Supplemental Data

Dysregulation of cholesterol homeostasis in human lung cancer tissue and tumour-associated macrophages

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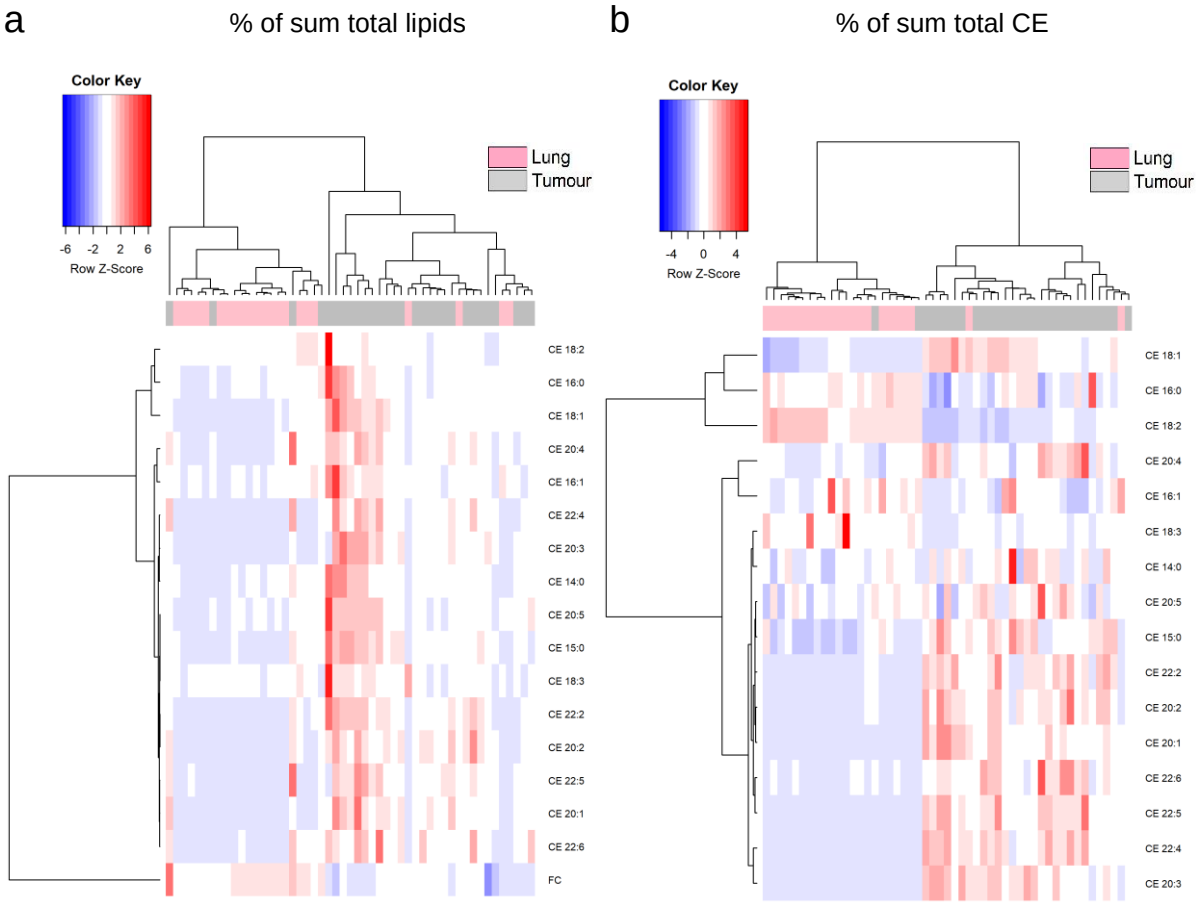
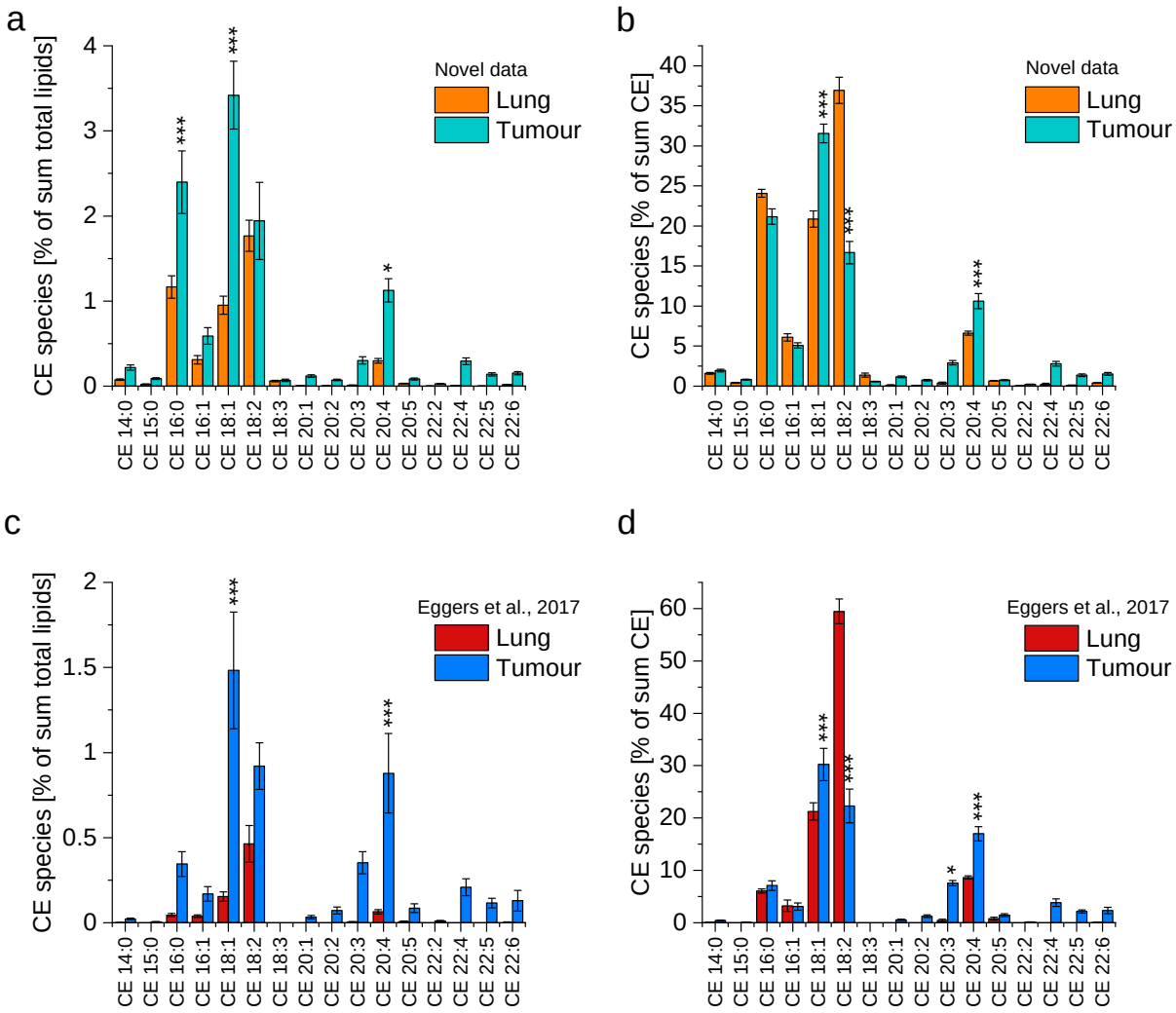
1. Supplemental Figures

