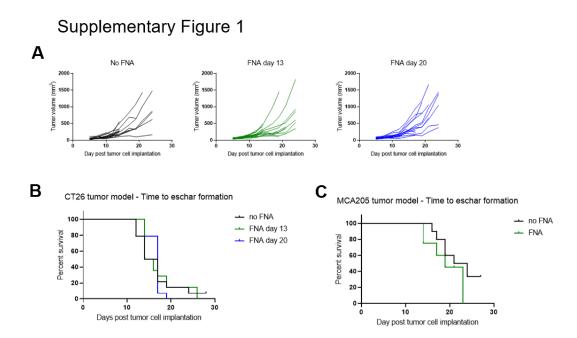
Title:

A novel non-terminal tumor sampling procedure using fine needle aspiration supports immuneoncology biomarker discovery in preclinical mouse models

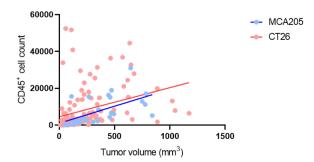
Supplementary figures and figure legends:



Supplementary Figure 1: Fine needle aspiration does not impact tumor growth or eschar formation

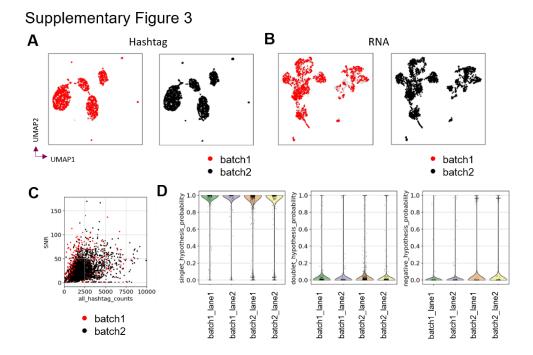
(A and B) Mice were subcutaneously implanted with CT26 tumors on the flank and the tumors were biopsied using FNA either 13 or 20 days later. (A) Tumor growth of individual mice over time. (B) Time to eschar formation was compared between groups using a log-rank test. No significant differences were seen between FNA-biopsied and control groups. (C) Mice were subcutaneously implanted with MCA205 tumors on the flank and the tumors were biopsied using FNA 18 days later. Time to eschar formation was compared between groups using a log-rank test. No significant differences were seen between FNA-biopsied and control groups.

Supplementary Figure 2



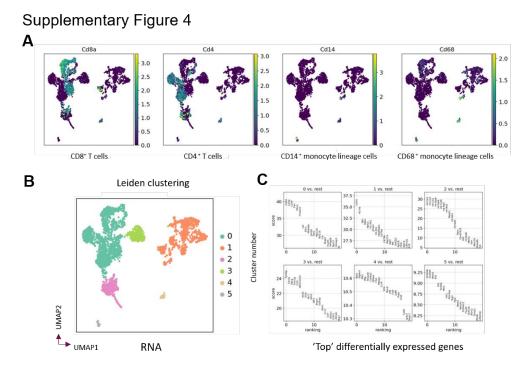
Supplementary Figure 2: Fine needle aspiration can be used to extract cells for flow cytometry at a range of tumor volumes

FNA biopsies were collected from mice bearing either CT26 or MCA205 tumors and analysed by flow cytometry to determine the total number of CD45⁺ tumor-infiltrating immune cells within the sample. This was plotted against the volume of the tumor on the day of FNA biopsying for each mouse and linear regression was used to calculate the correlation between the CD45⁺ cell count and tumor volume for both tumor models, indicated by the solid lines. 27-124 mice per group.



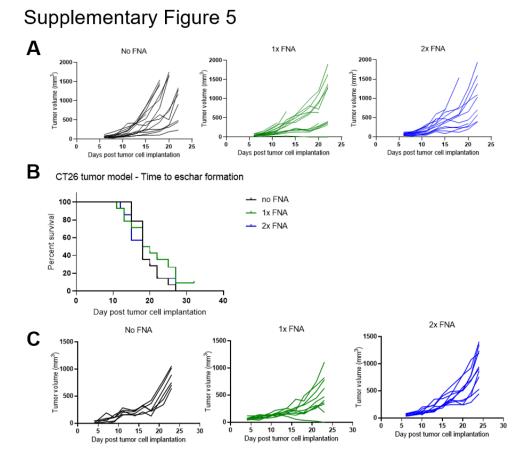
Supplementary Figure 3: Optimising single cell RNA sequencing parameters for use with fine needle aspiration samples

(A) Hashtag-embedded UMAP colored by batch. (B) RNA-embedded UMAPs colored by batch. (C) Number of hashtag counts per cell versus Signal-Noise Ratio (SNR) per cell. SNR was calculated for each cell as the most frequent hashtag count divided by all other hashtag counts. (D) Comparison of hashsolo probabilities of singlet, doublet/multiplet or negative assignment across the different batches and lanes.



