Maintaining the tumor microenvironment

Serum-free cell culture for an advanced 3D *in vitro* tissue model

Poster ID: 6781

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Introduction

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. This heterogeneity is one of the main challenges in the field of cancer. Thus, understanding the TME promises to reveal new findings in tumor biology that can improve cancer diagnosis and treatment. 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. 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 at resonable scale. But only carefully chosen culture conditions can preserve the *in vivo*–like environment of PCS. This poster 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, the new serum-free Lung TumorMACS Medium and a home-brew medium containing human serum were compared.

Materials & Methods

Figure 1

Experimental workflow from precision cut slice (PCS) preparation to downstream analysis

PCS preparation and cultivation
At the day of surgery, lung tumor tissue was delivered to Miltenyi Biotec, Bergisch Gladbach, Germany. The tumor was cut into pieces and embedded in low-melting agarose gel, before slicing the tissue into 200–300 µm thick PCS using a Krumdieck tissue slicer. After PCS randomization, PCS were transferred into cell culture inserts placed in 6-well plates. PCS were either cultivated using Lung TumorMACS Medium or a home-brew medium containing human serum. After 7–16 h recovery at 37 °C and 5% CO_2, stress-induced cytokines were removed by media exchange and cultivation was continued for 48 h.

PCS dissociation
A custom protocol, optimized for the dissociation of PCS, on the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, human was developed. After dissociation, the single-cell suspension was filtered using a MACS SmartStrainer (70 µm).

Flow cytometry analysis
Cells were stained with the desired antibodies to determine viability, yield, and frequency. The stained samples were analyzed using the MACSQuant Analyzer 10.

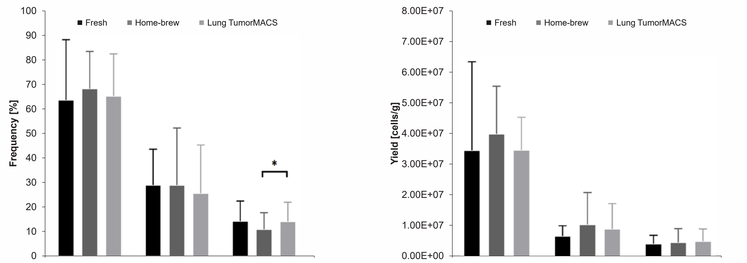
Bulk and single-cell RNA sequencing
RNA for bulk RNA sequencing was extracted using the RNeasy® Kit (QIAGEN). Library preparation was performed following the instructions of the QIAseq® Stranded mRNA Kit (QIAGEN) and libraries were sequenced using the NextSeqR 550 (Illumina). Data analysis following sequencing was performed via CLC Genomics Workbench/Server version 21. 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 (10x Genomics) to generate 5' gene expression (GEX) libraries. 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

1 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. PCS cultivated for 48 h in both media exhibit preserved cellular (e.g. immune and tumor cells) composition as shown via flow cytometry analysis (Fig. 2). PCS cultivated in Lung TumorMACS Medium show significantly higher frequency of EpCAM⁺ tumor cells compared to the home-brew medium.

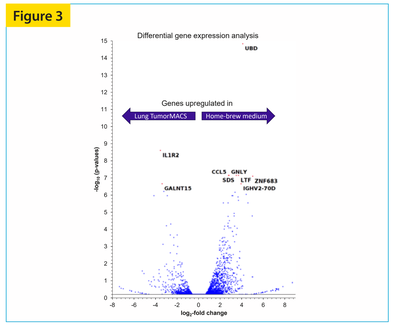
Figure 2



2 Higher differential gene expression in cells cultured in home-brew medium

Next, stress gene induction in cells derived from PCS cultivated in different media (Lung TumorMACS Medium versus homebrew medium) were 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 are significantly up-regulated in homebrew medium. Among these categories are response to stress and cell death, which are both not found to be affected when PCS are cultured in Lung TumorMACS Medium (data not shown and Fig. 3).

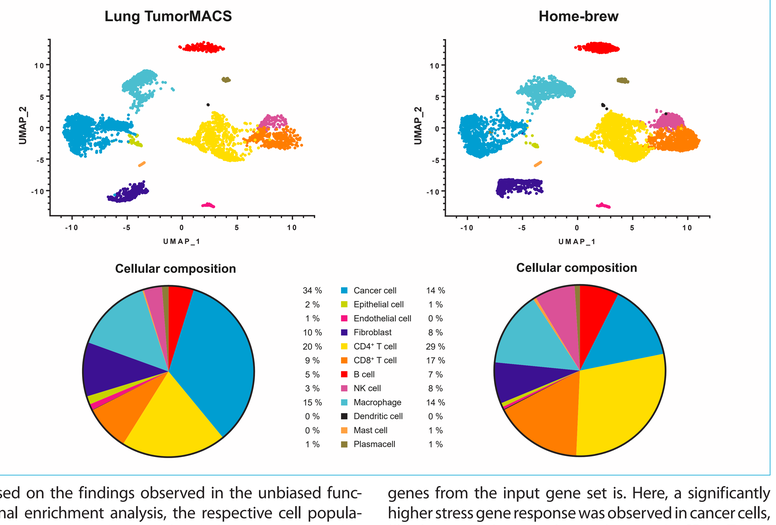
Figure 3



3 scRNAseq confirms differences in tumor cell content

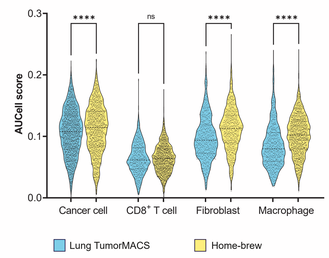
To validate the findings observed in flow cytometry and whole transcriptome analyses, PCS cells were analyzed by single-cell RNA sequencing. The UMAP plot shows clear cluster formation of cell populations including tumor cells, CD4+ T cells , CD8+ T cells, macrophages, and NK cells (Fig. 4; upper panels). In line with the flow cytometry results, single-cell RNA analysis shows a higher percentage of tumor cells in samples obtained from PCS cultivated in Lung TumorMACS Medium (Fig. 4; lower panels). 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 (Fig. 5). While these categories were not detected in cancer cells from PCS cultivated in Lung TumorMACS Medium, genes belonging to these categories were differentially expressed in the remaining cell populations examined from PCS cultivated in either media, but to a greater extend for home-brew medium (data not shown).

Figure 4



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 RT2 Profiler PCR Arrays (PAHS-019Z). 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 is. Here, a significantly higher stress gene response 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 and well maintained in PCS cultured in Lung TumorMACS Medium.

Figure 5



Conclusion

Lung TumorMACS Medium preserves the *in vivo*–like TME in lung tissue PCS and is suitable for the generation of reliable and 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 homebrew medium containing serum shows reduced induction of stress-related gene expression in tumor cells, fibroblasts, and macrophages.