

Supplementary Information

Sculpting the tumour microenvironment by combining radiotherapy and ATR inhibition for curative-intent adjuvant immunotherapy

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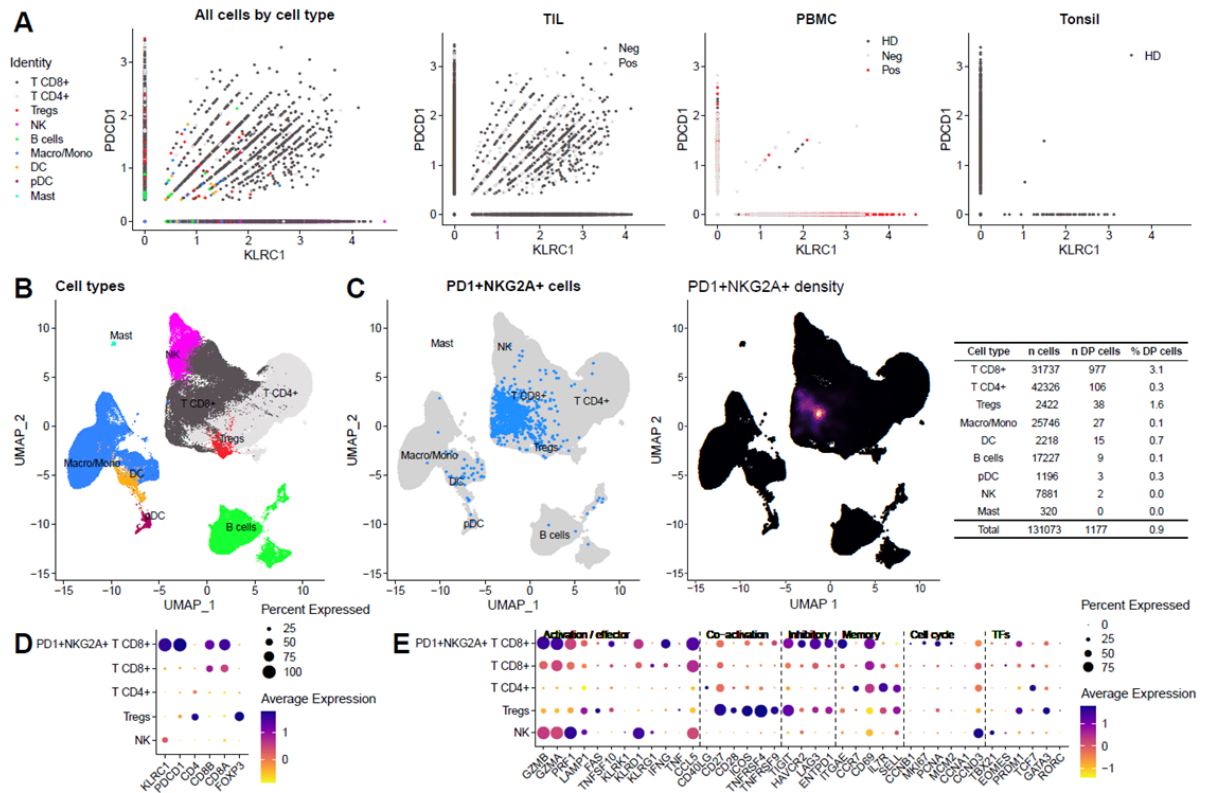


Figure S1. Highly activated NKG2A and PD-1 double-positive T cells are detected in head and neck cancer patients. (A) Scatter plots showing expression of *KLRC1* and *PDCD1* in, left panel; various cell population and other panels; tumours versus blood and tonsil (B) UMAP plot showing distribution of identified cell clusters distribution. (C) Left panel; UMAP plot depicting NKG2A/PD-1 double positive cells in the different identified cell clusters, right panel; UMAP plot depicting intensity expression of NKG2A/PD-1 double positive cells with absolute numbers for each identified cell populations (right table). Dot plots showing average and percentage expression of the *KLRC1*, *PDCD1* and pan-cell markers (D) and activation/effector, co-activation, inhibitory, memory, cell cycle and transcription factors (TFs) markers (E) in NKG2A⁺/PD-1⁺ CD8⁺ T cells versus CD8⁺T, CD4⁺T, Tregs and NK cells. Datasets from Cillo A et al., 2020.