Supplemental Figure 1



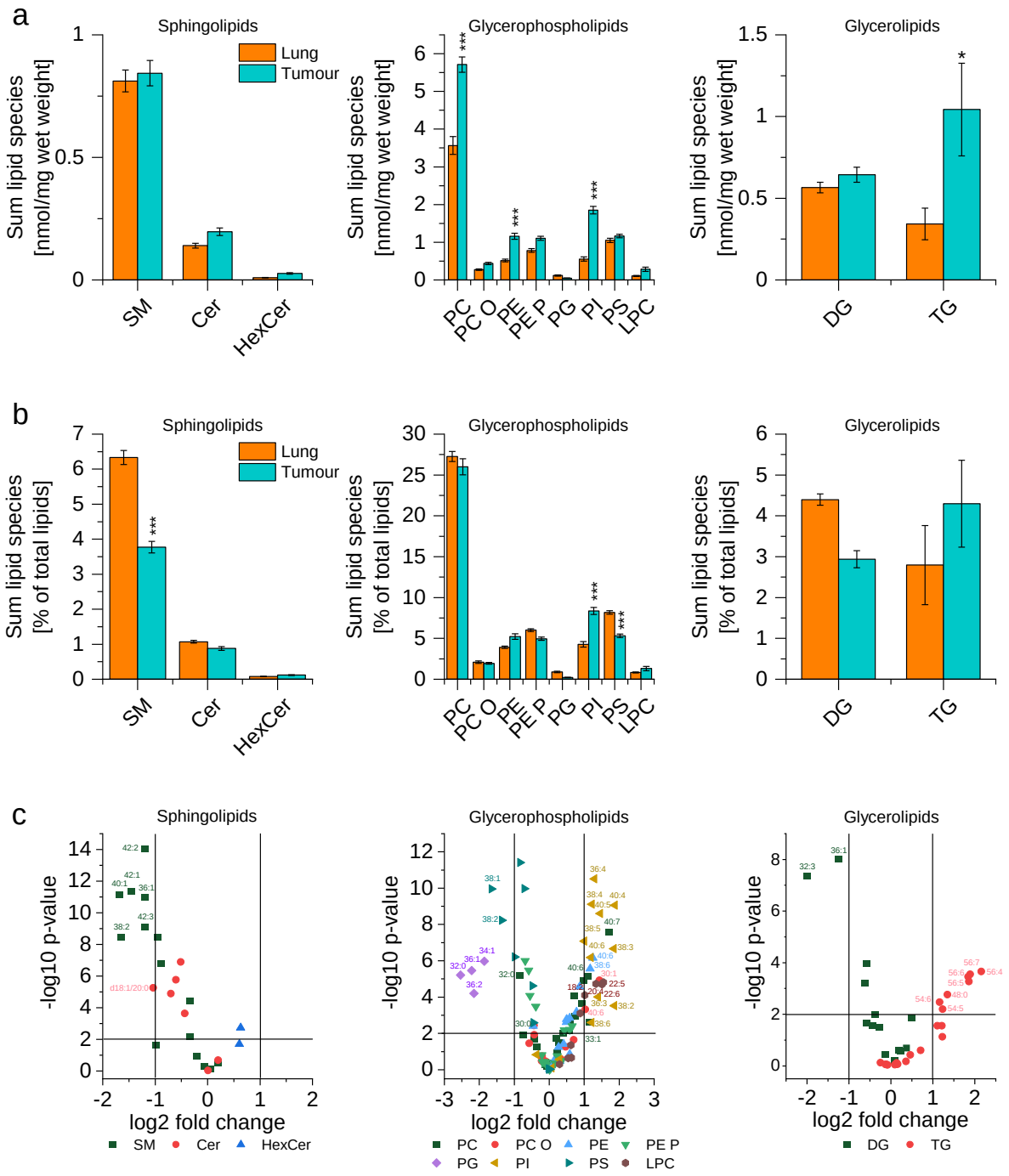
Supplemental Figure 1: *Sterol lipid content of human lung adenocarcinoma* vs. *adjacent non-tumour tissue.* Sterol lipid content of human lung adenocarcinoma *vs.* adjacent non-tumour tissue was determined in 22 normal and 29 tumour tissues by mass spectrometry and either expressed as percentage of total lipid concentration (a) or percentage of total cholesteryl ester concentration (b) within each tissue sample. A, B: Hierarchical clustering using Ward's linkage and Euclidean distances. Normal tissues are highlighted as grey and tumour samples as pink. FC: free cholesterol, CE: cholesteryl ester.

