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ORIGINAL ARTICLE

Immune activation of the monocyte-derived dendritic cells using patients own circulating tumor cells

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Abstract

Background Dendritic cell (DC) therapy counts to the promising strategies how to weaken and eradicate cancer disease. We aimed to develop a good manufacturing practice (GMP) protocol for monocyte-derived DC (Mo-DC) maturation using circulating tumor cells lysates with subsequent experimental T-cell priming in vitro.

Methods DC differentiation was induced from a population of immunomagnetically enriched CD14 + monocytes out of the leukapheresis samples ($n = 6$). The separation was provided automatically, in a closed bag system, using CliniMACS Prodigy® separation protocols (Miltenyi Biotec). For differentiation and maturation of CD14 + cells, DendriMACs® growing medium with supplements (GM-CSF, IL-4, IL-6, IL-1B, TNFa, PGE) was used. Immature Mo-DCs were loaded with autologous circulating tumor cell (CTCs) lysates. Autologous CTCs were sorted out by size-based filtration (MetaCell®) of the leukapheresis CD14-negative fraction. A mixture of mature Mo-DCs and autologous non-target blood cells (NTBCs) was co-cultured and the activation effect of mature Mo-DCs on T-cell activation was monitored by means of multimarker gene expression profiling.

Results New protocols for mMo-DC production using automatization and CTC lysates were introduced including a feasible in vitro assay for mMo-DC efficacy evaluation. Gene expression analysis revealed elevation for following genes in NTBC (T cells) subset primed by mMo-DCs: CD8A, CD4, MKI67, MIF, TNFA, CD86, and CD80 ($p \leq 0.01$).

Conclusion Summarizing the presented data, we might conclude mMo-DCs were generated using CliniMACS Prodigy® machine and CTC lysates in a homogenous manner showing a potential to generate NTBC activation in co-cultures. Identification of the activation signals in T-cell population by simple multimarker-qPCRs could fasten the process of effective mMo-DC production.

Keywords Dendritic cells · Immunotherapy · T cells · Personalized medicine · MetaCell · Circulating tumor cells

Introduction

Dendritic cells (DCs) are a particularly interesting immunotherapeutic tool given their ability to uptake and present tumor-associated antigens (TAAs) through a variety of mechanisms [1, 2]. DCs play a significant role in priming process of lymphocytes to potent effector responses against the tumor cells [1–3].

The character of DC vaccines is being extensively studied, comprising patient-monocyte-derived DCs

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manipulated ex vivo [4]. The summary on research progress and clinical testing of DCs vaccines has been recently reviewed in work of Yu et al. [5].

We present for the first-time immunization of the monocyte-derived DCs (Mo-DCs) using patient own circulating tumor cells (CTCs). CTCs are representing the tumor messengers floating from the primary tumor to the metastatic locations to set up new cancer cell colonies [6]. CTCs lysates are presented here as a new source of patient specific TAAs. Viable CTCs were enriched from patient's peripheral blood using size-based separation method MetaCell® [7, 8] in parallel with leukapheresis sampling for monocyte-culture setup.

The concept of in vitro Mo-DC activation by CTCs and the subsequent evaluation has been tested using patients own lymphocyte population. Multimarker gene expression profiling has enabled to describe the process of the Mo-DC activation and T-cell priming on the molecular level.

To meet the increasing regulatory requirements for cell-based therapeutics, all manufacturing steps in the presented vaccine preparation protocol of Mo-DCs were integrated in a closed system operated by an automated cell processing instrument, the CliniMACS Prodigy® (Fig. 1), [9, 10].

Although the principle of anti-tumor vaccines based on DCs has been known for a long time, prior-art studies always dealt with the preparation of anti-tumor vaccines derived from tumor cell cultures or autologous disseminated tumor cells, which are available in a sufficient quantity, but especially in more advanced stages of a tumor disease [11].

The primary idea of presented study was, that the immune reaction against the tumor could be induced with the use of the CTCs isolated from the patient in a time period when patient health condition is satisfactory (i.e., no sign of tumor progression is present: the tumor is not growing, metastases are not detected, protein biomarkers are in norm). The only thing that reflects ongoing activation of tumor processes during this period is a growing number of CTCs in a drawn sample of the patient's peripheral blood.

Presence of CTCs in the patient's blood in the remission period has already been described, but so far nobody has used these cells for immunization in the time frame when the patient is in the state of remission according to the clinical terminology. Autologous biological material is exclusively used, so there is no additional risk for the patient. A robust therapeutic anti-tumor intervention suitable also for routine and repeated use in oncology can be based on only some or even a single tumor cell on condition, it is conducted in the exact time when the number of CTCs in peripheral blood is growing.

CTCs can be used to detect an incoming relapse of the disease and to start to direct the immune answer against something that de facto does not exist to a greater extent yet—i.e., personalized therapeutically preventive vaccines.

CTCs appearing in the patient's blood during the remission period can be assumed to have been present in the patient's body in the period of the previous conducted therapy, which means that they are uniquely and individually modified by therapeutic interventions, which also contributes to specificity of their antigenic portfolio.