Supplementary Figure 4: Fine needle aspiration enables single cell RNA sequencing

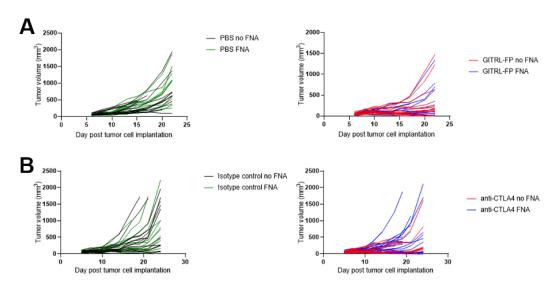
Expression of putative marker genes of immune cell types. (B) RNA UMAP colored by clustering annotation. (C) Top ranked (based on Z-score) differentially expressed genes between each cluster versus all other clusters.



Supplementary Figure 5: Repeated fine needle aspiration does not impact tumor growth or eschar formation

(A and B) Mice were subcutaneously implanted with CT26 tumors on the flank and the tumors were biopsied using FNA either 12 days later (1x FNA group) or 12 and 14 days later (2x FNA group). (A) Tumor growth of individual mice over time. (B) Time to eschar formation was compared between groups using a log-rank test. No significant differences were seen between FNA-biopsied and control groups. (C) Mice were subcutaneously implanted with A20 tumors on the flank and the tumors were sampled using FNA either 15 days later (1x FNA group) or 13 and 15 days later (2x FNA group). Tumor growth of individual mice over time.

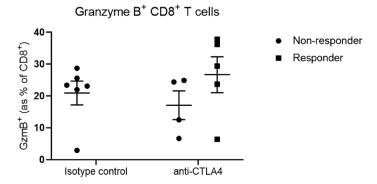
Supplementary Figure 6



Supplementary Figure 6: Fine needle aspiration does not affect response to immuno-oncology treatments

(A) CT26 tumor-bearing mice were treated with 0.2mg/kg GITRL-FP on day 6 and half of the mice were biopsied using FNA on day 14. Tumor growth of individual mice over time. (B) CT26 tumor-bearing mice were treated with 10mg/kg anti-CTLA-4 antibody or isotype control twice weekly from day 7 for 6 doses and half of the mice were biopsied using FNA on day 14. Tumor growth of individual mice over time.

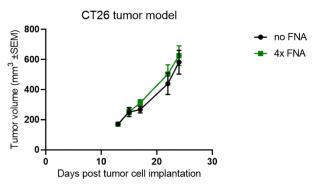
Supplementary Figure 7



Supplementary Figure 7: Fine needle aspiration enables intracellular cytokine staining

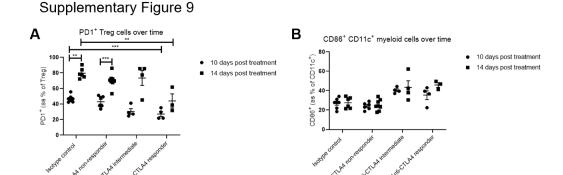
CT26 tumor-bearing mice were treated with 10mg/kg anti-CTLA-4 antibody or isotype control twice weekly from day 7 for 6 doses and the tumors were biopsied using FNA on day 17. Flow cytometric analysis of the FNA biopsies was used to quantify the frequency of Granzyme B-expressing CD8⁺ T cells after subdividing the treated group into responder and non-responder groups *post hoc* based on the outcome of the tumor growth inhibition study.