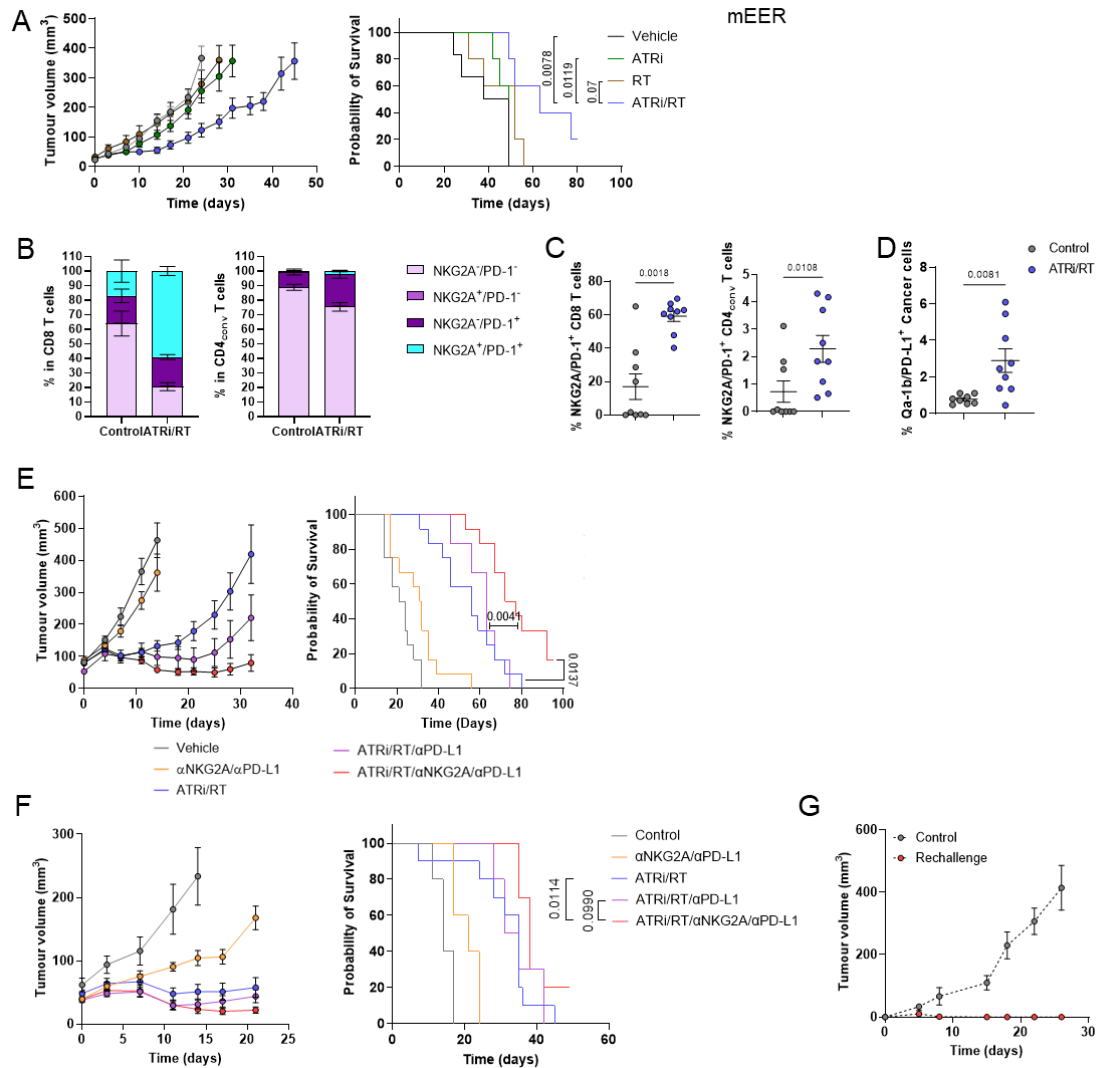


Figure S2. NKG2A and PD-L1 dual immune checkpoint blockade improves the therapeutic outcome of radiation and DDRi. Experiments in this figure were performed in the mEER model. (A) Tumour growth and survival curves across the different conditions (Control n = 6, ATRi n = 5, RT n = 5, ATRi/RT n = 6; from one experiment). (B) % of NKG2A and/or PD-1 positive populations in CD8 and CD4_{conv} T cells (n = 9 for each group; from one experiment). (C) % of NKG2A/PD-1 double positive populations in CD8 and CD4_{conv} T cells (n = 9 for each group; from one experiment). (D) % surface expression of Qa-1b/PD-L1 double positive cancer cells in the different conditions (n = 9 for each group; from one experiment). Tumour growth and survival curves across the different conditions in ectopic (E; Control n = 12, αNKG2A/αPD-L1 n = 12, ATRi/RT n = 12, ATRi/RT/αPD-L1 n = 6, ATRi/RT/αNKG2A/αPD-L1 n = 12; combined from two independent experiments) and orthotopic (F; Control n = 5, αNKG2A/αPD-L1 n = 5, ATRi/RT n = 10, ATRi/RT/αPD-L1 n = 10, ATRi/RT/αNKG2A/αPD-L1 n = 10; from one experiment). (G) Tumour growth in control versus rechallenged mice (Control = 6, Rechallenge n = 3; from one experiment). Results are shown as means ± SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent P values and were determined by Two-tailed Mann-Whitney test (C) and Unpaired t-test (D), and Log-Rank Mantel-Cox test (E and F). Significant outliers were removed using the Grubb's test.

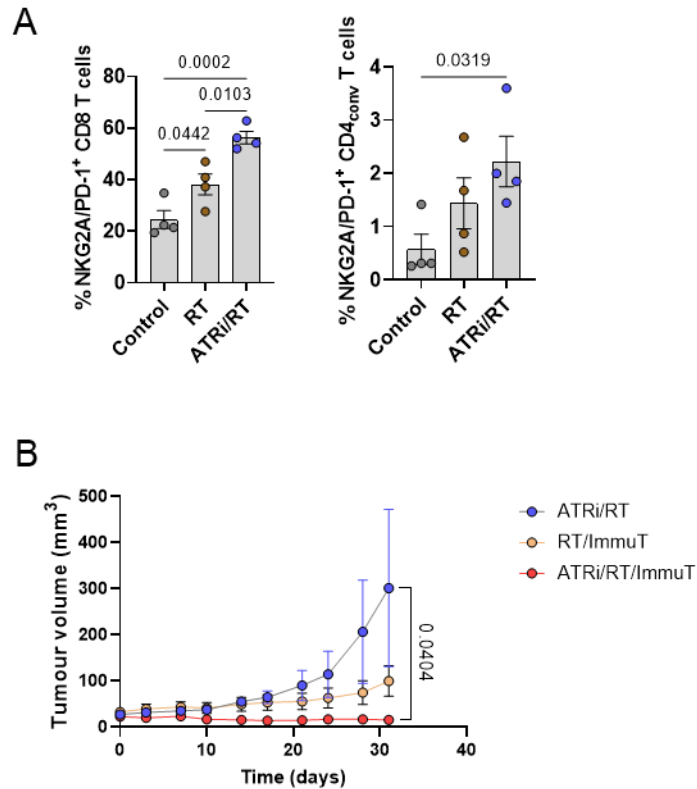


Figure S3. ATRi is critical for full treatment therapeutic efficacy. Experiments in this figure were performed in the MOC1 model. (A) % of NKG2A and/or PD-1 positive populations in CD8 and CD4_{conv} T cells (n = 4 for each group; from one experiment). (B) Tumour growth across the different conditions (ATRi/RT n = 6, RT/ImmuT n = 8, ATRi/RT/ImmuT n = 8). Results are shown as means $\pm$ SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent *P* values and were determined by ordinary one-way ANOVA with Tukey's multiple comparison test (A; CD8 T cells), Kruskal-Wallis test with Dunn's multiple comparison test (A; CD4_{conv} T cells) and Kruskal-Wallis test with Dunn's multiple comparison test from area under curve (B). Significant outliers were removed using the Grubb's test.

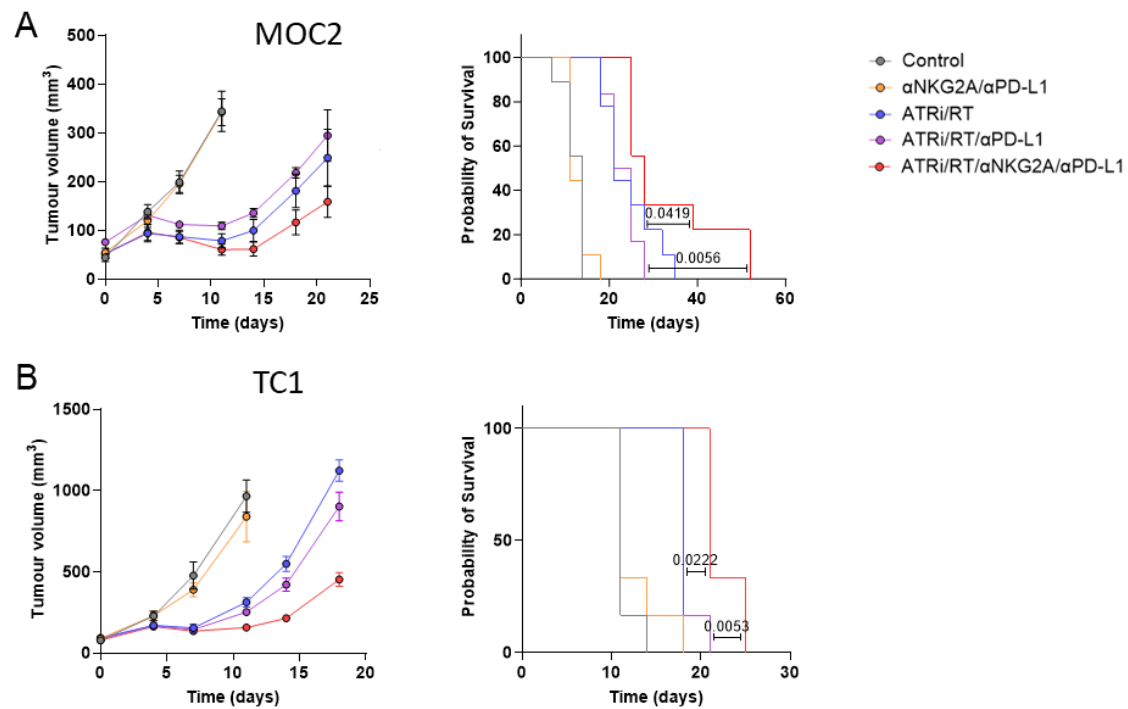


Figure S4. NKG2A and PD-L1 dual immune checkpoint blockade improves the therapeutic outcome of radiation and DDRi in aggressive models of head and neck cancer. Experiments in this figure were performed in the MOC2 and TC1 models. Tumour growth and survival curves across the different conditions in the (A) MOC2 (Control n = 9, αNKG2A/αPD-L1 n = 9, ATRi/RT n = 9, ATRi/RT/αPD-L1 n = 6, ATRi/RT/αNKG2A/αPD-L1 n = 9; combined from two independent experiments) and (B) TC1 (Control n = 6, αNKG2A/αPD-L1 n = 6, ATRi/RT n = 6, ATRi/RT/αPD-L1 n = 6, ATRi/RT/αNKG2A/αPD-L1 n = 6; from one experiment) models. Results are shown as means ± SEM and n represents number of mouse/groups. Numbers on graphs represent *P* values and were determined by Log-Rank Mantel-Cox test (A and B).