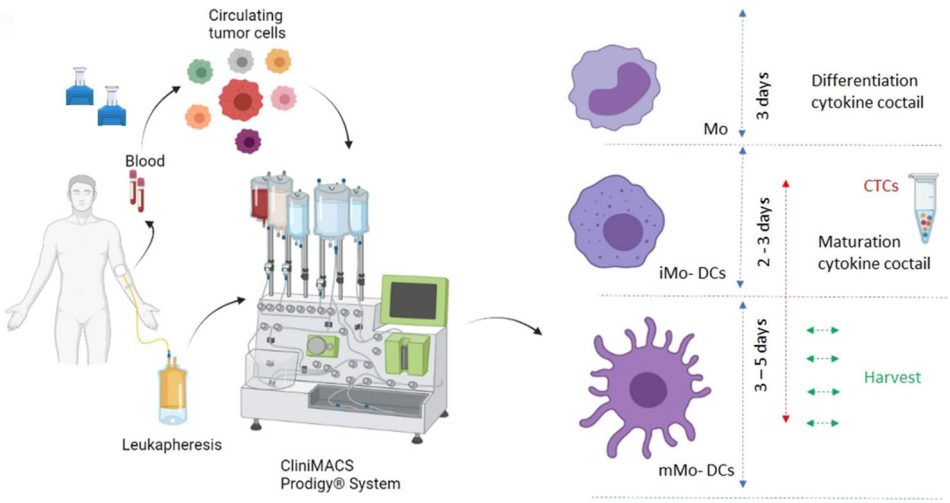


Fig. 1 The study outline summarizes the monocyte-derived dendritic cells (Mo-DCs) production in CliniMACS Prodigy® machine in a closed tubes system under sterile conditions. CliniMACS Prodigy® technology is used for automatic immunomagnetic antibody-based separation of CD14 + cells out of the leukapheresis (up to 100 mL). CD14 + cells are further cultured in vitro under the "controlled" in tube/bag conditions to generate immature Mo-DCs (iMo-DCs) and mature Mo-DCs (mMO-DCs). Protocol for mMo-DCs production included several modifications in comparison to the standard CliniMACS Prodigy® procedures. The changes were considering different cultivation length, different "activation strategies" using circulating tumor cell lysate (CTC lysate) to produce mMo-DCs

Tumor cells are in general a heterogeneous population of cells, are not genetically stable and in the course of an oncological disease in the patient's body, various variants of tumor cells are produced and spread in the course of the disease while these cells are different both from the original cells of the primary tumor and from each other [12, 13]. The production of changed tumor cells is further induced by the ongoing therapy, both on the level of chemotherapy and on the level of biological therapy. DC vaccines can be advantageously prepared repeatedly in short interval thanks to an available source of antigens, which is represented by CTCs.

Current vaccines based on DCs are most frequently produced from tumor cells of a cell culture, e.g., commercial cell lines, or from cells of the primary tumor [10, 11, 14, 15].

With regard to the relatively low number of CTCs in peripheral blood (approx. 1 CTC per 10^6–10^9 blood cells), it is very unlikely that an antigen presenting cell and a CTC will meet in the blood, inducing an immune response of the organism. In addition, CTCs are considerably differentiated so an antigen-presenting cell of the immune system frequently does not recognize a CTC and does not identify it as a potential threat.

The aim of effective cell-based immune therapy is to achieve ideally a long-term and individually specific response to immunization. The effect of the vaccine, or efficiency of the immunization process can be then (repeatedly) monitored during the time period following the administration of the vaccine.

In standardized protocols of DC vaccination clinical trials, the direct effect is measurable by an increase of CD8 + IFNγ + [16], however, repeated tests have not confirmed correlation between these cells and the real clinical impact.

Molecular testing of the characteristics of primary tumors and CTCs shows that besides antigens of the primary tumor, CTCs also express antigens that can be considered as new signs of aggressiveness of the tumor disease. Thus, CTCs are used as a source of new targeted antigens, i.e. antigens that treatment can be directed against.

Herein, CTCs refer to cells being released from the primary or secondary tumor to the bloodstream. Determination of CTCs can be carried out within liquid biopsy, referring here to collection of peripheral blood and its analysis for CTCs, fragments of circulating free DNA (cfDNA) from tumor cells, exosomes etc., wherein the result of the examination can be specification of the ongoing oncological disease, especially its development dynamics.

In these situations, an increased number of CTCs present in the blood should be a marker that could differentiate patients that need further additional treatment after passing a certain line (stage) of treatment (e.g., adjuvant). Therefore, personalization of oncological care is mentioned in the context of CTCs and fluid biopsy, namely on the basis of characteristics of CTCs.

The strategy of using CTCs for the production of personalized vaccines is based on the possibility of monitoring the course of standard treatment of patients with the use of CTCs. In case of repeatedly increasing number of CTCs, CTCs can be isolated and used for the production of a vaccine that will be unique for this time period (i.e., currently in the real time) and will induce an immune response especially against antigens present in CTCs.

In summary, we aimed to develop a good manufacturing practice (GMP) protocol for monocyte-derived DCs (Mo-DCs) maturation induced by circulating tumor cells lysates.