Supplemental Figure 2



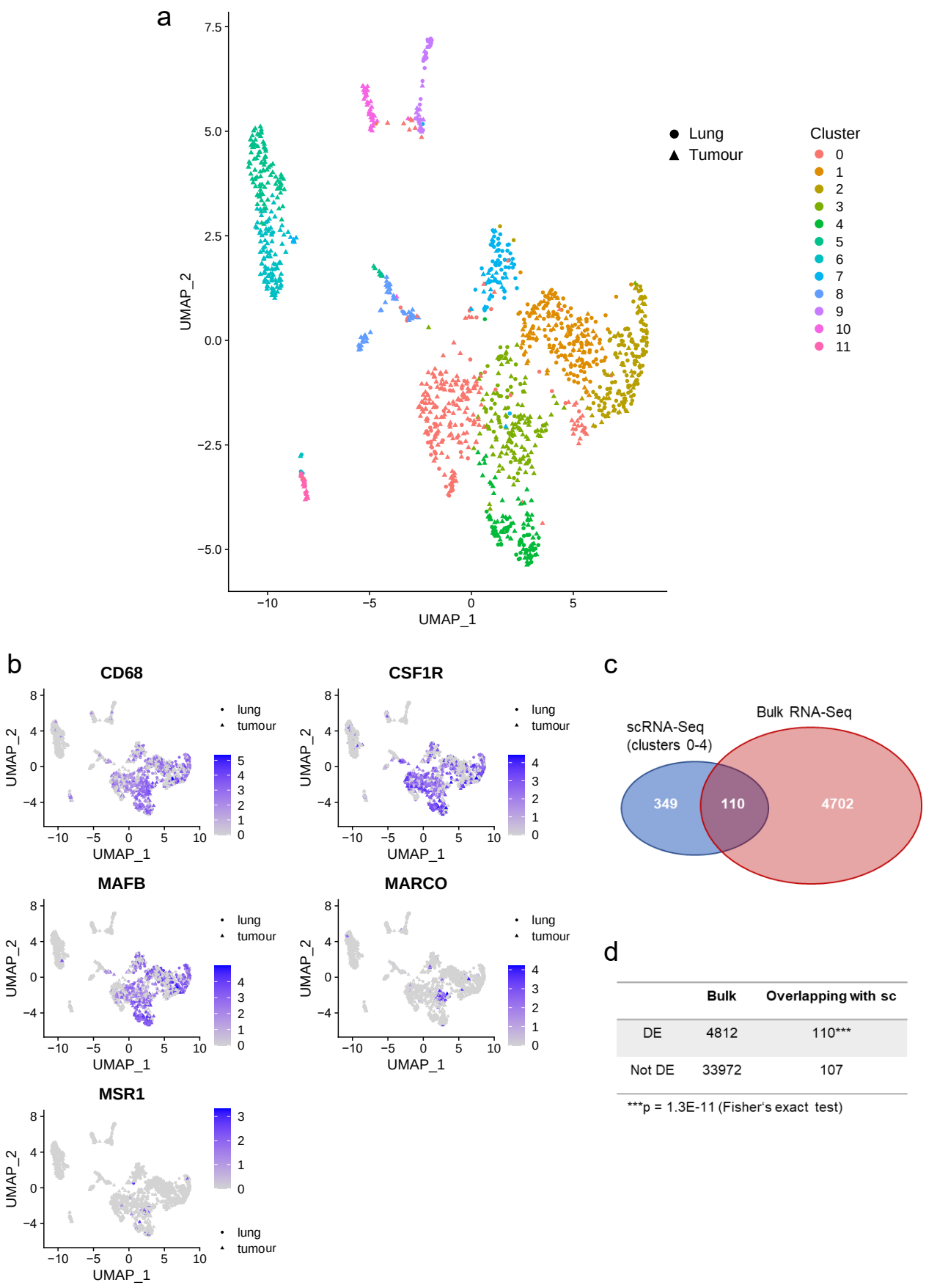
Supplemental Figure 2. *Content of individual cholesteryl ester species in human lung adenocarcinoma vs. non-tumour tissue: comparison previously published data.* The cholesteryl ester (CE) content was determined by mass spectrometry an*d* either expressed as percentage of total lipid concentration (a, c) or percentage of total cholesteryl ester concentration (b, d) within each sample. a, b: Cholesteryl ester concentrations were determined in 22 normal and 29 tumour tissues. c, d: Cholesteryl ester concentrations in 7 normal and 10 tumour tissue samples obtained from lung adenocarcinoma patients according to a previously published data set.[1] *p<0.05, ***p<0.001 *vs.* normal lung (ANOVA with Bonferroni's post-hoc test).