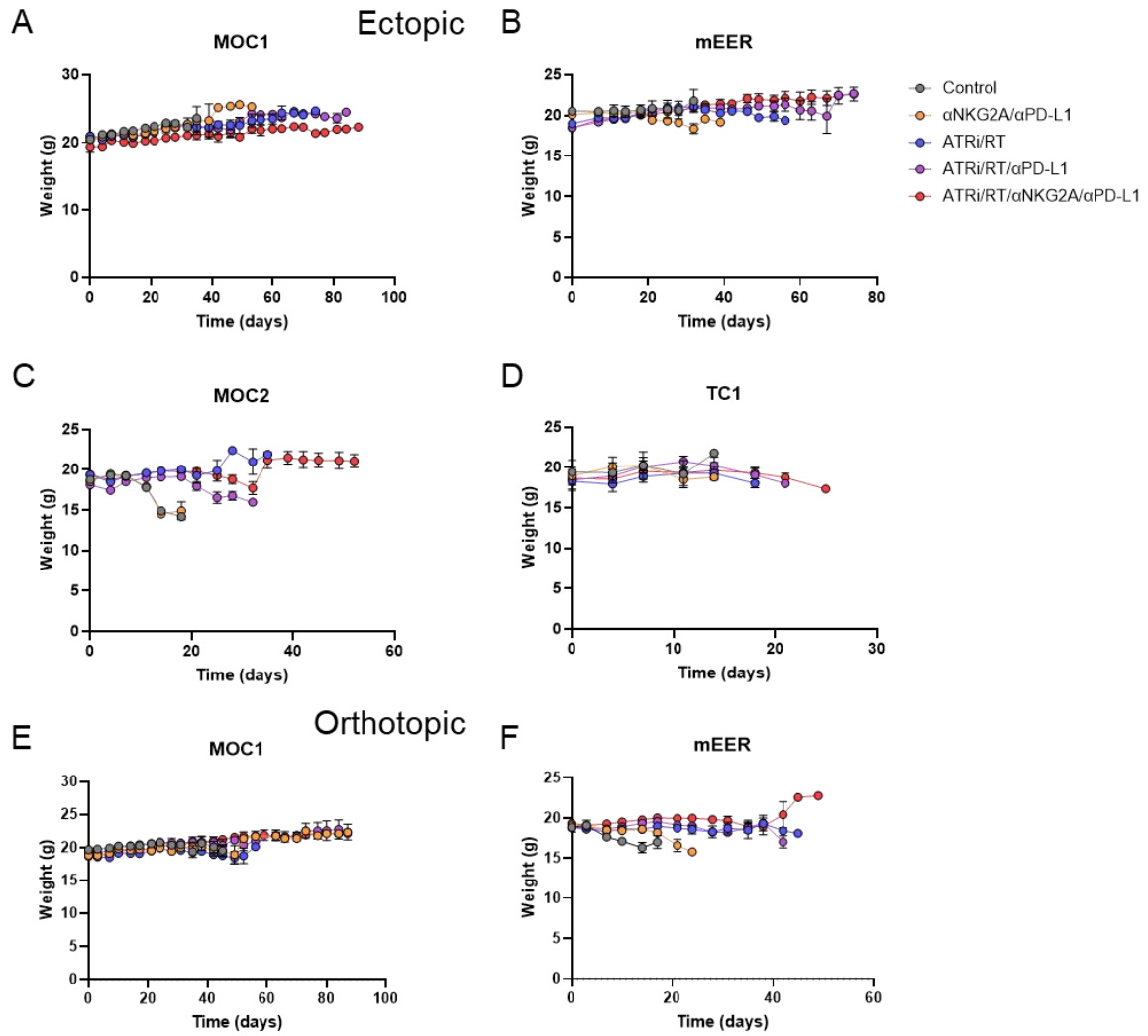


Figure S5. ATRi/RT and dual anti-NKG2A/PD-L1 immunotherapy do not trigger significant toxicity. Body weight measurements over time after the beginning to the treatment in ectopic (A) MOC1, (B) mEER, (C) MOC2, (D) TC1 and orthotopic (E) MOC1 and (F) mEER ($n = 6$ for each group). Results are shown as means $\pm$ SEM and n represents number of mouse/groups.

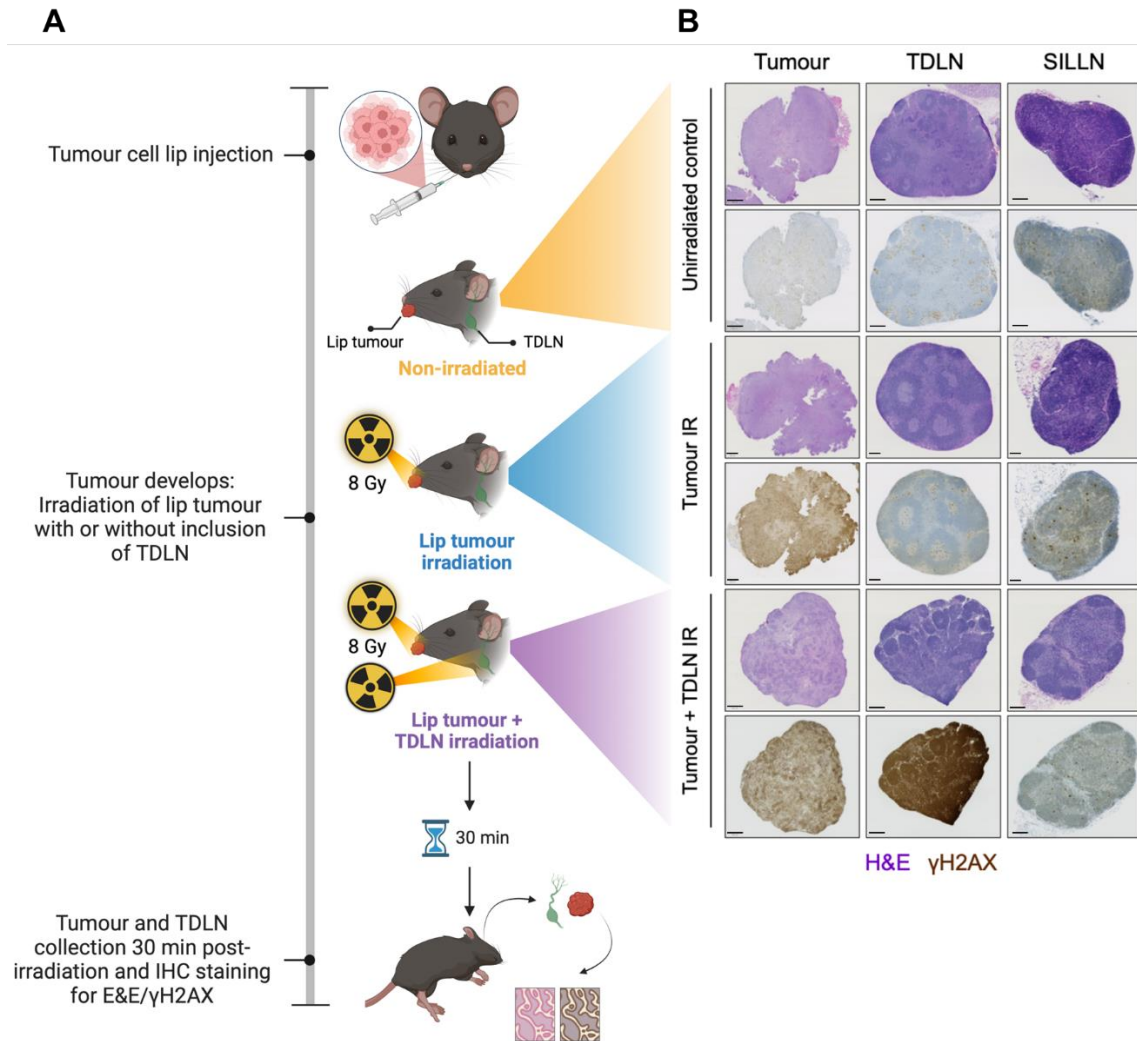
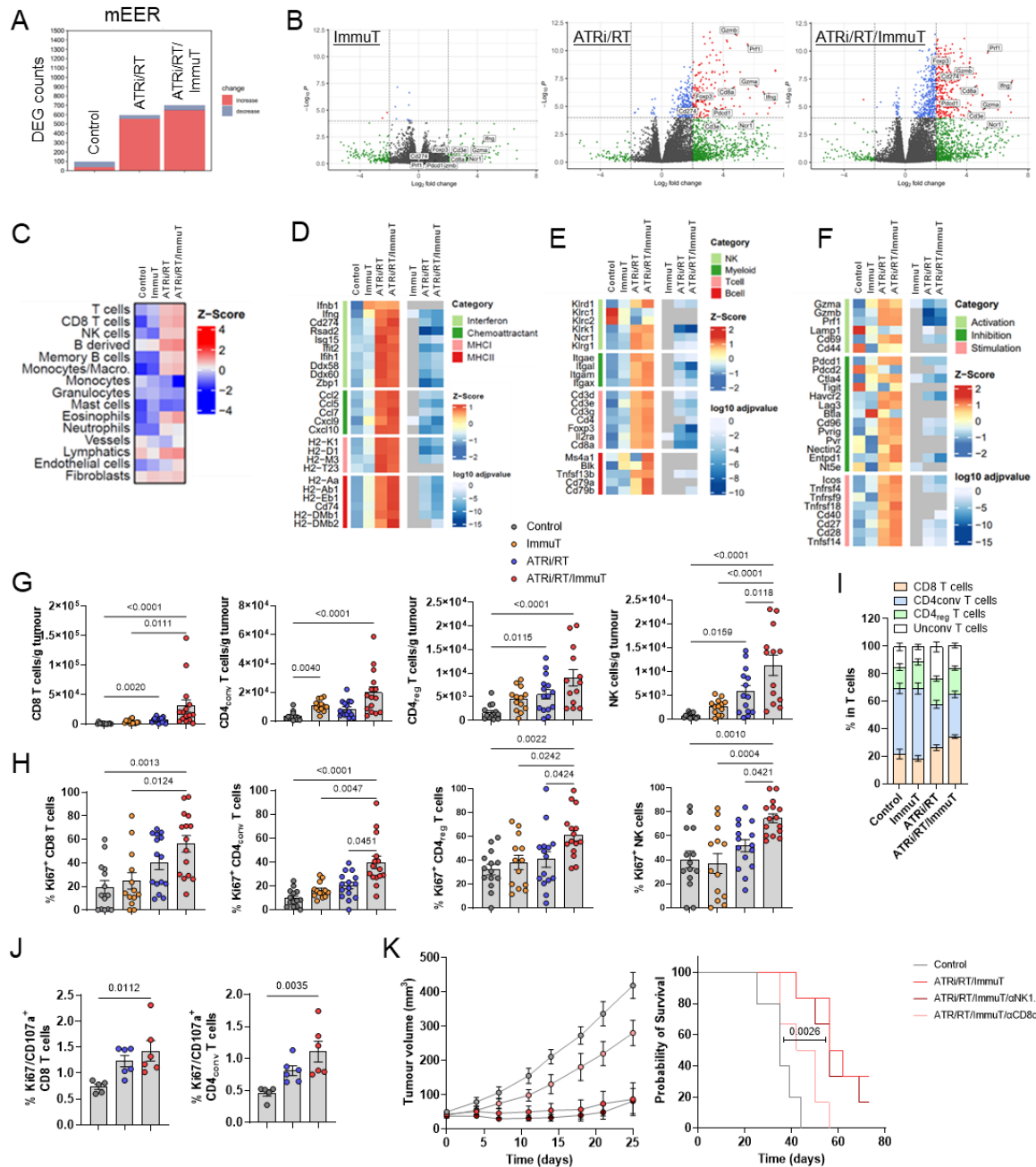