Methods

Patients

Patients ($n = 6$, average age 40,4 years) enrolled into the study were undergoing palliative treatment of the metastatic breast cancer disease in the University Hospital Wroclaw. First, two tubes of peripheral blood (VACUETTE® 9 ml K3 EDTA) were obtained to evaluate CTC presence/absence. Patients with CTCs (≥ 100 CTCs/1 mL) were included into study after signing informed consent. Informed consent was obtained after possible consequences of the leukapheresis have been fully explained. The experiments were performed with an agreement of local ethical committee (Krajská zdravotní a.s., Ústí nad Labem, Dept. of Thoracic Surgery, Czech Republic and Ethical Committee of Wroclaw Medical University, Nr.KB-94/2018). The short outline of the study can be seen in Table 1 General procedures during Mo-DCs production, Fig. 1 and in Supplementary file 1.

Enrichment of CTCs

CTCs are enriched during the study at two time points. First, appx. 14 days before study enrollment. Only patients with present CTCs (≥ 100 CTCs/1 mL) are included into the study. Second, CTCs are isolated in parallel with Mo-DC production protocol. At this point CTCs are used for CTC lysate production. More details on CTC enrichment processes are described in Supplementary file 2.

CTC lysate preparation

For every patient, two types of samples were used for CTCs enrichment: venous blood (4 × 8 mL) and leukapheretic CD14-negative cell fraction (20 mL). Approximately 4 × 8 mL of venous blood was drawn from the cubital veins and placed into Vacuette EDTA tubes (Greiner Bio-One, Kremsmuenster,

Table 1 General procedures during Mo-DCs production

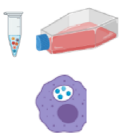
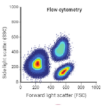
STEP			PROTOCOL PART		ACTIVITY	TIMING	FREQUENCY	EXPECTED OUTCOME	Process lenght (Days)/action wanted	NOTES
1		SAMPLING	**Inclusion criteria - Test of CTCs presence**		**2x8 ml** of blood withdrawal (EDTA tubes) for CTCs separation	Before DC-vaccine study inclusion	1	Inclusion into the DC vaccination study if CTCs are present (e.g 100 CTCs /8mL)	<14	If no CTCs are present, please repeat the blood withdrawal in 4- 6 weeks 
2	2.1		Starting material for Mo-DC - vaccine production		**4x8 ml** of blood withdrawal (EDTA tubes) for CTCs separation	**DAY 0**	1	Isolation of CTCs (MetaCell®) and preparing the **CTCs- lysate** for immature Mo-DCs activation (see Step 3.4.)	<24 hours	For CTC- lysate preparation - appx. 1mL RNA-free water with BSA (1%) in 2-3 thaw- freeze cycles is used
	2.2				**20-100mL** of leukapheresis product		1	Source of monocytes (Mo), /CD14+ cells/ for immature monocyte- derived dendritic cells (iMo-DCs) production (see Step 3.)	<24 hours	
3	3.1.	Mo-DCs PRODUCTION	**Monocyte (Mo) separation**		Immunomagnetic separation of monocytes / CliniMACs®	**DAY 1**	1	Isolation of monocytes (Mo) for immature monocyte- derived dendritic cells (iMo-DCs) production (see Step 4.)		Monocytes are cultured with differentiation media including cytokines in the cultivation chamber of Clinimacs®
	3.2.		**Mo- DCs differentiation**		Monocytes in vitro culture set up (media and cytokine cocktail preparation incl. **IL4, GM- CSF)**	**DAY 1**	1	Differentiation of monocytes (Mo) to immature monocyte-derived dendritic cells (iMo-DCs) production		More details on cytokines activity during the differentiation process are shown on LIST **CYTOKINE MIX RECIPE 3**
	3.3.		**Immature (i)Mo-DC maturation**		immature Mo-DC in vitro culture plus maturation process set up (media and cytokine cocktail preparation incl. **IL1B, IL6, TNFA, PGE**)	**DAY 4**	1	Maturation process set up (media and cytokine cocktail preparation)		More details on cytokine activity during maturation process are shown in LIST **CYTOKINE MIX RECIPE 3**
	3.4.		**CTC - lysate**		CTC lysate preparation from viable CTCs size-based enriched by MetaCell® tube	**DAY 4**	1	Lysate of CTCs in RNA-free water solution (1%BSA) (4x1mL) after several freeze-thaw cycles		More details on CTCs-enrichment are shown in LIST **CTCs for iMo-DCs maturation**
	3.4.1.				**Storage of CTCs-lysates/month**		1	CTCs - lysate dose (vial) might be available also for future DC-vaccine generation		Storage: ~frozen (-20/80°C)
	3.5.				Administration of CTC-"maturation" lysate to iMo-DCs + adding of cytokine mix (IL-6, TNFa, IL-1B, PGE)	**DAY 5**		mature Mo-DCs product	± 24 hours	More details on cytokine activity for maturation process are shown on LIST **CYTOKINE MIX RECIPE 3**
	3.6.				Mo-DCs- maturity control - microscopy - real time	**DAY 7**	1	mMo-DCs show typical signs of dendritic cells morphology	± 24 hours	

Table 1 (continued)

