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Maintaining the tumor microenvironment in a 3D tissue model using TumorMACS™ Medium

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Background

Advances in cancer research open new avenues for treatment options, such as immunotherapies using immune modulators. Most of these novel therapeutic approaches focus on targeting the tumor microenvironment (TME), which consists of a variety of cellular (e.g., cancer cells, infiltrating and resident immune cells) and structural (e.g., secreted factors, extracellular matrix proteins) components[1,2]. This heterogeneity is one of the main challenges in the field of cancer research. Thus, understanding the TME promises to reveal new findings in tumor biology that can improve cancer diagnosis and treatment[3]. Several experimental murine models have been generated throughout the years to represent human cancer; however, these models exhibit limitations in fully mimicking the human TME[4,5].

Recently, increasing attention has been given to the *ex vivo* culture of tumor tissues such as precision-cut slices (PCS) as they combine several advantages for personalized therapy testing. They recapitulate the TME in a 3D format and allow for time-efficient experiments[6,7]. But only carefully chosen culture conditions can preserve the *in vivo*–like environment of PCS.

This application note summarizes the data generated to test suitable culturing conditions that maintain TME composition *ex vivo* in PCS prepared from fresh non-small cell lung carcinoma (NSCLC) tissue. For this purpose, Lung TumorMACS Medium and a home-brew medium containing human serum were compared (entire experimental workflow is shown in fig. 1).

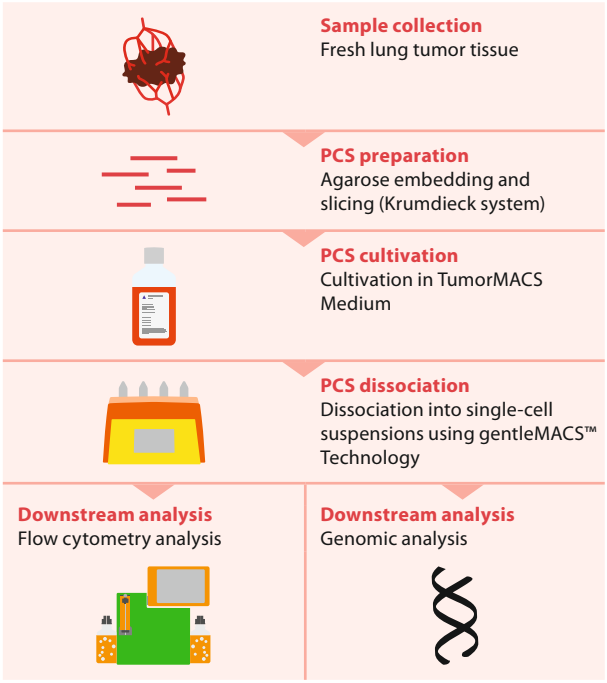

Figure 1: Experimental workflow from PCS preparation to downstream analysis. Workflow steps include agarose embedding of freshly collected lung tumor, PCS preparation using the Krumdieck Tissue Slicer, PCS cultivation, PCS dissociation, and subsequent downstream analysis by flow cytometry and RNA sequencing.

Materials and methods

Ethics statement

The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

PCS preparation and cultivation
PCS were prepared from fresh (day of surgery) lung tumor tissue. In brief, lung tumor tissue was cut into cylinders, embedded in low-melting agarose (Sigma-Aldrich®), and sliced into 200–300 µm thick slices (Krumdieck, TSE systems). During preparation, tissues were kept in ice-cold DMEM + 1× Pen/Strep.
After media exchange, four slices were transferred into culture inserts placed in 6-well plates and a total of 12 PCS were used per condition. Here, the Lung TumorMACS Medium was compared to a home-brew medium containing human serum. After 48 hours incubation, PCS were collected for dissociation.