Figure S6. Selective field irradiation of lip tumours excludes cervical tumour-draining lymph node targeting. (A) Study design schematic. (B) Representative (of $n = 3$ mice/group) immunohistochemistry images of lip tumours, tumour-draining lymph nodes (TDLN) and uninvolved sub-iliac lymph nodes (SILLN) stained with H&E and γ -H2AX upon 30 min exposure to 8 Gy X-ray radiation. Black scale bars represent scales at 800 μ m for all unirradiated control images and 500 μ m for all tumour IR images and tumour plus TDLN IR images.



Non-significant adjusted p -values > 0.05 are indicated as grey. (G) Absolute number/gram of tumour of the indicated lymphocytes in the various conditions (Control $n = 14$, ImmuT $n = 13$, ATRi/RT $n = 15$, ATRi/RT/ImmuT $n = 15$; combined from two independent experiments). (H) % Ki67 positive cells in the indicated lymphocytes in the different conditions (Control $n = 14$, ImmuT $n = 13$, ATRi/RT $n = 15$, ATRi/RT/ImmuT $n = 15$; combined from two independent experiments). (I) % of indicated lymphocytes in the total T cell population across all treatment conditions (Control $n = 14$, ImmuT $n = 13$, ATRi/RT $n = 15$, ATRi/RT/ImmuT $n = 15$; combined from two independent experiments). (J) % Ki67/CD107a double positive CD8 and CD4_{conv} T cells in splenocytes from mice treated with the indicated conditions restimulated with an HPV peptide (Control $n = 5$, ATRi/RT $n = 6$, ATRi/RT/ImmuT $n = 6$; from one experiment). (K) Tumour growth and survival curves across the different conditions (Control $n = 5$, ATRi/RT/ImmuT $n = 6$, ATRi/RT/ImmuT/aNK1.1 $n = 6$, ATRi/RT/ImmuT/aCD8 $n = 6$; from one experiment). Results are shown as means $\pm$ SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent P values and were determined by Kruskal-Wallis test with Dunn's multiple comparison test (G; CD8 T cells, CD4_{conv} T cells and CD4_{reg} T cells; H; CD8 T cells and CD4_{conv} T cells; J; CD4_{conv} T cells), ordinary one-way ANOVA with Tukey's multiple comparison test (G; NK cells; H; CD4_{reg} T cells and NK cells; J; CD8 T cells) and Log-Rank Mantel-Cox test (K). Significant outliers were removed using the Grubb's test.