4.	4.1.	QUALITY CONTROL	**Counting of mature Mo-DCs in generated batches**		Microscopy evaluation of Mo-DCs maturity/ cytomorphology/ functional assays (e.g. Mitotracker, Zymosan)	**DAY 7**	Information on Mo-DCs vaccine cellularity	<8 hours	Storage: ~frozen (-20/80°C)
	4.2.		**Freezing of mature Mo-DCs batches**		Controlled freezing of Mo-DCs cells	**DAY 7**	Viable matured Mo-DCs are detected in re- frozen sample	<8 hours	Storage: ~frozen (-20/80°C)
	4.3.		**Flow Cytometry of mature Mo-DCs**		Flow cytometry testing *	**DAY 7**	CD14, CD 83, HLADR, CD209, CD11 (described on LIST QUALITY TESTS 2 in detail), viability testing	<3	* Outsourced
	4.4.		**Re- freezing**		retesting of viability Flow cytometry testing *	x x	**~60%** CD14, CD 83, HLADR, CD209, CD11 (or alternative antibodies of Quality tests)	<14 <14	* Outsourced (details are reported in Supplementary File 1/ List 4 Quality Tests)

Genes relevant for mMo-DC generation are marked by "mMo-DCs". Genes relevant for T-cell activation are marked by "Activation"

*Relative gene expression is presented for all samples in comparison to the starting leukapheresis cell-mix

Austria). CTCs were enriched using MetaCell® size-based separation tube (MetaCell, Prague, Czech Republic). Directly after CTC enrichment, CTCs were lysed in a RNAse-free water solution with BSA (1%). The lysis process was supported by thaw–freeze cycles (3x) to ensure the cells lysis. The CTC lysate was then stored at minus 20 °C.

Mo-DC production (low-scale protocol)

For the primary in vitro experiments, low-scale, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Merck KGaA, Darmstadt, Germany) gradient centrifugation. One half of isolated PBMC was stored at − 80 °C for T-cell restimulation. Monocytes from the remaining PBMC were separated using $CD14^+$ isolation kit (EasySep™, Human CD14 Positive Selection Kit, Stemcell Technologies, U.S.) according to the manufacturer's instructions. 2.5×10^6/well $CD14^+$ cells were seeded into the six-well plate (Merck KGaA, Darmstadt, Germany) in 4 ml of X-VIVO 15 medium (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with GM-CSF and IL-4 as described in detail in Supplementary file 1/List 5: Cytokine mix recipe. On day 4, DC were co-cultured in six-well plate with a mixture of frozen–thawed CTC lysate. CD14 cells were harvested in serum free GMP-compliant media X-VIVO 15 at 37 °C. Monocyte-derived DC were generated in X-VIVO 15 for 7–12 days in the presence of 500 IU/ml of GM-CSF and 20 ng/ml of IL-4 (Milteniy Biotec, Bergisch Gladbach, Germany).

Mo-DC production using CliniMACS Prodigy® (high-scale protocol)

For the introductory Mo-DC-activation experiments, high-scale protocols were applied (see more details in Table 1 and Supplementary file 1/List 2: Protocol Mo-DCs). In short monocytes were selected out from leukapheresis products ($n = 6$, 80–100-ml of blood product) by semi-automated magnetic bead separation of $CD14 + cells_n$ (CliniMACS Prodigy®, Miltenyi Biotec, Bergisch Gladbach, Germany). The CD14-negative cell fraction was used in experiments assigned as Non-Target Blood Cells (NTBCs) fraction.

CD14 + cells were further kept in "closed-bag" culture in Clinimacs Prodigy® machine chamber under standard culturing conditions (38 °C, 5%CO_2) enabling differentiation into immature Mo-DC (iMo-DCs) and maturation into mature Mo-DCs (mMo-DCs). The process of differentiation lasted alternatively 3–5 days, the process of maturation lasted 3–6 days, the whole process took 7–12 days in bag-culture in total. Immature Mo-DCs, cells were loaded using patients autologous CTC lysates and matured by the addition of Prostin (PGE2) (Pfizer, New York, U.S.). After 40–72 h in bag-culture, the mature mMo-DCs are harvested (See Fig. 1).

The effect on iMo-DC maturation induced by co-culture with CTC-antigens was evaluated by flow cytometry, and by the multimarker gene expression profiling. DC phenotype was determined by staining with antibodies anti-CD80-FITC, -CD86-PE, -CD83-PE-Cy5.5 (Mo-DC Differentiation Inspector®, Miltenyi Biotec, Bergisch Gladbach, Germany), and HLA-DR-PE-Cy7 (BD Biosciences, U.S.) for 20 min. Cell's viability was detected with 7AAD-staining, which was

as part of Mo-DC differentiation inspector. $CD11c^{+}7AAD$ negative DC were analyzed for mean fluorescence intensity (MFI) of the particular maturation marker. DC maturation process was monitored on the functional level using phagocytosis of Zymosan particles (ThermofisherScientific, Whatam, U.S.),

Mo-DC induction of NTBC activation

A portion of mature Mo-DCs (mMo-DCs) is used to stimulate non-target blood cells (NTBCs) consisting of a mixture of CD4 + /CD8 + lymphocytes. Co-culture lasting 1, 3, 7, 14 days generated two cells fractions, adherent and floating NTBC cells. Multimarker gene expression has been tested in the NTBC-adherent and floating cells co-cultured 7 and 14 days by means of qPCR after mMo-DC priming.

