murine model of lung cancer. A) Representative IVIS pictures from one out of three experiments are shown on day 16 and day 20 with the region of interest (ROI). **B)** Quantitative analysis of in vivo bioluminescence imaging results on day 20. Data are shown as average radiance (p / s / cm2 / sr) of an area 3,17 cm x 33,17 cm. **C)** Tumor development (%) was calculated by the ratio of the average radiance (p / s / cm2 / sr) on day 20 and day 16. **D)** Semiquantitative analysis of total tumor load shown in histology of three independent experiments, analyzed in a blinded study of a pathologist from the Department of Pathology of the University hospital Erlangen ($n^{\text{breeding diet}}=9$, $n^{\text{high carbohydrate diet}}=12$, $n^{\text{low carbohydrate diet}}=12$), p (high carbohydrate vs. low carbohydrate diet) =0,0128. **E)** Experimental design of isolated murine total lung cells culture for 96h with 0mg/dl glucose or 200mg/dl glucose, depending on mice´s diet. Cells from mice fed with carbohydrate containing diet were cultured with 200 mg/dl glucose in the cell culture medium. Cells from mice fed with the low carbohydrate diet were cultured with 0 mg/dl glucose in the cell culture medium. Additionally, cells were either left unstimulated, treated with αIgG2a or αPD1. **F)** CD3+ CD8+ Perforin+% T-cells of total lung lymphocytes after 5 days cell culture as described above, treated with anti-PD-1 antibody or anti-IgG2a. Cells were collected, stained with antibodies and analyzed by flow cytometry. **G)** Murine IFN-γ concentration of the total lung cell culture supernatant after 5 days either stimulated with αCD3/αCD28, treated with anti-PD-1 antibody or treated with anti-IgG2a antibody and analyzed by ELISA. **H)** Murine IL-2 concentration in total lung cell culture supernatant after 5 days cell culture, measured by ELISA; ($n^{\text{breeding diet}}=3$, $n^{\text{high carbohydrate diet}}=3$, $n^{\text{low carbohydrate diet}}=5$). **I)** Murine IL-2 concentration in total lung cell culture supernatant after 5 days cell culture and stimulation von αCD3/αCD28 antibodies, measured by ELISA; ($n^{\text{breeding diet}}=3$, $n^{\text{high carbohydrate diet}}=4$, $n^{\text{low carbohydrate diet}}=3$). One-way ANOVA test was used for figure D.

Supplementary Figure S4



Supplementary Figure S4. Gating strategy for different lymphocyte subpopulations in murine lung and lymph node. A) Representative flow cytometry staining of a lung sample from a naïve C57BL/6 mouse with tumor. For lung preparation, the tissue was digested with DNase I/Collagenase D for the isolation of lung single cell suspension. Next, cell suspension was filtered and treated with ACK lysis buffer. Total lung cells were stained with fluorochrome labeled antibodies for the identification of the different lymphocytes subsets. Cells that are shown were gated to identify the TCM, TEM, TRM ($CD3^+CD8^+CCR7^{high}CD62L^{high}$ for TCM), ($CD3^+CD8^+CCR7^{low}CD62L^{low}$ for TEM) for TCM) and ($CD3^+CD8^+CD103^+$ for TRM). Therefore, doublets were excluded gating FSC-A against FSC-H. Afterwards, the lymphocytes are gated in FSC-A against SSC-A. Next, T cells were identified gating on $CD3^+$ cells. Then, $CD4^+$ and $CD8^+$ T cells were identified gating on CD4 and CD8. To identify TCM T cells, we next gated in $CCR7^{high}$ and $CD62L^{high}$. For TEM we gated on $CCR7^{low}$ and $CD62L^{low}$. To identify PD1 positive CD8 T cells we gated from $CD3^+$ on $CD8^+$ and $PD1^+$ cells. We also gated for TRM T cells from $CD3^+$ on $CD8^+$ and $CD103^+$ cells. Finally, data were further analyzed and evaluated with the program FlowJo.