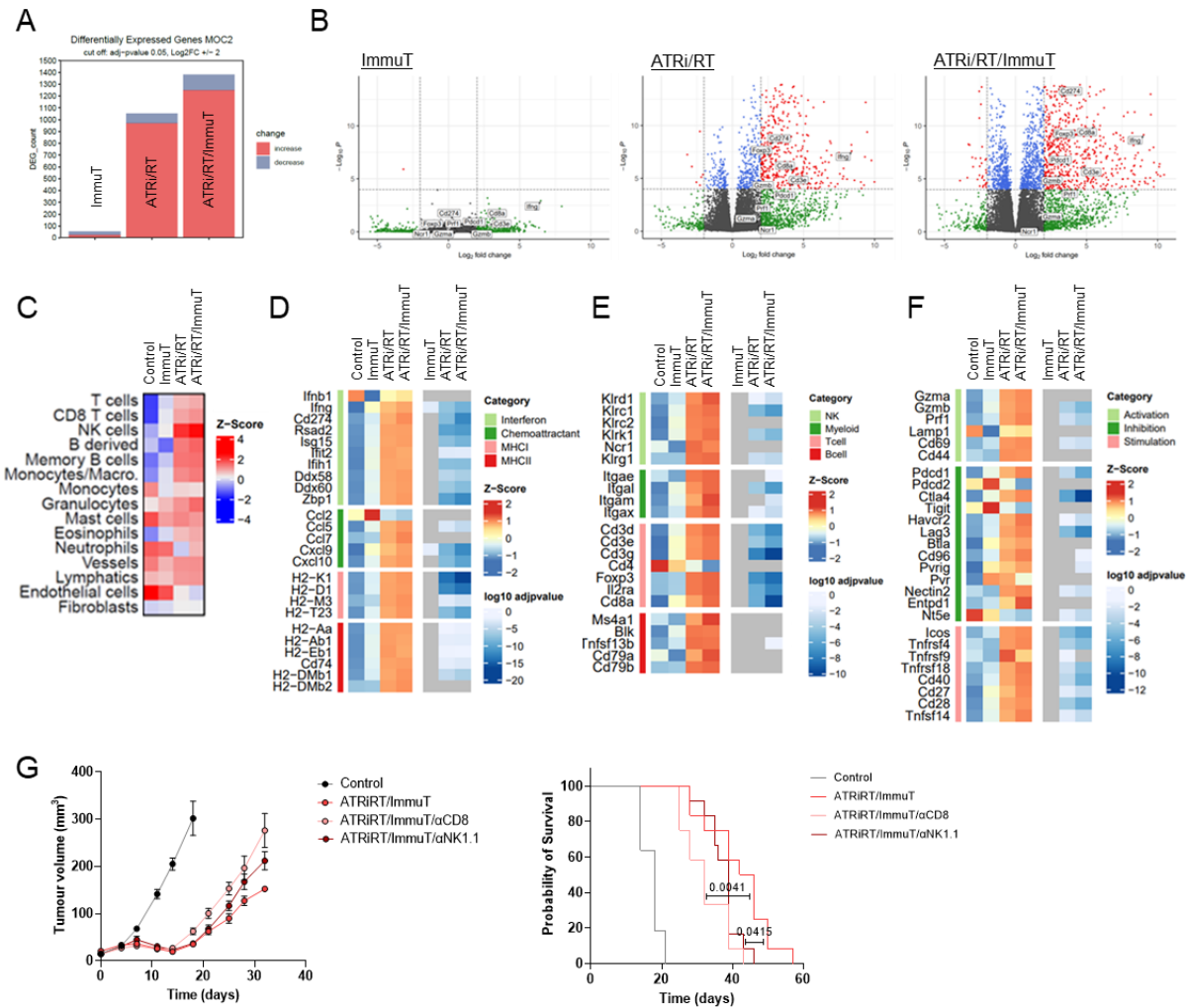


Figure S8. ATRi/RT and dual anti-NKG2A/PD-L1 immunotherapy trigger a potent CD8 T-cell antitumour immune response in an aggressive HPV-negative head and neck cancer model. Experiments presented in this figure were performed in the MOC2 model. RNAseq experiments were performed with $n = 4$ for each group (A) Number of differentially expressed genes (DEGs) across the different conditions calculated by DESeq2 using Wald test. (B) Volcano plots showing expression of the different genes in the various conditions (genes characteristic of an immune response are named). (C) Immune cell population estimates. Immune cell scoring was performed on normalised RNAseq counts using the mMCP-counter package. Heatmaps corresponding to interferon and cytokine signalling, chemoattractant, MHC I and MHC II (D); immune cell populations (E); and immune cell activation status (F). Data shown are z-scores of log2 transformed normalised counts for the treatment conditions shown. This plotted alongside log10 adjusted p -value for each gene calculated from DEG analysis using Wald test. Non-significant adjusted p -values > 0.05 are indicated as grey. (G) Tumour growth and survival curves across the different conditions (Control $n = 11$, ATRi/RT/ImmuT $n = 12$, ATRi/RT/ImmuT/aNK1.1 $n = 12$, ATRi/RT/ImmuT/aCD8 $n = 12$; combined from two independent experiment). Results are shown as means $\pm$ SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent P values and were determined by Log-Rank Mantel-Cox test (G).

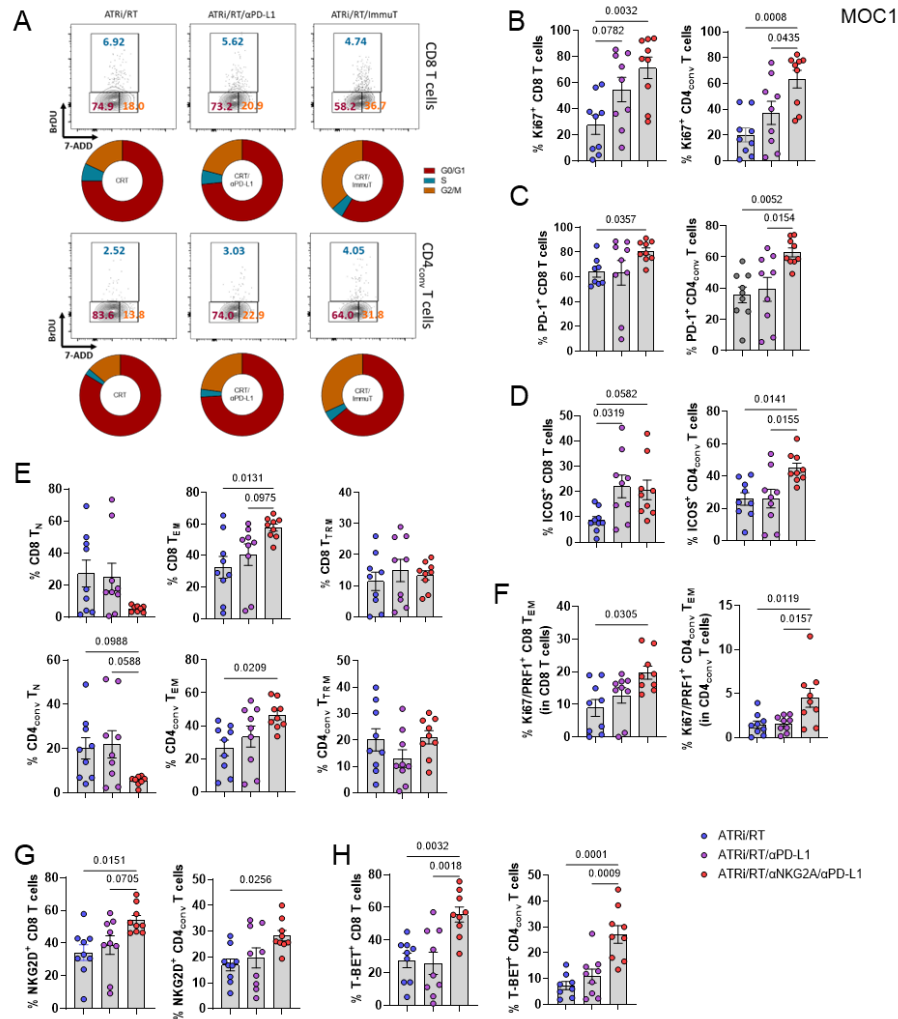


Figure S9. ATRi/RT and dual anti-NKG2A/PD-L1 immunotherapy induce the proliferation of PD-1⁺ effector memory cytotoxic CD8 and CD4 T-cells in the tumour microenvironment. Experiments presented in this figure were performed in the mEER model. (A) Dot plot and donut chart representing % of CD8 and CD4_{conv} T cells in the different cell cycle phases across all conditions (concatenated from ATRi/RT n = 6, ATRi/RT/αPD-L1 n = 5, ATRi/RT/ImmuT n = 6; from one experiment)). Across all conditions the following graphs show % (B) Ki67⁺, (C) PD-1⁺, (D) ICOS⁺, (E) naïve (N), effector (EM) and tissue-resident (TRM) memory, (F) Ki67/PRF1⁺ EM, (G) NKG2D⁺ and (H) T-BET⁺ CD8 and CD4_{conv} T cells (n = 9 for each group; from one experiment). Results are shown as means ± SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent P values and were determined by Kruskal-Wallis test with Dunn's multiple comparison test (C; CD8 T cells; E; CD8 T cells and F; CD8 T cells) or ordinary one-way ANOVA with Tukey's multiple comparison test (B; C; CD4_{conv} T cells; D; E; CD4_{conv} T cells; F; CD4_{conv} T cells; G and H). Significant outliers were removed using the Grubb's test.