Multimarker gene expression analysis

Total RNA was isolated from cell lysates (CTCs, mMo-DCs, NTBC -adherent cells, NTBC -floating cells and their co-cultures—see more in Supplementary file 1/ List 7 Multimarker gene expression) using RNeasy mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The RNA purity and concentration was determined spectrophotometrically using a Nanodrop 2000c (Thermo Fisher Scientific, Whatam, U.S.).

Reverse transcription was performed from 100 ng of isolated RNA using High RNA to cDNA synthesis kit. Taqman-based qPCR chemistry (Taqman FAST qPCR solution) combined with gene specific Taqman® probes (22 genes in total) was used. Taqman probes are listed in Supplementary file 1/ List 7 Multimarker gene expression.

Real-time PCR was executed on Cobas Z480 Real-Time PCR Detection System (Roche, Basel, Switzerland). The 16 µl reaction contained 8 µl of Taqman FAST qPCR Master Mix, 1 µL of TaqMan probe (ThermofisherScientific, Whatam, U.S.), 4.0 µl of Dnase-free water and 3 µl of cDNA.

The relative gene expression of the studied genes was calculated with GenEx software (MultiD Analyses AB, Goeteborg, Sweden). The relative gene expression ratios were compared between the iMo-DCs, mMo-DCs, and NTBCs vs. non-treated NTBCs cells.

Statistical analysis

Two-tailed paired *t* test or unpaired, nonparametric Mann–Whitney test were applied for data analysis using GraphPad PRISM 9 (San Diego, California, USA). The results were considered statistically significant if $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

Results

mMo-DC production summary

The used automatized protocol (CliniMACSs Prodigy®) for Mo-DC production resulted in robust and reproducible upregulation of DC maturation markers such as cluster of differentiation (CD) CD80, CD83, CD86, human leukocyte antigen-DR (HLA-DRA) and DC-SIGN (CD209), which have been tested by flow cytometry (Fig. 2) and gene expression profiling in parallel (Table 2). Functionality of these Mo-DCs was shown by phagocytosis activity of Zymosan® particles (Fig. 2) and corresponding mMo-DCs morphology (See Supplementary File 1/List 2 Protocol for MO-DC production), positive T-cell stimulatory capacity and the ability to upregulate the proliferation capacity in the mixture of naive NTBCs towards the group of T cells expressing CD8 marker (Table 2).

Performance of the automated CD14 + cell separation and Mo-DC generation process

Using the CliniMACS CD14® Reagent in 6 testing procedures, the monocytes were routinely enriched out of the 80-100 mL leukapheresis (cellularity WBC = $74–75 \times 10^9$/L) to a purity of 97% and a recovery of 81%. There were $8.7–19.5 \times 10^9$ /L monocytes (CD14 +) enriched by CD14 antigen. Viability of the enriched cells before culture was 98% on average.

Isolated monocytes were cultured for a minimum of 6 days in total. First, cells were cultured 3–4 days (differentiation period) in the presence of GM-CSF (500 IU/mL) and IL-4 (1000 IU/L), yielding immature Mo-DCs (iMo-DCs), which were subsequently matured for an additional 24/48 h (maturation period) in the presence of IL-1β, IL-6, TNF-α, and PGE_2 (see more in Supplementary file 1/List 4 Cytokine Recipe). Maturation was completed by addition of CTC lysate (up to 4 mL in total). Mature Mo-DCs (mMo-DCs) were generated with recoveries of 20% on average, calculated from the number of initially seeded monocytes. The viability of mMo-DCs was evaluated by flow cytometry, i.e., exclusion of PI + dead cells, reaching up to 72 ± 13.9%.

In detail, CD14 + cells were after automated magnetic bead separation cultured with GM-CSF and IL-4 in DendriMACS® applying four different protocols (see Supplementary File 1 in detail). The protocols differed in differentiation and maturation period length in particular.

For the iMo-DCs 3–4 days of culture were used and finally, mMo-DCs were collected after 6–7 days of culture in total. Mature Mo-DCs were analyzed for their morphological appearance and expression levels of CD14, CD15, CD45, HLA_DRB1, and CD209 by flow cytometry and gene

Table 2 Data from gene expression profiling of the different cell groups included into the process of mMo-DCs differentiation and subsequent NTBCs activation during Mixed Lymphocyte reaction are presented

GE analysis	CD14+ separated Day 0	CD14+ cultured Day 7	NTBC separated Day 0	NTBC cultured Day 7	NTBC floating + mMo-DC	NTBC adherent+ mMo-DC
CD45						
INFG						
CD86		mMo-DCs				
CCR7						
CD14						
IL-12B						
CD68						
MIF						Activation
CDX2						
CD56						
CD4					Activation	Activation
CD8A					Activation	Activation
KI67					Activation	Activation
CLDN18						
CD80		mMo-DCs				
IL17						
HLA_DRA		mMo-DCs				
HLA_DRB1		mMo-DCs				
CD 209		mMo-DCs				
CD 209C		mMo-DCs				
CD83		mMo-DCs				
FOXO3p						
TNFA						Activation
FOXO3p2						
IFNG 2						
CD56 2						
CD274 2		mMo-DCs?				