Supplemental Figure 3



Supplemental Figure 3: *Non-sterol lipids in human lung adenocarcinoma* vs. *adjacent non-tumour tissue.* The lipid content of human lung adenocarcinoma *vs.* adjacent non-tumour tissue was determined in 22 normal and 29 tumour tissues by mass spectrometry. a: Concentration of individual lipid classes. b: Individual lipid classes were expressed as a percentage of the total lipid concentration within each tissue sample. a, b: *p<0.05, ***p<0.001 *vs.* normal lung (ANOVA with Bonferroni's post-hoc test). c: Fold change of individual lipid species (as % sum of total lipids) in tumour *vs.* normal lung tissue. Log2 fold change is plotted against –log10 p-value (horizontal lines: p=0-01; vertical lines: 2-fold change.). SM: sphingomyelin; Cer: ceramide; HexCer: hexosylceramide; PC: phosphatidylcholine; PC O: PC ether; PE: phosphatidylethanolamine; PE P: PE-based plasmalogens; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine; LPC: lyso-phosphatidylcholine; DG: diacylglycerol, TG: triacylglycerol.

Supplemental Figure 4



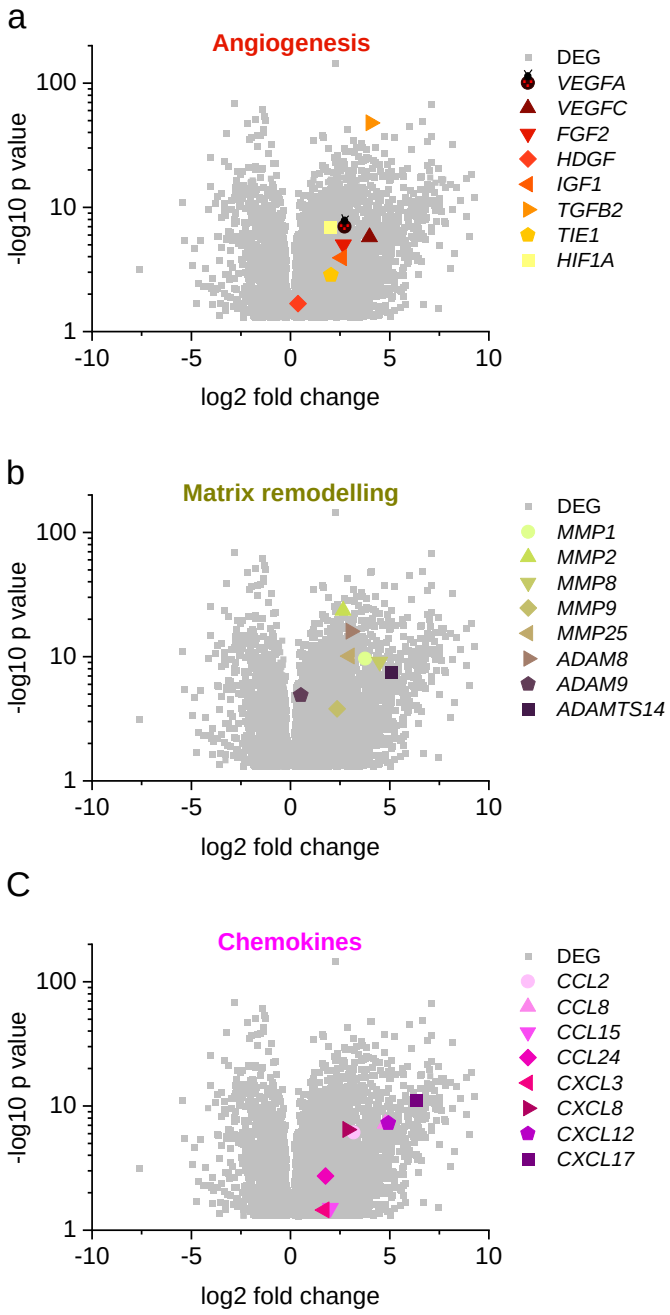
d

	Bulk	Overlapping with sc
DE	4812	110***
Not DE	33972	107

***p = 1.3E-11 (Fisher's exact test)

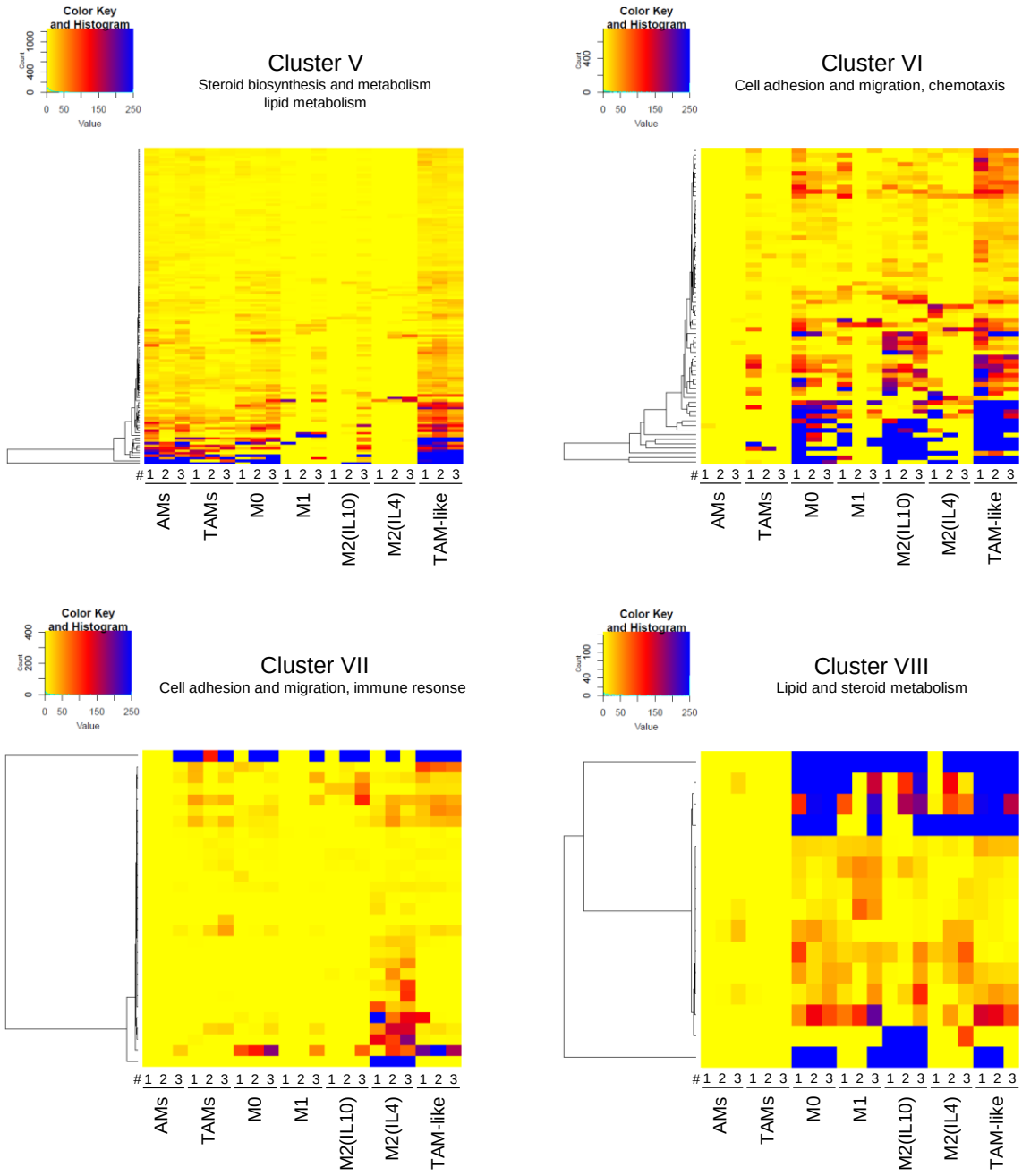
Supplemental Figure 4: *Comparison of bulk RNA-Seq and publicly available scRNA-Seq data.* a, b: Single cell RNA-seq (scRNA-Seq) on non-lymphocytic immune cells sorted from human early-stage lung adenocarcinoma lesions and from adjacent non-involved lung tissue (lung: n=4, tumour: n=6, GEO dataset GSE97168).