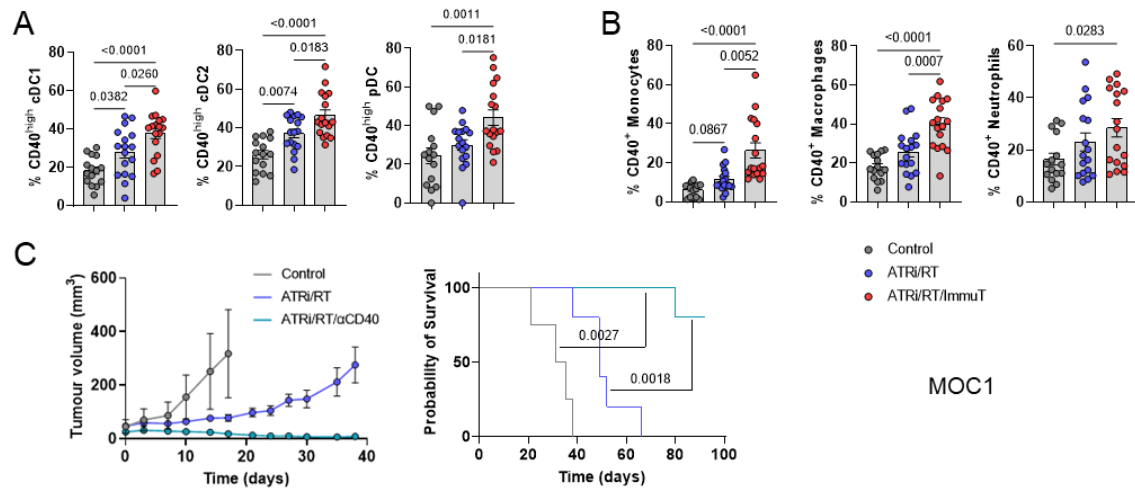


Figure S10. CD40 stimulation promotes potent antitumour response following ATRi/RT. Experiments presented in this figure were performed in the MOC1 model. (A) % of CD40^{high} cDC1, cDC2 and pDCs and (B) CD40⁺ monocytes, macrophages and neutrophils across all treatment groups (Control n = 15, ATRi/RT n = 17, ATRi/RT n = 17; combined from two independent experiments). (C) Tumour growth and survival curves across the different conditions (Control n = 4, ATRi/RT n = 5, ATRi/RT/αCD40 n = 5; from one experiment). Results are shown as means ± SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent *P* values and were determined by Kruskal-Wallis test with Dunn's multiple comparison test (B; Monocytes and Neutrophils), ordinary one-way ANOVA with Tukey's multiple comparison test (A and B; Macrophages) and Log-Rank Mantel-Cox test (G).

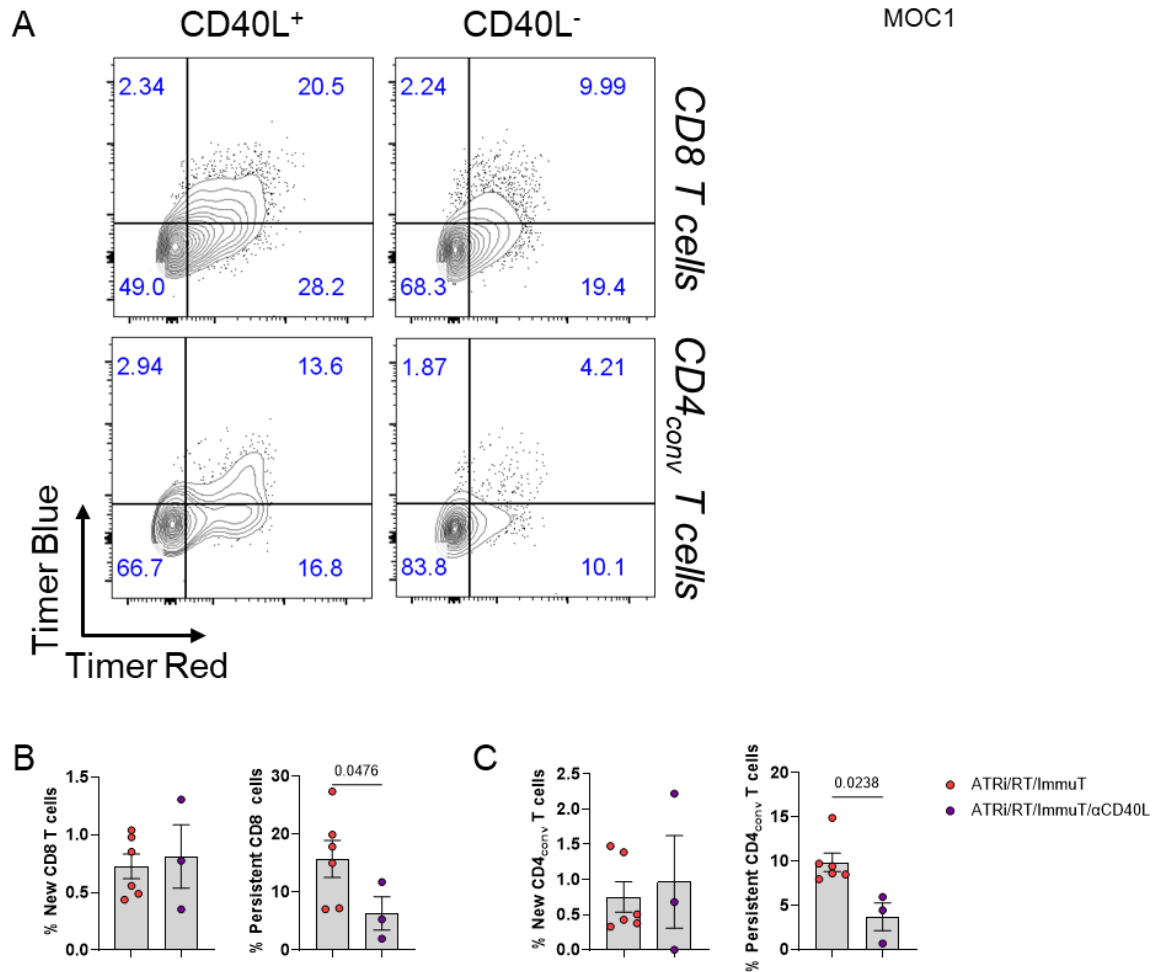


Figure S11. CD40/CD40L axis mediates the persistence of TCR signalling in both CD8 and CD4_{conv} T cells following ATRi/RT and anti-NKG2A/PD-L1 immunotherapy. Experiments presented in this figure were performed in the MOC1 model. (A) Dot plots showing the % of the different Timer population in CD40L⁺ versus CD40L⁻ CD8 and CD4_{conv} T cells (concatenated from n = 6 from the ATRi/RT/ImmuT group). % of the indicated Timer population in CD8 (B) and CD4_{conv} (C) T cells in both conditions (ATRi/RT/ImmuT n = 6; ATRi/RT/ImmuT/αCD40L n = 3; from one experiment). Results are shown as means ± SEM and n represents number of mouse/groups. Numbers on graphs represent P values and were determined by Two-tailed Mann Whitney test.