↑↑	Significantly elevated GE
↑	Elevated GE
	No change in the gene expression level
↓	Decreased GE
↓↓	Significantly decreased GE

Table 2 (continued)

Genes relevant for mMo-DC generation are marked by "mMo-DCs". Genes relevant for T-cell activation are marked by "Activation"

*Relative gene expression is presented for all samples in comparison to the starting leukapheresis cell-mix

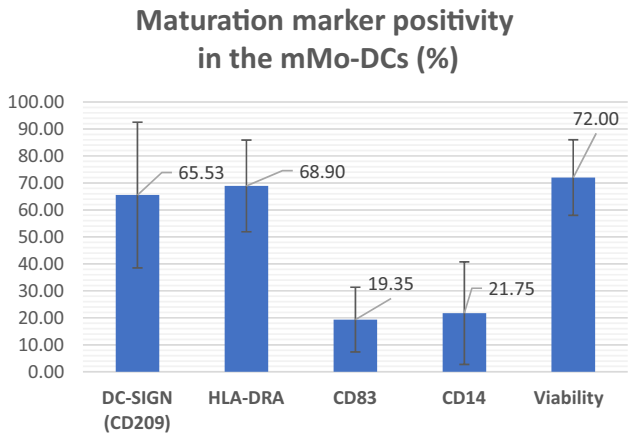


Fig. 2 Flow cytometry evaluation of the maturation markers on the Mo-DCs after the CliniMACS Prodigy® culture

expression. On average, the yield of maturation as well as the viability (~80%) was similar between all four tested protocols. Differentiation of monocytes towards mMo-DC phenotype was observed for all the tested protocols as proved by the downregulation of CD14 (≤10.4%) and CD15 (≤0.01%) and the increased expression of the DC markers HLA-DRB1 (68.9%) and CD209 (65.53%) shown by flow cytometry. CD83 was expressed only in 19.83% of the tested mMo-DCs.

As confirmed by flow cytometry, during the Mo-DCs production, the enriched and culture monocytes (CD14+) diminished the expression of CD14. In contrast, mMo-DCs expressed various DC-markers involved in the formation of immunological synapse between DCs and naive T cells, including CD80, CD83, CD209, and MHC II (HLA-DR). The flow cytometry results were confirmed by gene expression analysis (Table 1). All together 22 markers were tested by qPCR. For more detailed data, please see the Table 2 summarizing relative gene expression data for all of the tested samples.

Gene expression of maturation-associated markers was tested in iMo-DCs and mMo-DCs cell fractions. Elevation of gene expression for following genes was confirmed in mMo-DCs fraction: CD80, CD83, CD86, HLA-DRA, HLA-DRAB1, CD209, MIF (Macrophage Migration Inhibitory Factor) ($p \leq 0.01$). Gene expression of following genes was undetectable: IFNG, IL12B, IL17.

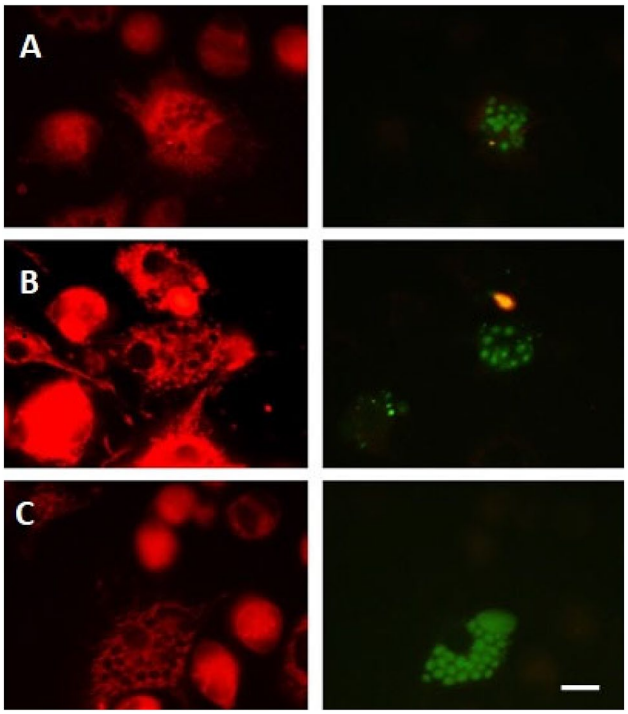


Fig. 3 Green Zymozan® (Thermofisher Scientific) particles are engulfed by Mo-DCs to test the endocyting capacity of generated Mo-DCs in time **a** 2 h, **b** 6 h, **c** 24 h of Zymosan particles co-culture. The Mo-DCs display significant mitochondrion activity shown by red fluorescence (Mitotracker®, ThermoFisher Scientific). The bar represents 10 µm