Supplementary Figure 8: Fine needle aspiration can be carried out four times without impacting tumor growth

Mice were subcutaneously implanted with CT26 tumors on the flank and the tumors were biopsied using FNA 12, 15, 18 and 21 days later (4x FNA group). Tumor growth over time was compared between groups using a mixed effects model. No significant differences were seen between FNA-biopsied and control groups. Error bars indicate the mean ± SEM. 10 mice per group.



Supplementary Figure 9: Repeated fine needle aspiration enables comparison of the TME at several timepoints after treatment

CT26 tumor-bearing mice were treated with 10mg/kg anti-CTLA-4 antibody or isotype control twice weekly from day 7 for 6 doses and the tumors were biopsied using FNA on day 17 (10 days post treatment initiation) and day 21 (14 days post treatment initiation). Flow cytometric analysis of the FNA biopsies was used to compare (**A**) the frequency of PD1-expressing Treg cells and (**B**) the frequency of CD86-expressing CD11c⁺ myeloid cells after subdividing the treated group into responder, intermediate and non-responder groups *post hoc* based on the outcome of the tumor growth inhibition study. The 2 timepoints were compared using Mann-Whitney tests with multiple comparison correction. For each timepoint, the treatment groups were compared using a one-way ANOVA with Tukey's multiple comparison testing. Error bars indicate the mean \pm SEM. ** P < 0.01, *** P < 0.001.

Median UMI Counts per Cell	5,296	5,269
Total Genes Detected	16,742	17,467
Fraction Reads in Cells	86.20% 16,742 5,296	88.10% 17,467
Reads Mapped Antisense to Gene	1.20%	1.10%
Reads Mapped Reads Mapped Confidently Confidently to to Exonic Transcriptome Regions	%08'95	%00'85
Reads Mapped Confidently to Exonic Regions	29.50%	%07.09
Reads Mapped Confidently to Intronic Regions	21.40%	21.80%
Reads Mapped Confidently to Intergenic Regions	4.00%	3.80%
Reads Reads Mapped Mapped to Confidently Genome to Genome	84.90%	86.40%
Reads Mapped N to Co Genome to	91.10%	92.20%
Q30 Bases in UMI	94.90%	94.90%
Q30 Bases Q30 Bases Q30 N in RNA in Sample Bases in Read Index UMI	95.70%	96.10%
Q30 Bases Q30 Bases in RNA in Sample Read Index	93.50% 95.70% 94.90% 91.10% 84.90%	93.60% 96.10% 94.90% 92.20%
Q30 Bases in Barcode	95.70%	95.70%
Valid Sequencing Q30 Bases Barcodes Saturation in Barcode	94.10%	79.40%
Valid		97.70%
Number of Reads	408,344,185 97.80%	207,618,619 97.70%
ean Median ads Genes Cell perCell	1,915	1,933
Re Per	371,898	101,129
Estimated Mean Number of Reads Cells per Cell	1,098	2,053
Library Type	RNA	RNA
Batch	1	2

Α

Median UMIs per Cell (summed over all recognized antibody barcodes)	1,586	2,072
Antibody Reads in Cells	%08.99	76.20%
Fraction h Unrecognized _R Antibody	12.70%	8.70%
Fraction Reads in Barcodes wit High UMI Counts	%00'0	%00:0
Antibody Reads Usable per Cell	3,759	685'5
Fraction Antibody Antibody Reads Usabl Reads Usable per Cell	28.00%	69.20%
Fraction Antibody Reads	87.30%	%06.16
Q30 Bases in UMI	%08'96	%07'96
Q30 Bases in Q30 Bases in Q30 Bases in Antibody Sample Index UMI	%08'06	91.30%
Q30 Bases in Antibody Read	%00'96	%00'96
Sequencing Q30 Bases in Saturation Barcode	96.10%	%00'96
	21.50%	27.10%
Valid Barcodes	99.10%	99.10%
Number of Mean Reads Reads per Cell	6,481	8,003
Number of Reads	7,116,195	ntibody 16,431,509
Library Type Ni	Antibody	Antibody
Batch	1	2

Supplementary Table 1: QC metrics for scRNAseq per batch

Quality control metrics from cellranger count for each batch for (A) RNA and (B) antibody hashtag.