PCS dissociation
3 wells with 4 PCS each were pooled per donor (n=6) and dissociated using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, human (1× enzyme R, 4× enzyme H, and 1× enzyme A in a total volume of 2.5 mL), as per manufacturer's instructions. Immediately following dissociation, all single-cell suspensions were filtered using a MACS® SmartStrainer (70 µm).

Flow cytometry analysis
Cells were stained with the desired antibodies to determine viability, yield, and frequency. Up to 10^6 cells were stained 1:50 using antibody conjugates from Miltenyi Biotec in 100 µL total staining volume and incubated for 10 min at 4 °C (see respective data sheet). Cells were then washed, resuspended in PBS containing bovine serum albumin (BSA), and analyzed using the MACSQuant® Analyzer 10. Data was analyzed considering cell viability, proportion of singlets, and number of red blood cells and dead cells (data not shown). Mean +/–SD and the statistical significance of differences was assessed by GraphPad Prism 6™ applying 2-way ANOVA or mixed effect model analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Bulk and single-cell RNA sequencing
RNA for bulk RNA sequencing was extracted using the RNeasy® Kit (QIAGEN®). Library preparation was performed following the instructions of the QIAseq® Stranded mRNA Kit (QIAGEN) and libraries were sequenced (NextSeq® 550, Illumina®) yielding at least 30 million clusters per sample using paired-end chemistry.

Data analysis following sequencing was performed via CLC Genomics Workbench/Server version 21 covering trimming of reads, read mapping, differential gene expression analysis, and generation of graphs (i.e., volcano plots). Genes with a false discovery rate of <0.05 and absolute fold changes >1.5 are considered to be differentially expressed.

To perform single-cell RNA sequencing, cells suspended in PBS + 0,04% non-acetylated BSA were processed using Chromium® Next GEM Single Cell V(D)J Reagent Kits v1.1 (10× Genomics®) to generate 5' gene expression (GEX) libraries. Pooled samples were sequenced on a high output cartridge aiming for a depth of 20.000 reads pers per cell (Nextseq 550, Illumina). Sequencing data was pre-processed using Cell Ranger software version 6 (10× Genomics) followed by downstream processing in python and R mainly relying on the Seurat package version 4.
Gene signature scores for stress-associated genes were produced with the AUCell R package based on genes derived from the RT² Profiler™ PCR Array Human Cellular Stress Responses (PAHS-019Z, QIAGEN). Functional enrichment analyses for bulk and single-cell RNA sequencing were performed via the gProfiler2 R package.

Results

Maintained cellular composition in PCS cultivated in Lung TumorMACS Medium
To determine which cell culture conditions maintain the composition of major cellular populations in tumor tissue, PCS from fresh lung tumors were cultivated in either Lung TumorMACS Medium or home-brew medium containing human serum. Athough the cellular composition (e.g., immune and tumor cells) of PCS cultivated for 48 h was preserved in both media, as shown via flow cytometry analysis (fig. 2), TumorMACS Medium supported the tumor cell population better in PCS in 4 out of 6 samples (no statistical significance detected due to donor variance). Additionally, PCS cultivated in Lung TumorMACS Medium showed significantly higher frequency of EpCAM⁺ tumor cells in all 6 samples (fig. 2B) compared to the home-brew medium.