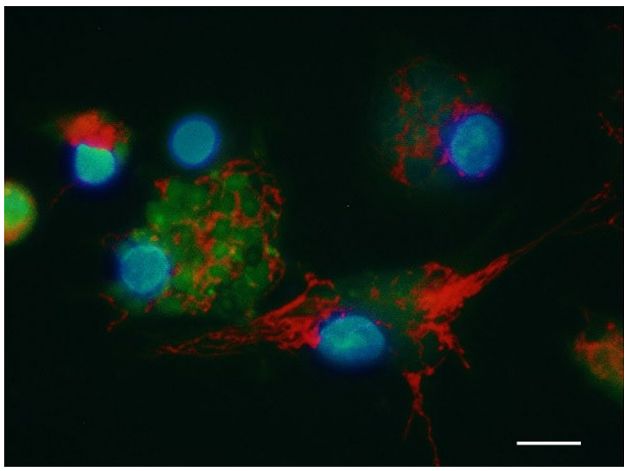


Fig. 4 Mature Mo-DCs (mMo-DCs) are shown after co-incubation with Zymosan® (2 h) with a special focus on massive mitochondrial network generated especially in the dendrites of the mMo-DCs. The bar represents 10 µm

Functional capacities of produced Mo-DCs

Differentiation of monocytes toward an mMo-DC phenotype was observed for all the tested protocols, as proven by the downregulation of CD14 and the increased expression of the DC markers HLA-DR and CD209. Importantly, all the protocols induced functionally active Mo-DCs populations regarding the phagocyting activity (Fig. 3) and massive mitochondrial network presence (Fig. 4).

The capacity of DCs to engulf extracellular material, such as apoptotic/necrotic tumor cells, is critical for their role in antitumor immunity and is an indicator of functional quality of *ex-vivo* differentiated Mo-DCs. Therefore, we tested the impact of the different protocols on iMo-DC/mMO-DCs phagocytosis capacity of Zymozan® particles.

The results indicate that Mo-DCs produced with the different protocols present a similar capacity to internalize apoptotic/necrotic tumor cells. Mo-DCs presented engulfed material after 2–24 h of co-culture with Zymozan® (Fig. 3). However, these values are significantly lower ($p < 0.05$; $p < 0.01$) for the cells tested during the protocol with prolonged culture in CliniMACs Prodigy® chamber. These results indicate that the length of cultivation in the cultivation chamber during DC differentiation modulates their endocytic capacity.

mMo-DCs are inducers of T cell activation

T cell priming capacity of mMo-DCs was assessed in a mixed lymphocyte reaction (MLR). To this end, autologous, naive T cells (Non target Blood cells—NTBCs) were co-cultured (7/14 days) with mMo-DCs in different ratios (2:1, 4:1, 5:1, 10:1).

The gene expression results were generated comparing gene expression data in NTBCs (day 0, day 7) and mMo-DCs co-cultured NTBCs fractions (after culture day7, day 14). There were two co-cultured NTBCs fractions in total: 1. Adherent NTBCs. 2. Non-adherent (floating) NTBCs.

NTBCs (T cell) proliferation as confirmed by gene expression was elevated when mMo-DCs were used as antigen-presenting cells and added to the NTBCs in the ratio 1:5/ 1:10. This observation was in line with the result showing that gene expression of markers reporting successful T cell priming was elevated for genes in the floating cell subset: CD8A, CD4, MKI67, TNFA ($p \leq 0.01$).

The gene expression data are summarized in Table 2 with intention to compare all the tested cell subsets side by side. The relative gene expression is presented for samples in comparison to the starting leukapheresis cell mix.

We also sought to address whether the capacity of mMo-DCs to stimulate T cells were affected by the length of the co-culture. In concordance with expectation, T cells that were stimulated with mMo-DCs proliferated more frequently than the T cells without stimulus (alone).

Despite not being statistically significant, mDCs co-cultured with T cells longer, induced higher KI67 gene expression, which possibly confirms the ongoing proliferation of T cells ($p \leq 0.001$) if compared to the shorter co-culture protocols. Looking at each lymphocyte population individually, mMo-DCs co-cultured longer induced superior proliferation of $CD8^+$ T cells ($p \leq 0.001$).

Discussion

Mo-DCs continue to be used in clinical trials to boost the immune response, but many difficulties remain and prevent the widespread of DC vaccine immunotherapy [5]. These limitations include weak cellular responses, high cost and time-consuming processes [17]. The good safety profile of Mo-DCs antitumor vaccines and parameters of effectivity in inducing therapeutic outcomes are not being consistent so far. Effective responses are observed in less than 15% of the cases [18].

Monocyte enrichment is a key step to generate Mo-DCs for downstream applications, and various methods for enrichment have been discussed till today. However, the method for monocyte enrichment appears to have little impact on the quality of the resultant DCs as confirmed in previous studies [10]. The decision of which method to use for enrichment should consider various factors, including quantity and quality of starting material, purity required, maturation status of the final product, experience of personnel manufacturing the DCs, equipment currently available, and phase of the clinical trial [10]. It has been shown that using automatized enrichment CliniMACS Prodigy® technology for monocytes, could be of advantage in producing more homogenous monocyte-precursors.

The non-existence of standard operating procedures (SOPs) for ex vivo manipulation of Mo-DCs [4] results in a plethora of protocols that differ in the source of precursors, differentiation and maturation stimuli, antigen nature and loading procedures and, finally, route of administration [18]. Additionally, there are more reasons of why cancer patients present different DCs functionality. One of them is the lack of proper tumor antigens to activate Mo-DCs [15]. While extensive research has been performed on the impact of cytokines and growth factors used for DC differentiation and maturation, the relevance of delivered maturation antigens to these processes, has been underestimated.