[2] a: Clustering. b: Expression of macrophage markers *CD68*, *CSF1R*, *MAFB*, *MARCO*, and *MSR1* identified clusters 0-4 as macrophage-enriched populations. c: Overlap of genes differentially expressed between lung and tumour tissue in scRNA-Seq (clusters 0-4) and bulk RNA-Seq. d: Clear enrichment of DE genes in the comparison of bulk and single cell data. Processed sequencing data are available in the Mendeley repository (doi:10.17632/c3ntj95zgg.1).

Supplemental Figure 5



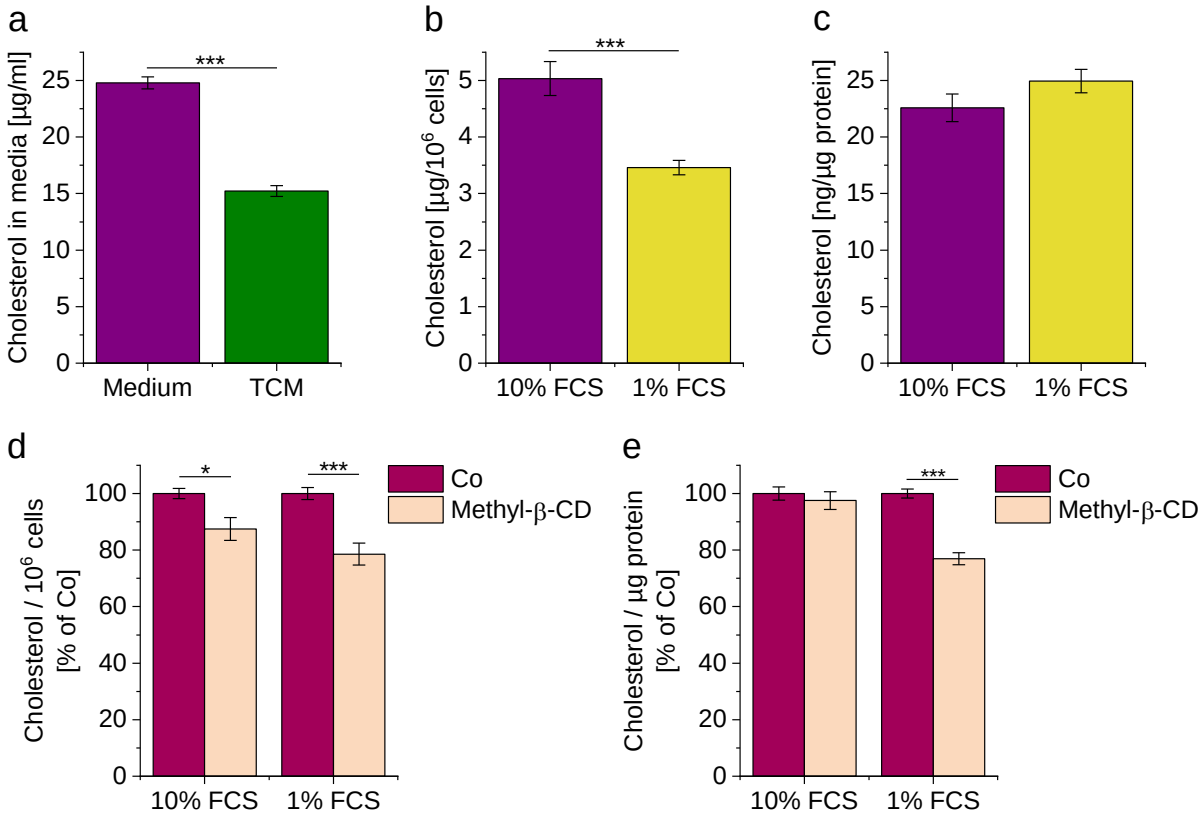
Supplemental Figure 5: *Gene expression in TAMs* vs. *AMs.* Differentially expressed genes (DEGs) in TAMs *vs*. AMs are shown as volcano plots. Log2 fold change is plotted against –log10 p-value. a: Angiogenesis-associated genes. *VEGFA/C*: vascular endothelial growth factor A/C; FGF*2*: fibroblast growth factor 2; *HDGF*: hepatoma-derived growth factor; *IGF1*: insulin-like growth factor 1; *TGFB2*: transforming growth factor beta 2; *TIE1*: tyrosine kinase with immunoglobulin-like and EGF-like domains 1; *HIF1A*: hypoxia-inducible factor 1-alpha. b: Genes involved in matrix remodelling. *MMP1/2/8/9/25*: matrix metalloproteinase 1/2/8/9/25; *ADAM8/9*: a disintegrin and metalloprotease 8/9; *ADAMTS14*: ADAM with thrombospondin motifs 14. c: Chemokine expression. *CCL2/8/15/24*: CC chemokine ligand 2/8/15/24; *CXCL3/8/12/17*: C-X-C motif ligand 3/8/12/17.