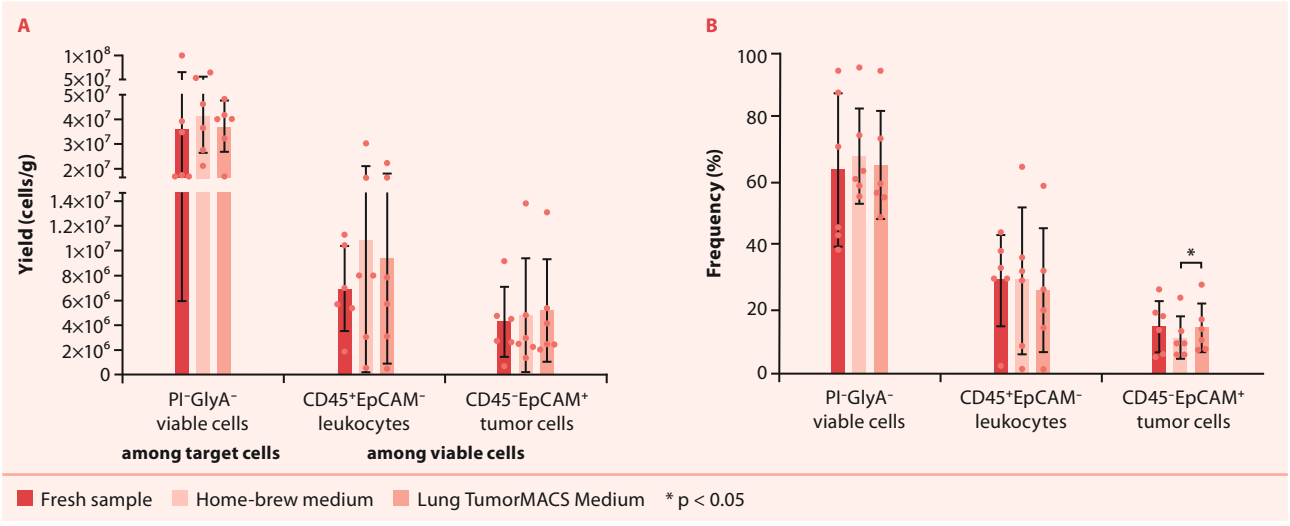

Figure 2: Maintained cellular composition of lung tumor PCS cultivated in TumorMACS Medium. Following 48 h cultivation in TumorMACS Medium or home-brew medium, PCS were dissociated using the gentleMACS Octo Dissociator with Heaters and the Tumor Dissociation Kit, human and cellular composition yield (A) and frequency (B) were analyzed by flow cytometry based on the expression of propidium iodide (PI), CD45, and EpCAM.

Higher induction of stress-related genes in cells from PCS cultivated in home-brew medium as compared to TumorMACS Medium

Next, induction of stress-related genes in cells derived from PCS cultivated in different media (Lung TumorMACS Medium versus home-brew medium) was examined. PCS were cultivated for 48 h, dissociated, and cells were analyzed using bulk (fig. 3) and single-cell RNA sequencing (fig. 4).

Whole transcriptome sequencing analysis showed 5-fold more up-regulated genes in cells from PCS cultivated in home-brew medium compared to PCS cultivated in Lung TumorMACS Medium. In line with the number of differentially expressed genes, gene set enrichment analysis revealed that more functional categories were significantly up-regulated in home-brew medium. Among these categories were response to stress and cell death, which were both not found to be affected when PCS were cultured in Lung TumorMACS Medium (data not shown and fig. 3).

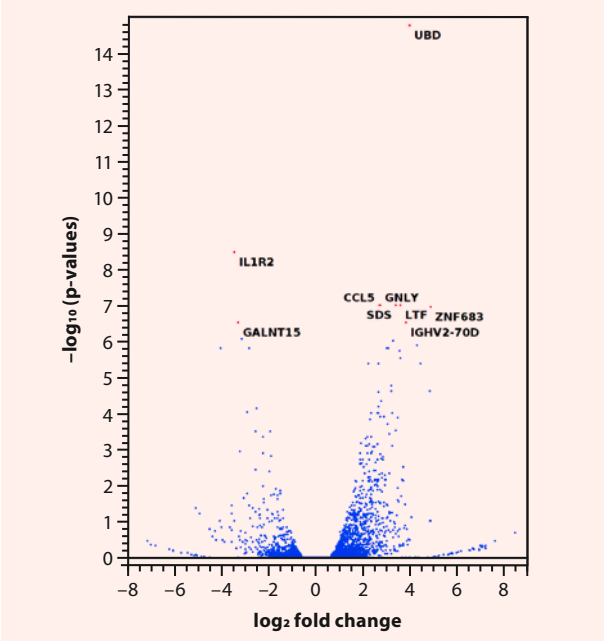

Figure 3: Bulk RNA sequencing shows higher induction of stress-related genes in cells from PCS cultivated in home-brew medium as compared to TumorMACS Medium. Volcano plot based on bulk RNA sequencing data shows 5-fold more up-regulated genes in cells from PCS cultivated in home-brew medium compared to PCS cultivated in Lung TumorMACS Medium.