Recently, cell lysates show the benefit of presenting a multitude of tumor protein both known and unknown, as well as mutated protein found in the tumor. These mutated proteins give rise to neo-antigens that overcome the problem of self-tolerance and thus are more immunogenic. Cell lysates seem

to have advantage over apoptotic bodies vaccines, presenting wider array of antigenic epitopes and stimulating a larger of cytotoxic lymphocyte repertoire [17, 19].

Circulating tumor cells were used to produce tumor lysates for several reasons. First, they are autologous, and to the point of the patient situation also real-time actual, we might call them personalized. "Personalized" antigen load was expected to generate "personalized" immune answer. For this type of study, it was crucial to show, that even the CTCs might produce immunogenic stimuli for Mo-DCs maturation process. A common way to produce cell lysate is to freeze/thaw cells for several cycles producing necrotic cell death. The manipulation with CTCs could be indeed very simplified, counting with CTCs enrichment and lysate preparation in time of Mo-DCs preparation. The process of cell lysis leaves cell membrane fragments, RNA and DNA in the lysate which provide danger signals promoting DC maturation [20]. As reported, cell lysate was added to culture media at ratios of 5:1 [21] and up to 1:1. This required access to a large amount of tumor material, which, particularly in the setting of small tumors, is difficult. We present application of CTC lysate (cellularity up to 1000 CTCs/1 ml lysate). The total volume of CTC lysate added to the cultivation chamber (400 ml) was relatively small (4 ml).

We cannot confirm that the real-time CTC-antigens were inducing the Mo-DC maturation towards generating active anti-CTC fighters, but we present a potential of the strategy to personalize anti-cancer effect influencing Mo-DCs maturation ex vivo using CTCs. From the literature, it has been known that even Mo-DCs without any loaded antigen helped patients to generate antitumor immune reactions if injected. Assuming this, we believe, only small numbers of CTCs might be a good antigenic source for mMo-DC generation.

It was observed that Mo-DCs expressing higher levels of CD80, CD83 and CD86 present superior capacity to polarize Th1 subset and to prime antigen-specific $CD8^+$ T cells [18]. In our study, the elevation of CD83 have been confirmed by flow cytometry only in 19% of the tested mMo-DCs, but the mMo-DCs were still able to prime T-cells in MLR. Gene expression of the co-cultured NTBCs and mMo-DCs cell mixture did not confirm elevation in CD83 expression, but CD80 and CD86 were significantly elevated, so one could expect that these co-stimulatory molecules would be sufficient for NTBCs and Mo-DCs interactions. Additionally, the gene expression data of "activated" NTBCs, co-cultured with mMo-DCs, confirmed the significant elevation in CD8, CD4, KI67 genes, which in turn of elevated TNFA might reflect the activation of the T-cell responses. In parallel with these genes, also MIF is elevated. Higher expression of MIF might reflect the proinflammatory situation in the in vitro co-culture. It has to be mentioned, that the data, even looking good, are generated in vitro, so that we have to discuss this success with caution. However, there is so far no better and faster option how to test the effect of mMo-DCs on autologous NTBCs, except multimarker gene expression analyses.

Inhibiting the programmed death-1 (PD-1) is one of the most effective approaches to enhance cancer immunotherapy, but its mechanistic basis remains incompletely understood. DCs represent a critical source of PD-L1, despite being vastly outnumbered by $PD\text{-}L1^+$ macrophages [22]. Considering the possibility of inhibiting (PD-1) pathway in presented gene expression analysis also PD-L1(CD274) expression was tested. Deletion of PD-L1 in DCs, but not macrophages, might restrict tumor growth and lead to enhanced antitumor $CD8^+$ T-cell responses [22]. Our data confirmed an elevated gene expression of PD-L1 in mMo-DCs fraction, signifying that mMo-DCs might play a significant role in the PD-L1/PD1 regulatory axis.

Considering the metabolic characters of the Mo-DCs differentiation processes it has been shown in previous work that differentiation of monocytes into Mo-DCs relies on the activation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) [23, 24]. This process is being supported by a catabolic metabolism dependent on oxidative phosphorylation (OXPHOS). As was shown, during the maturation process the DC transpose their mitochondrial network towards massively fused mitochondria, what would definitely stress the role of mitochondria in the process of DC-maturation. Opposite to OXPHOS, it has been previously reported, that upon activation of DCs, DCs were shifted to an aerobic glycolytic state contributing to the upregulation of co-stimulatory surface molecules, cytokine production and T cell stimulatory capacity [25, 26], this process might be associated with fused mitochondria too. We have observed massively fused mitochondria in mMo-DCs as shown in Fig. 4. Summarizing the data, one might expect, that massive mitochondrial network could be a pre-requisite of stimulated DCs as presented in our study.

Summarizing our data and doubts, we might conclude, mMo-DCs were generated using CliniMACS Prodigy® machine and CTC lysates in a homogenous manner showing a potential to generate NTBC activation in co-cultures.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00262-022-03189-2.

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