Supplemental Figure 6



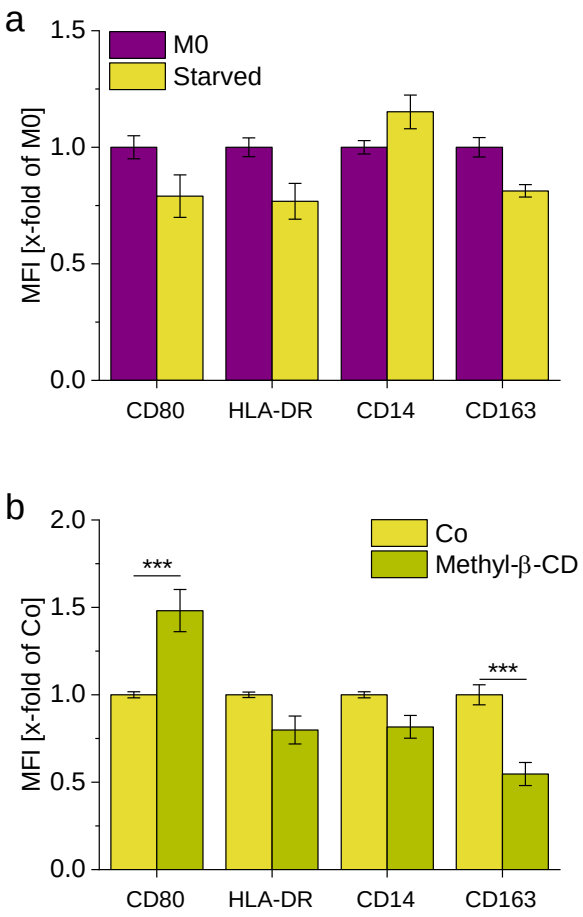
Supplemental Figure 6: *Comparison of gene expression signatures of TAMs/AMs and* in vitro *polarized macrophages, clusters V-VIII.* Genes differentially expressed in TAMs *vs.* AMs were clustered according to their expression pattern, and gene expression in *in vitro* polarized cells and *ex vivo* macrophages is visualized as one heatmap per cluster. Clusters were linked to distinct functions based on the predominant GO terms associated with genes contained within each cluster. TAMs/AMs: mean values of technical replicates for each donor are shown. MDMs: Individual values per donor are shown.

Supplemental Figure 7



Supplemental figure 7: *Cholesterol content in media and macrophages.* a: Cholesterol concentration in standard macrophage medium containing 10% FCS and A549 tumour cell-conditioned medium (TCM) (n=11, each). b, c: Cellular cholesterol content of human MDMs cultured in medium containing 10% FCS (normal medium) or 1% FCS (starving medium) for 24 hours. Values were normalized to cell count (b) or total cellular protein (c) (n=5, triplicates). d, e: MDMs were incubated with PBS (Co) or Methyl-β-cyclodextrin (Methyl-β-CD, 5 mM in PBS) for one hour, followed by incubation in media containing either 10% or 1% FCS for 24 hours. Cholesterol concentrations were measured in MDM lysates, either normalized to cell count (d) or protein content (e) and are expressed as percentage of the respective control (n=4, triplicates). *p<0.05, ***p<0.001 (a-c: t-test, d-e: ANOVA with Bonferroni's post-hoc test).

Supplemental Figure 8



Supplemental Figure 8: *Surface marker expression upon starving and cholesterol depletion*. Surface marker expression was quantified by flow cytometry. a: Human MDMs were cultured in medium containing 10% FCS (normal medium, M0) or 1% FCS (starving medium) for 24 hours. Mean fluorescence intensities (MFIs) are expressed as x-fold of M0 cells (n=4, duplicates). b: MDMs were incubated with PBS (Co) or Methyl-β-cyclodextrin (Methyl-β-CD, 5 mM in PBS) for one hour, followed by incubation in media containing 1% FCS for 24 hours. MFIs are expressed as x-fold of Co (n=3, duplicates). ***p<0.001 (ANOVA with Bonferroni's post-hoc test).