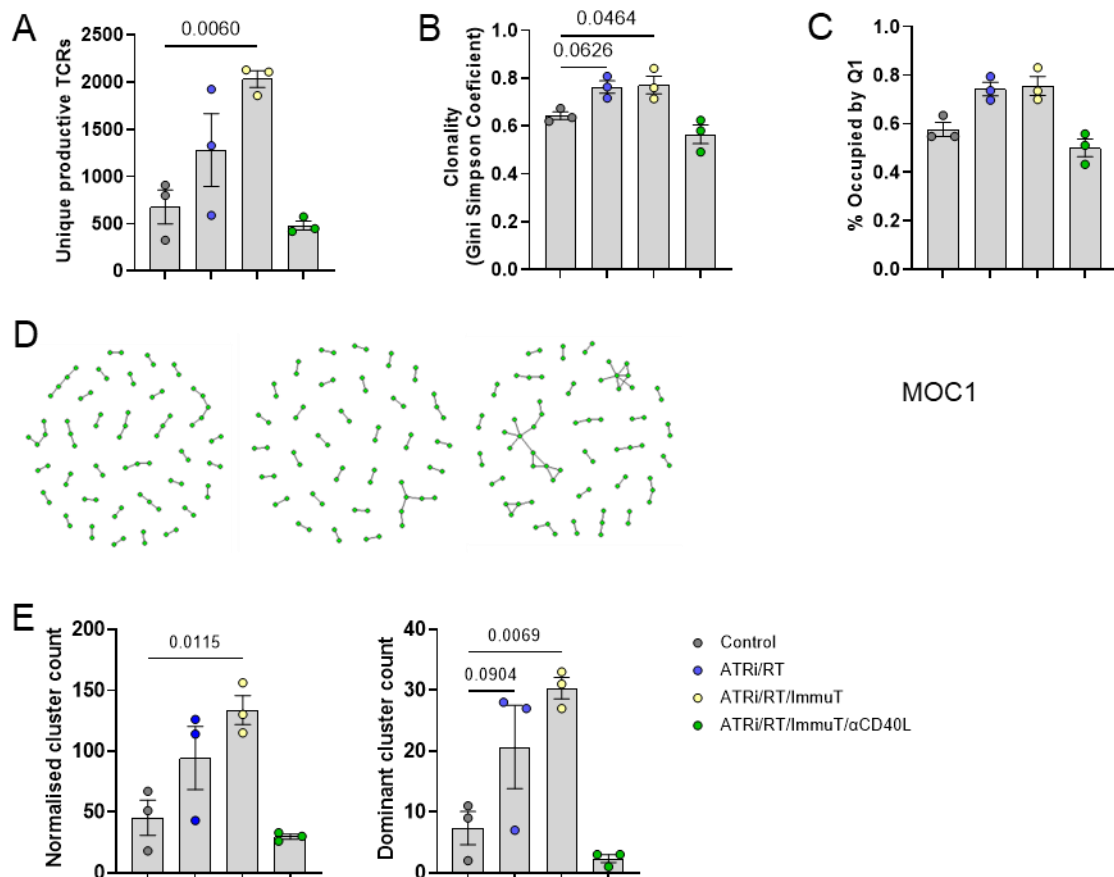


Figure S12. CD40/CD40L axis mediates the variations in tumour TCR repertoire following ATR/RT and anti-NKG2A/PD-L1 immunotherapy. One single experiment was performed with $n = 3$ for each group in the MOC1 model. (A) Absolute number of unique productive clonotypes across the different conditions. (B) Clonality (Gini Simpson coefficient) across different conditions. (C) Comparison of the proportion (in percentage) of the TCR repertoire occupied by the first quintile of clonotypes. (D) Network diagrams of CDR3B amino acid triplet clusters for ATRi/RT/ImmuT/αCD40L condition. Clusters containing expanded CDR3s are shown. (E) Comparison of normalized cluster count and dominant cluster count for each condition. Results are shown as means $\pm$ SEM and n represents number of mouse/groups. Parametric statistics were only applied to normally distributed data. Numbers on graphs represent *P* values and were determined by ordinary one-way ANOVA with Tukey's multiple comparison test (A, B, C and E).

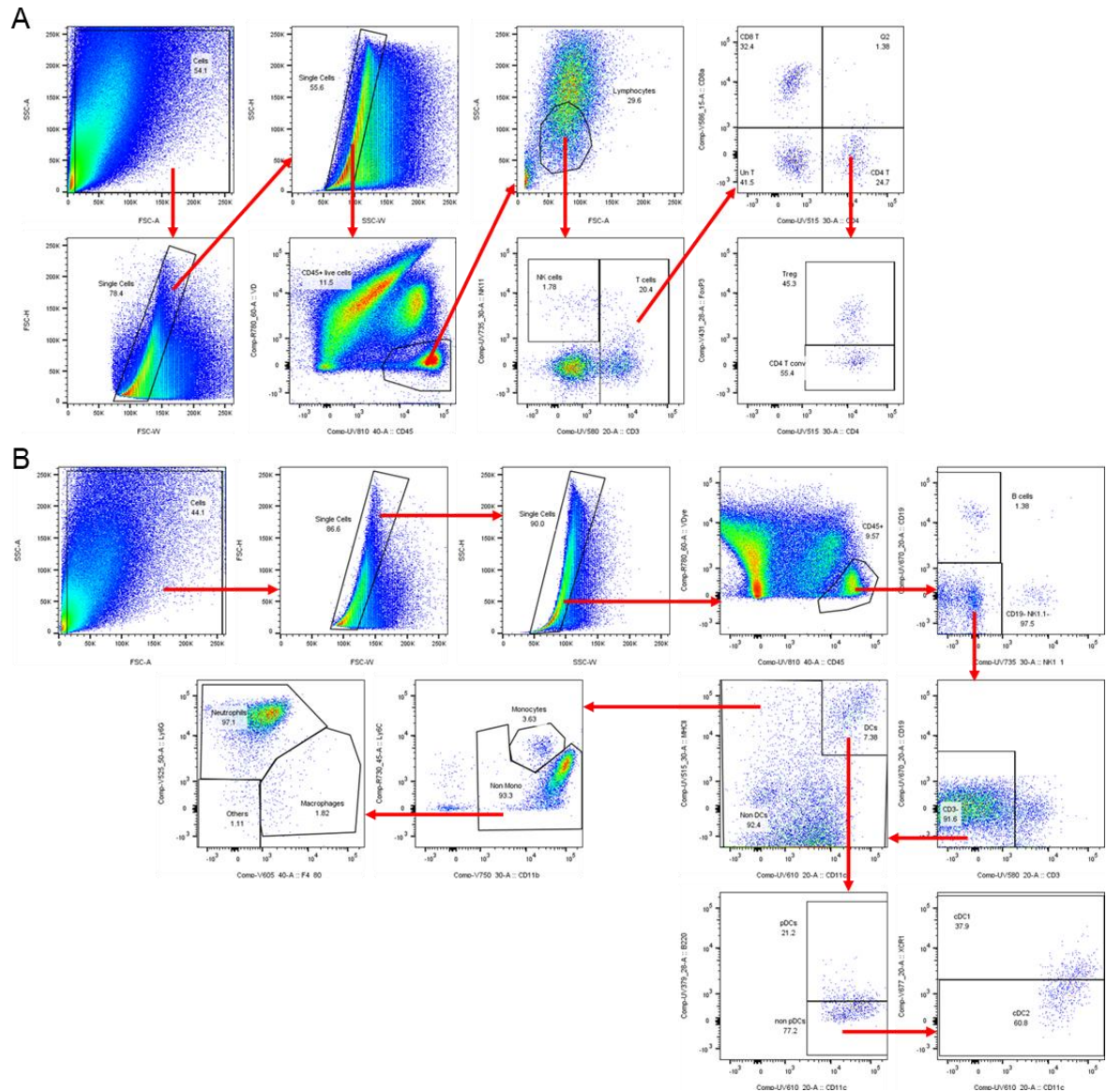


Figure S13. Gating strategy. The figure shows an example of gating strategy in MOC1 tumours for lymphocytes (A) and myeloid cells (B). The same strategy was used for all the different tumour models and tissues.