To validate the findings observed in flow cytometry and whole transcriptome analyses, PCS cells were analyzed by single-cell RNA sequencing. The UMAP plot shows clear cluster formation of cell populations including tumor cells, CD4$^+$ T cells, CD8$^+$ T cells, macrophages, and NK cells (fig. 4A). In line with the flow cytometry results, single-cell RNA analysis showed higher percentage of tumor cells in samples obtained from PCS cultivated in Lung TumorMACS Medium (fig. 4B).

Functional enrichment analysis of four individual cell types (i.e., cancer cells, CD8$^+$ T cells, fibroblasts, and macrophages) revealed that more genes of the functional categories cell death and stress response are higher expressed in cells from PCS cultivated in home-brew medium. For cancer cells from PCS cultivated in Lung TumorMACS medium, genes belonging to these categories were not detected at all in the functional enrichment analysis (data not shown).

Based on the findings observed in the unbiased functional enrichment analysis, the respective cell populations were selected to examine their response towards a selected stress gene panel derived from RT^2 Profiler PCR Arrays (PAHS-019Z). To evaluate the response of stress-related genes in cells, gene set signatures were determined and compared. The scoring method applied (AUCell) relies on expression-based gene ranking to define scores describing the enrichment of an input gene set among the expressed genes in each cell.

Considering the comparison of multiple samples, the higher the AUCell score, the higher the proportion of highly expressed genes from the input gene set. Here, significantly higher response of stress-related genes in cells was observed in cancer cells, fibroblasts, and macrophages, but not in CD8$^+$ T cells from PCS cultivated in home-brew medium as compared to Lung TumorMACS Medium (fig. 4C).

Overall, single-cell RNA sequencing confirmed the results obtained by whole transcriptome sequencing and showed that especially cancer cells are less stressed in PCS cultured in Lung TumorMACS Medium.

Conclusion

Lung TumorMACS Medium preserves the *in vivo*–like TME in lung tissue PCS and is suitable for the generation of reliable, time-efficient 3D tumor models.

- The cellular TME composition is maintained in lung tumor PCS cultivated in Lung TumorMACS Medium.
- The tumor cell population in lung tumor PCS is better maintained in Lung TumorMACS Medium as compared to home-brew medium containing serum.
- PCS cultivation in Lung TumorMACS Medium versus home-brew medium containing serum shows reduced induction of stress-related gene expression in tumor cells, fibroblasts, and macrophages.

Product	Order no.
Cell culture	
Lung TumorMACS Medium	130-134-218
Sample preparation	
gentleMACS Octo Dissociator with Heaters	130-096-427
gentleMACS C Tubes	130-096-334
Tumor Dissociation Kit, human	130-095-929
MACS SmartStrainers (70 µm)	130-110-916
Flow cytometry	
MACSQuant Analyzer 10	130-096-343
Propidium Iodide Solution	130-093-233
CD45 Antibody, anti-human, VioGreen™, REAfinity®	130-110-638
CD326 (EpCAM) Antibody, anti-human, APC, REAfinity	130-111-000