2. Supplemental Tables

Supplemental Table 1: Patient data.

Gender	Age (years)	TNM (8th ed.)	Tissue		Used for		
			Lung	Tumour	Lipidomics (Donor No.)	TAM/AM RNA-Seq (Donor No.)	TAM/AM Cholestrol (Donor No.)
female	57	T2aN0	x	x	1		
male	54	T2bN1	x	x	2		
male	64	T2aN1	x	x	3		
female	85	T3N0	x	x	4		
female	71	T3N0	x	x	5		
female	74	T3N0	x	x	6		
female	74	T2aN0	x	x	7		
male	66	T4N1	x	x	8		
male	73	T1aN0	x	x	9		
female	65	T2aN2	x	x	10		
male	69	T3N1	x	x	11		
male	70	T2aN1	x	x	12		
male	73	T3N1	x	x	13		
male	64	T2aN2	x	x	14		
female	75	T1aN0	x	x	15		
female	53	T2bN2	x	x	16		
female	76	T2aN0	x	x	17		
female	42	T3N0	x	x	18		
male	68	T2bN0	x	x	19		
male	63	T3N2	x	x	20		
male	46	T3N2	x	x	21		
male	71	T2aN0	x	x	22		
female	66	T4N2	-	x	23		
male	70	T2aN0	-	x	24		
female	61	T2aN0	-	x	25		
male	73	T4N0	-	x	26		
female	62	T1bN0	-	x	27		
female	77	T3N0	-	x	28		
male	78	T2bN1	-	x	29		
male	70	T2aN1	x	x		1	
female	74	T1aN0	x	x		2	
female	75	T2aN0	x	x		3	
male	53	T2aN0	x	x			1
male	68	T2aN1	x	x			2
female	76	T2aN0	x	x			3
male	75	T2aN0	x	x			4

Supplemental Table 2: Primer sequences.

Gene	Forward	Reverse
ABCA1	CATCTGGTTCTATGCCCGCT	TCTGCATTCCACCTGACAGC
ABCG1	GCGCCAAACTCTTCGAGCTG	CGGATGCAACCTCCATGACAAA
ACTB	TGCGTGACATTAAGGAGAAG	GTCAGGCAGCTCGTAGCTCT
CCL2	TTGATGTTTTAAGTTTATCTTTCATGG	CAGGGGTAGAACTGTGGTTCA
CXCL8	GAGAAGTTTTTGAAGAGGGCTGA	GCTTGAAGTTTCACTGGCATCT
HIF1A	CGGGGACCGATTCACCAT	TTTCGAACGTTCAGAACTTATCTTTT
HMGCR	GCAGGACCCCTTTGCTTAGA	GCACCTCCACCAAGACCTAT
MMP2	CGTCGCCCATCATCAAGTTC	GAAGGTGTTCAGGTATTGCACTG
MMP9	CTTTGAGTCCGGTGGACGAT	TCGCCAGTACTTCCCATCCT
MVK	GGAGCCATGTTGTCAGAAGTC	TACAGCCAGTGCTACCTTGC
VEGFA	CGCTTACTCTCACCTGCTTCTG	GGTCAACCACTCACACACACAC

Supplemental Table 3: Sample sizes and statistical tests.

Title	Sample size	Data reported as	Unit	Statistical test	Figure
Total lipids in lung and tumour tissues	lung = 22, tumour = 29	Mean ± SD	nmol/mg wet weight	t-test	1b
Sterol lipids in lung and tumour tissue	lung = 22, tumour = 29	Mean ± SEM	nmol/mg wet weight	ANOVA with Bonferroni's post-hoc test	1c
Sterol lipids in lung and tumour tissue	lung = 22, tumour = 29	Mean ± SEM	% of total lipids	ANOVA with Bonferroni's post-hoc test	1e
CE species in lung and tumour tissue	lung = 22, tumour = 29	Mean ± SEM	nmol/mg wet weight	ANOVA with Bonferroni's post-hoc test	1f
Intracellular cholesterol in AMs and TAMs	n = 4 (duplicates or triplicates)	Mean ± SEM	µg/10^6 cells	t-test	2a
Intracellular cholesterol in AMs and TAMs	n = 4 (duplicates or triplicates)	Mean ± SEM	ng/µg protein	t-test	2b
Enriched GO terms	n = 3 (triplicates)	N/A	N/A	Mann-Whitney U test with Bonferroni correction	2c, 2g
DEGs in TAMs *vs.* AMs (RNA-Seq)	n = 3 (triplicates)	log2 fold change *vs.* Log10 p-value	N/A	Wald-test with Bonferroni correction	2f, 2i
Surface marker expression in *in vitro* polarized macrophages	n = 4-7 (duplicates)	Mean ± SEM	x-fold of M0	ANOVA with Bonferroni's post-hoc test	3b
DEGs in *in vitro* polarized macrophages (RNA-Seq)	n = 3	No. of DEGs	N/A	Mann-Whitney U test with Bonferroni correction	3c
Comparison of DEGs in TAMs *vs.* AMs to *in vitro* polarized macrophages (RNA-Seq)	n = 3	N/A	TPM	Mann-Whitney U test with Bonferroni correction	4
Enriched GO terms (overlapping DEGs between TAMs *vs.* AMs and TAM-like *vs.* M0)	n = 3	N/A	N/A	Mann-Whitney U test with Bonferroni correction	5a, 5b
DEGs in TAM-like *vs.* M0 cells (RNA-Seq)	n = 3	log2 fold change vs. Log10 p-value	N/A	Wald-test with Bonferroni correction	5e
Intracellular cholesterol in *in vitro* polarized macrophages	n = 3 (triplicates)	Mean ± SEM	µg/10^6 cells	ANOVA with Bonferroni's post-hoc test	5f
Gene expression in M0, TAM-like and starved macrophages (qPCR)	n = 4 (triplicates)	Mean ± SEM	x-fold of M0	ANOVA with Bonferroni's post-hoc test	6a
Intracellular cholesterol upon cholesterol depletion	n = 4 (triplicates)	Mean ± SEM	ng/µg protein	t-test	6b
Gene expression in cholesterol-depleted macrophages	n = 3 (triplicates)	Mean ± SEM	x-fold of Co	ANOVA with Bonferroni's post-hoc test	6c
Intracellular cholesterol in ATR-101-treated macrophages	n = 4 (triplicates)	Mean ± SEM	ng/µg protein	ANOVA with Bonferroni's post-hoc test	6d
Gene expression in ATR-101-treated macrophages	n = 3 (duplicates)	Mean ± SEM	% of TAM-like	ANOVA with Bonferroni's post-hoc test	6e
CE species in lung and tumour tissue (% of sum total)	lung = 22, tumour = 29	Mean ± SEM	% of total lipids	ANOVA with Bonferroni's post-hoc test	S2a
CE species in lung and tumour tissue (% of sum CE)	lung = 22, tumour = 29	Mean ± SEM	% of sum CE	ANOVA with Bonferroni's post-hoc test	S2b
CE species in lung and tumour tissue (% of sum total)	lung = 7, tumour = 10	Mean ± SEM	% of total lipids	ANOVA with Bonferroni's post-hoc test	S2c