Median UMI Counts per Cell	5,133	5,153	4,681	4,732
Total Genes Detected	16,448	16,479	16,954	17,001
Reads lapped Fraction Reads Total Genes rtisense in Cells Detected o Gene	86.10%	86.20%	88.00%	88.10%
2 2 4	1.20%	1.20%	1.10%	1.20%
Reads Reads Mapped Mapped Confidently Confidently Confidently to to Intronic to Exonic Transcriptome Regions	26.90%	%08:95	%00'85	906'25
Reads Reads Mapped Mapped I ConfidentlyConfidently to Intronic to Exonic Regions Regions	29.60%	29.50%	%08'09	%02'09
Reads Mapped Confidently to Intronic Regions	21.30%	21.40%	21.70%	21.80%
Reads Mapped Confidently to Intergenic Regions	4.00%	4.00%	3.80%	3.80%
Reads Reads Mapped Mapped Confidently to Confidently Genome to Genome Regions	84.90%	82.00%	86.40%	86.40%
Reads Mapped to Genome	91.10%	91.10%	92.20%	92.20%
Q30 ases ir UMI	92.00%	94.70%	%00.56	94.70%
is in	92.90%	95.50%	%08.36	92.90%
Q30 Bases in RNA Read	93.70%	93.30%	93.80%	93.40%
Q30 Bases in Barcode	%06'56	%09'56	%08'56	92.50%
7 Valid Sequending Bases in Bases in Base Saturation Barcode RNA Sam Barcode Read Ind	88.80%	89.40% 95.60% 93.30% 95.50% 94.70% 91.10% 85.00%	65.20% 95.80% 93.80% 96.30% 95.00% 92.20%	66.00% 95.50% 93.40% 95.90% 94.70% 92.20% 86.40%
Valid Barcodes	%08'26	%01.76	%01.76	97.70%
Number of Reads	198,321,366 97.80% 88.80% 95.90% 93.70% 95.90% 95.00% 91.10% 84.90%	210,022,819 97.70%	101,931,446 97.70%	105,687,173 97.70%
Median Genes per Cell		1,870	1,757	1,780
모모	184,313	195,370 1,870	52,460 1,757	54,562
Batch Lane Type Of Cells perCell p	1,076 184,313 1,863	1,075	1,943	2 RNA 1,937 54,562 1,780
Library Type	RNA	RNA	RNA	RNA
Lane	1	2	1	2
Batch	1	1	2	2

Antibody Fraction Reads Fraction Antibody Cell (summed over Unrecognized Reads in Burcognized Unrecognized Per Cell Counts Antibody Cells antibody barcodes)	1,309	724	1,765	1,083
Antibody Reads in Cells	%08'99	%08'99	%09'5/	%01.51
Fraction Antibody Unrecognized Reads in Antibody Cells	12.70%	12.60%	%02'8	8.60%
Q30 Bases Q30 Bases in Jum Reads In Jum Read	%00'0	%00'0	%00'0	%00'0
Antibody Reads Usable per Cell	2,661	1,172	3,945	1,863
Fraction Antibody Reads Usable	27.90%	58.00%	%09.89	68.70%
Fraction Antibody Reads	87.30%	87.40%	91.30%	91.40%
Q30 Bases in UMI	96.40%	96.10%	%08.36	96.10%
Q30 Bases in Sample Index	96.00% 91.00% 96.40% 87.30% 57.90%	95.90% 90.40% 96.10% 87.40% 58.00% 1,172	%05.16	91.00%
Q30 Bases in Antibody Read	%00'96	92.90%	96.00% 91.50% 96.30% 91.30% 68.60%	96.00% 91.00% 96.10% 91.40% 68.70% 1,863
	96.20%	92.90%	99.10% 47.40% 96.10%	92.80%
Valid Sequencing Q30 Bases i Barcodes Saturation Barcode	99.10% 42.70%	99.00% 26.90%	47.40%	99.10% 31.00%
Valid Barcodes	99.10%	%00.66	%01.66	99.10%
Mean Reads per Cell	4,593	2,021	5,753	2,711
Number of Reads	4,942,840	2,173,355	Antibody 11,179,458 5,753	2 Antibody 5,252,051 2,711
stch Lane Library Type	1 Antibody	Antibody	Antibody	Antibody
Lane	1	2 A	1	2
atch	H	H	2	2

Α

Supplementary Table 2: QC metrics for scRNAseq per lane

Quality control metrics from cellranger count for each batch split by lane for (A) RNA and (B) antibody hashtag.