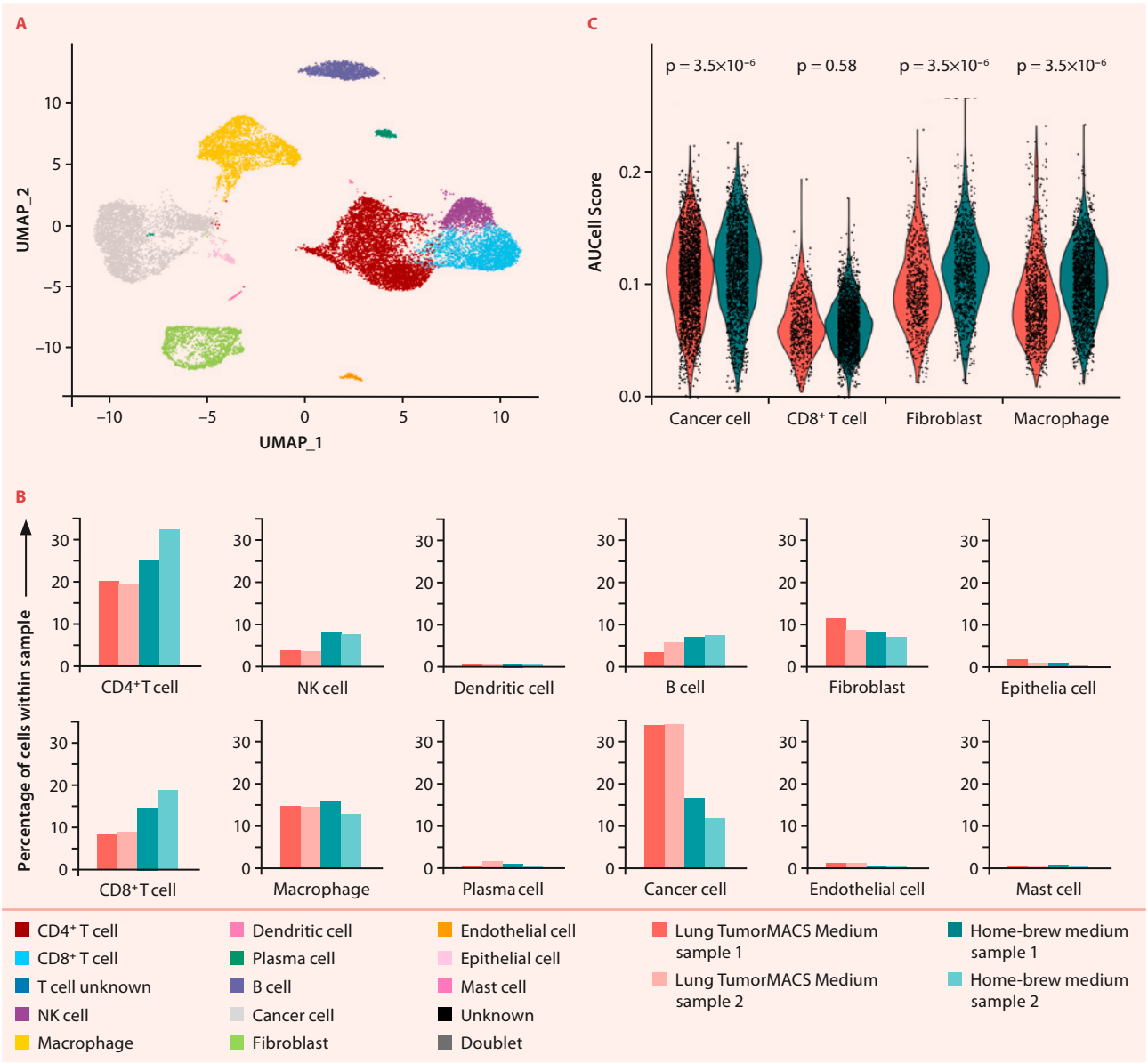

Figure 4: Singe-cell RNA sequencing analysis confirms higher stress gene response in PCS cultivated in home-brew medium as compared to TumorMACS Medium. (A) UMAP plot showing cluster formation of the analyzed cell types. (B) Percentage of cell types identified in the respective samples. (C) AUCell score of response of stress-related genes in cancer cells, CD8⁺ T cells, fibroblasts, and macrophages.

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