A

cluster_man	n cells			n DP cells			% DP cells		
	HPV-	HPV+	Total	HPV-	HPV+	Total	HPV-	HPV+	Total
T CD8+	15,840	9,892	25,732	496	335	831	3.1%	3.4%	3.2%
T CD4+	12,313	12,554	24,867	31	27	58	0.3%	0.2%	0.2%
Macro/Mono	14,829	6,827	21,656	27	7	34	0.2%	0.1%	0.2%
Tregs	4,322	2,386	6,708	13	17	30	0.3%	0.7%	0.4%
B cells	2,149	7,058	9,207	2	2	4	0.1%	0.0%	0.0%
NK	3,342	2,323	5,665	2	1	3	0.1%	0.0%	0.1%
Epithelial	3,983	9,970	13,953	1		1	0.0%	0.0%	0.0%
pDC	621	394	1,015	1		1	0.2%	0.0%	0.1%
Endothelial	6,327	798	7,125				0%	0%	0%
T cells	1	28	29				0%	0%	0%
Mast	214	76	290				0%	0%	0%
Pericytes	674	26	700				0%	0%	0%
Fibroblasts	3,377	495	3,872				0%	0%	0%
Grand Total	67,992	52,827	120,819	573	389	962	0.8%	0.7%	0.8%

B

cluster_man	n cells			n DP cells			% DP cells		
	HPV-	HPV+	Total	HPV-	HPV+	Total	HPV-	HPV+	Total
T CD8+	22,269	9,468	31,737	644	333	977	2.9%	3.5%	3.1%
T CD4+	30,458	11,868	42,326	79	27	106	0.3%	0.2%	0.3%
Tregs	1,735	687	2,422	16	22	38	0.9%	3.2%	1.6%
Macro/Mono	20,988	4,758	25,746	24	3	27	0.1%	0.1%	0.1%
DC	1,717	501	2,218	12	3	15	0.7%	0.6%	0.7%
B cells	11,537	5,690	17,227	7	2	9	0.1%	0.0%	0.1%
pDC	859	337	1,196	3	0	3	0.3%	0.0%	0.3%
NK	5,445	2,436	7,881	1	1	2	0.0%	0.0%	0.0%
Mast	262	58	320	0	0	0	0%	0%	0%
Grand Total	95,270	35,803	131,073	786	391	1,177	0.8%	1.1%	0.9%

Table S1. Proportions of NKG2A and PD-1 double-positive cells in head and neck cancer patients according to HPV status. Absolute numbers of NKG2A/PD-1 double positive cells for each identified cell populations. Datasets from Kurten CHL et al., 2021 (A) and Cillo A et al., 2020 (B).

Epitope	Clone	Fluorophore	Manufacturer	Reference number	Lot number	Dilution
Anti-mouse B220	RA3-6B2	BUV395	BD	563793	121784	1/200
Anti-mouse CD107a	1D4B	BUV395	BD	565533	126192	1/200
		PE	Biolegend	121612	296413	1/200
Anti-mouse CD44	IM7	BUV395	BD	740215	3142888	1/100
		Pacific Blue	Biolegend	103019	237528	1/100
Anti-mouse CD25	PC61	BUV395	BD	564022	1277848	1/100
		BV650	Biolegend	102038	324012	1/100
		APC	Biolegend	102012	276009	1/100
Anti-mouse CD4	RM4-4	BUV496	BD	741051	3269088	1/100
Anti-mouse MHCII	RM4-5	PerCp	Biolegend	100538	368727	1/100
	M5/114.15.2	BUV496	BD	750281	4080119	1/200
		PE-Dazzle 594	Biolegend	107648	340469	1/300
		AF700	Biolegend	107622	264454	1/300
Anti-mouse CD3	17A2	BUV563	BD	741319	4012564	1/100
		BUV737	BD	612803	2129948	1/100
		BV650	Biolegend	100229	261371	1/100
		AF700	Biolegend	100216	263796	1/100
		PerCp Cy5.5	Biolegend	100218	279058	1/100
Anti-mouse TCRδ	GL3	BUV615	BD	751183	1327787	1/100
Anti-mouse CD11c	N418	BUV615	BD	751222	1029930	1/100
		BV750	Biolegend	117357	344140	1/100
		PerCp Cy5.5	Biolegend	117328	256419	1/100
Anti-mouse PD-1	29F.1A12	Pe-Cy7	Biolegend	135216	227806	1/200
		BV605	Biolegend	135220	355299	1/200
Anti-mouse CD103	2E07.	BUV615	BD	751631	3146223	1/100
		BV711	Biolegend	121435	286529	1/100
		BV785	Biolegend	121439	353449	1/100
		BV510	Biolegend	121423	299061	1/100
Anti-mouse CD69	H1.2F3	BUV661	BD	741478	2161836	1/100
		BV785	Biolegend	104543	309634	1/100
		PE-Dazzle 594	Biolegend	104536	252250	1/100
Anti-mouse CD19	1D3	BUV661	BD	612971	1284531	1/200
	6D5	APC	Biolegend	115512	252571	1/200
Anti-mouse NK1.1	PK136	BUV737	BD	741715	3104716	1/100
		BV421	Biolegend	108741	267090	1/100
Anti-mouse TCRβ	H57-597	BUV737	BD	612821	1315739	1/100
Anti-mouse CD45	30-F11	BUV805	BD	748370	2161908	1/300
Anti-mouse F4/80	BM8	Pacific Blue	Biolegend	123124	217178	1/100
Anti-mouse FoxP3	FJK-16s	eFluor 450	Thermofisher Scientific	48-5773-82	2401080	1/100
Anti-mouse NKG2A	20D5	BV421	BD	740065	315172	1/100
	16A11	PE	Biolegend	142804	236468	1/100
		Pe-Cy7	Biolegend	142810	293737	1/100
Anti-mouse PD-L1	MIH5	BV421	BD	564716	0023985	1/100
	10F.9G2	PerCp Cy5.5	Biolegend	124334	286738	1/100
Anti-mouse Ly6G	1A8	BV510	Biolegend	127633	266675	1/300
Anti-mouse CD62L	MEL-14	BV510	Biolegend	104441	403539	1/100
		Pe-Cy5	Biolegend	104410	316174	1/300
		PE-Dazzle 594	Biolegend	104447	252447	1/300
		PerCp Cy5.5	Biolegend	104432	289782	1/100
		AF700	Biolegend	104426	333915	1/100
Anti-mouse CD8α	53.-6.7	BV570	Biolegend	100740	361198	1/200
		BV711	Biolegend	100759	357159	1/200
		BV785	Biolegend	100750	369774	1/200
Anti-mouse CD27	LG.3410	BV650	Biolegend	124233	281805	1/100
		PE-Dazzle 594	Biolegend	124228	323766	1/100
		PerCp Cy5.5	Biolegend	124214	239197	1/100
	LG-7F9	AF700	Thermofisher Scientific	56-0271-82	2152316	1/100
Anti-mouse TIM-3	RMT3-23	BV711	Biolegend	119727	362528	1/100
		APC	Biolegend	119705	265068	1/100
Anti-mouse ICOS	C398.4A	BV711	Biolegend	313548	355933	1/100
		BV750	Biolegend	313558	400930	1/100
Anti-mouse CD11b	M1/70	BV750	Biolegend	101267	317458	1/200
Anti-mouse Ly6C	HK1.4	APC	Biolegend	128016	234315	1/200
		AF700	Biolegend	128024	388651	1/200
		BV785	Biolegend	128041	276551	1/200
Anti-mouse Qa-1b	6F10.1A6	PE	BD	566641	1273601	1/100
Anti-mouse Ki67	B56	BV786	BD	563756	3145783	1/100
	16A8	AF488	Biolegend	652418	297510	1/100
		PE	Biolegend	652404	287649	1/100
		PE-Dazzle 594	Biolegend	652428	335444	1/100
Anti-mouse NKG2D	CX5	PE	Biolegend	130208	221128	1/100
		PE-Dazzle 594	Biolegend	130214	336666	1/100
Anti-mouse CD28	37.51	PE	Biolegend	102106	324383	1/100
		PE-Dazzle 594	Biolegend	102124	328810	1/100
		PerCp Cy5.5	Biolegend	102114	313345	1/100
Anti-mouse Perforin	S16009B	PE	Biolegend	154405	335441	1/100
Anti-mouse Grzmb	GB11	AF647	Biolegend	515406	341642	1/100
Anti-mouse CD40L	SA047C3	APC	Biolegend	157010	337879	1/100
Anti-mouse CD40	3/23	Pe-Cy7	Biolegend	124622	376123	1/100
		PE-Dazzle 594	Biolegend	124630	306497	1/100
Anti-mouse T-bet	4B10	Pe-Cy7	Thermofisher Scientific	25-5825-82	2410093	1/100
Anti-mouse EpCAM	G8.8	FITC	Biolegend	118208	337007	1/100

Table S2. List of flow cytometry antibodies used in the study.