CE species in lung and tumour tissue (% of sum CE)	lung = 7, tumour = 10	Mean ± SEM	% of sum CE	ANOVA with Bonferroni's post-hoc test	S2d
Non-sterol lipids: concentration	lung = 22, tumour = 29	Mean ± SEM	nmol/mg wet weight	ANOVA with Bonferroni's post-hoc test	S3a
Non-sterol lipids: % of total lipids	lung = 22, tumour = 29	Mean ± SEM	% of total lipids	ANOVA with Bonferroni's post-hoc test	S3b
Non-sterol lipids: individual lipid species	lung = 22, tumour = 29	log2 fold change *vs.* Log10 p-value	nmol/mg wet weight	Mann-Whitney U test with Bonferroni correction	S3c
DEGs in TAMs *vs.* AMs (bulk RNA-Seq *vs.* scRNA-Seq)	bulk: n = 3 (triplicates), sc: normal =4, tumour = 6	No. of DEGs	N/A	Wald-test with Bonferroni correction	S4c
DEGs in TAMs *vs.* AMs (bulk RNA-Seq *vs.* scRNA-Seq): gene enrichment	bulk: n = 3 (triplicates), sc: normal =4, tumour = 6	No. of overlapping genes	N/A	Fisher's Exact test	S4d
DEGs in TAMs *vs.* AMs (RNA-Seq)	n = 3 (triplicates)	log2 fold change vs. Log10 p-value	N/A	Wald-test with Bonferroni correction	S5
Comparison of DEGs in in TAMs *vs.* AMs to vitro polarized macrophages (RNA-Seq)	n = 3	N/A	TPM	Mann-Whitney U test with Bonferroni correction	S6
Cholesterol in media	n = 11	Mean ± SEM	µg/ml	t-test	S7a
Cholesterol in starved macrophages	n = 5 (triplicates)	Mean ± SEM	µg/10^6 cells	t-test	S7b
Cholesterol in starved macrophages	n = 5 (triplicates)	Mean ± SEM	ng/µg protein	t-test	S7c
Cholesterol in cholesterol-depleted macrophages kept in different media	n = 4 (triplicates)	Mean ± SEM	µg/10^6 cells	ANOVA with Bonferroni's post-hoc test	S7d
Cholesterol in cholesterol-depleted macrophages kept in different media	n = 4 (triplicates)	Mean ± SEM	ng/µg protein	ANOVA with Bonferroni's post-hoc test	S7e
Surface marker expression upon starving	n = 4 (duplicates)	Mean ± SEM	x-fold of M0	ANOVA with Bonferroni's post-hoc test	S8a
Surface marker expression upon cholesterol depletion	n = 3 (duplicates)	Mean ± SEM	x-fold of Co	ANOVA with Bonferroni's post-hoc test	S8b

3. References

1. Eggers LF, Müller J, Marella C, Scholz V, Watz H, Kugler C, Rabe KF, Goldmann T, Schwudke D. Lipidomes of lung cancer and tumour-free lung tissues reveal distinct molecular signatures for cancer differentiation, age, inflammation, and pulmonary emphysema. Sci Rep. 2017;7(1):11087.
2. Lavin Y, Kobayashi S, Leader A, Amir ED, Elefant N, Bigenwald C, Remark R, Sweeney R, Becker CD, Levine JH, Meinhof K, Chow A, Kim-Shulze S, Wolf A, Medaglia C, Li H, Rytlewski JA, Emerson RO, Solovyov A, Greenbaum BD, Sanders C, Vignali M, Beasley MB, Flores R, Gnjatic S, Pe'er D, Rahman A, Amit I, Merad M. Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses. Cell. 2017;169(4):750-765 e717.