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An optimized method for the manufacture of a dendritic cell–based vaccine for translational glioblastoma research

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Introduction

Glioblastoma (GB) is the most prevalent and aggressive malignancy in the central nervous system (CNS). Immunotherapy represents a promising approach to support the immune system in the elimination of tumors, and dendritic cells (DCs) hold great potential for the induction of anti-tumor responses as shown in animal models[1-3] as well as various clinical trials[4,5]. DCs are professional antigen-presenting cells playing a central role in the regulation of the adaptive immune response via T cells and B cells. Remarkably, DCs incubated with tumor lysates or peptides generate specific anti-tumor responses in the CNS[6-10].

For the manufacture of DC products that are appropriate for translational research towards immunotherapy, it is mandatory to follow good manufacturing practice (GMP) guidelines. Moreover, a high level of standardization is required to ensure maximum reproducibility.

We developed an optimized method for the production of a DC-based vaccine, involving i) the automated isolation of $CD14^+$ monocytes from peripheral blood mononuclear cells (PBMCs) using the closed CliniMACS® System, ii) differentiation of monocytes into immature DCs (iDCs), iii) standardized and reproducible preparation of autologous tumor lysates using the gentleMACS™ Dissociator, and iv) efficient maturation of iDCs into mature DCs (mDCs) using the tumor lysates.

The resulting mDCs were functional as evaluated using a mixed lymphocyte reaction (MLR). This method is reproducible and conforms to GMP guidelines for pharmaceutical products, as assessed by microbiological safety, viability, phenotype, and functionality of mDCs produced.[11]

Materials and methods

The study was approved by the local institutional review board of the Fondazione IRCCS Istituto Neurologico Carlo Besta (Milan, Italy), and informed written consent was obtained from all patients. The whole process was performed in the clean-room facility of the "Cell Therapy Production Unit" in the Istituto Neurologico Carlo Besta.

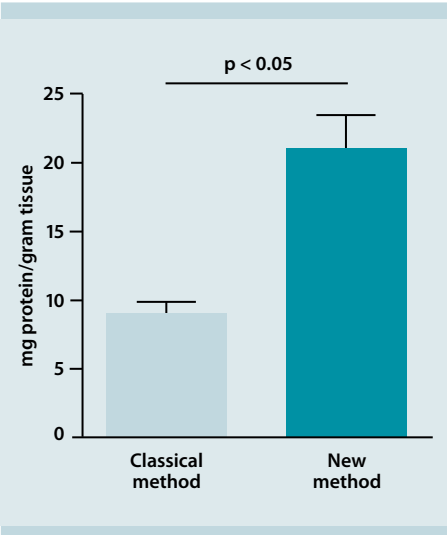

Figure 1 Comparison of the new tumor lysis protocol vs. a traditional standard method. Lysates were prepared as described in the materials and methods section. Data indicate means±sd from 12 and 19 preparations using the classical and new protocol, respectively. Data were adapted from ref. 11.

DC culture

$CD14^+$ cells were isolated from leukapheresis products from GB patients using the CliniMACS System (Miltenyi Biotec) according to the manufacturer's guidelines. The positive fraction was cultured at $3–5\times10^6$ cells/mL in a VueLife® Closed Culture Systems (Afc) in CellGRO® Medium (CellGenix), supplemented with 20 ng/mL IL-4 and 50 ng/mL GM-CSF. All reagents were of clinical grade. On day 5 of culture, iDCs were pulsed with autologous tumor lysate (see below) at the concentration of 50 µg protein/10^6 living cells plus 50 µg/mL keyhole limpet hemocyanin (KLH, Calbiochem), and 10 ng/mL IL-4 and 25 ng/mL GM-CSF for 24 hours. On day 6, antigen-loaded DCs (aDCs) were cultured with a pro-inflammatory cocktail including 10 ng/mL of TNF-α, IL-1β, IL-6, and 1 µg/mL PGE_2. After 24 hours, mDCs were collected and frozen. All samples were stored in a GMP-dedicated area and managed with a Good Automated Manufacturing Practices-4 (GAMP 4) software.[11]

Preparation of tumor lysates

GB specimens removed during surgery were used for the preparation of tumor lysates. Samples were washed in a sterile 0.9% NaCl solution, weighed, and snap-frozen in nitrogen gas until use. For tumor lysate preparation two alternative methods were applied.[11]

The "classical" method was previously described by Ashley and colleagues[12]. In brief, tumor samples were minced and mechanically dispersed using syringes with decreasing

needle sizes (18G, 20G, and 21G). The single-cell suspension was diluted in PBS and centrifuged at 300×g for 5 minutes. The pellet was resuspended in PBS and filtered through 70 µm and 30 µm filters. The suspension was centrifuged at 300×g for 5 minutes and sonicated in a bath for at least 1 hour.

In our new method tumor samples were minced, transferred to a gentleMACS C Tube (Miltenyi Biotec) and mechanically dissociated with the gentleMACS Dissociator (Miltenyi Biotec) using the installed software program "m_spleen04". The resulting single-cell suspension was centrifuged at 1000×g for 10 minutes. The pellet was resuspended in PBS, transferred to a gentleMACS M Tube (Miltenyi Biotec) and homogenized with the gentleMACS Dissociator using program "protein_01". The homogenate was filtered through 70 µm and 30 µm filters and then sonicated for 30 minutes.[11]

For both methods the presence of latent live tumor cells was determined by trypan blue exclusion. If viable cells were present, further sonication steps were performed until 0% viability was obtained. Protein content was determined by reaction with bicinchoninic acid (BCA, Pierce Biotechnology/Thermo Fisher Scientific) following the manufacturer's instructions.

Flow cytometry

PBMCs from healthy donors and DCs harvested at different culture steps (iDCs day 5, aDCs day 6, mDCs day 7) were analyzed by flow cytometry using CD14-PE, CD80-FITC, CD83-PE, CD86-PE, and HLA-DR-FITC antibodies. Specific staining was determined with appropriate isotype controls.[11]

Mixed lymphocyte reaction (MLR)

PBMCs were isolated from patient or unrelated healthy donor blood by centrifugation over a Ficoll-Paque™ gradient and resuspended in CellGRO medium (CellGenix). For unidirectional MLRs 2×10^5 responder cells (PBMCs) were co-cultured with stimulating cells in a 96-well plate (Corning). Stimulating cells were 1×10^4 DCs, 2×10^5 autologous PBMCs (for auto-MLR, i.e., negative control), or 2×10^5 allogeneic PBMCs (for allo-MLR, i.e., positive control). Stimulating cells were pre-treated with mitomycin C (50 µg/mL, Sigma Aldrich®) for 20 minutes at 37 °C and used after extensive wash. After 5 days, 1 µCi

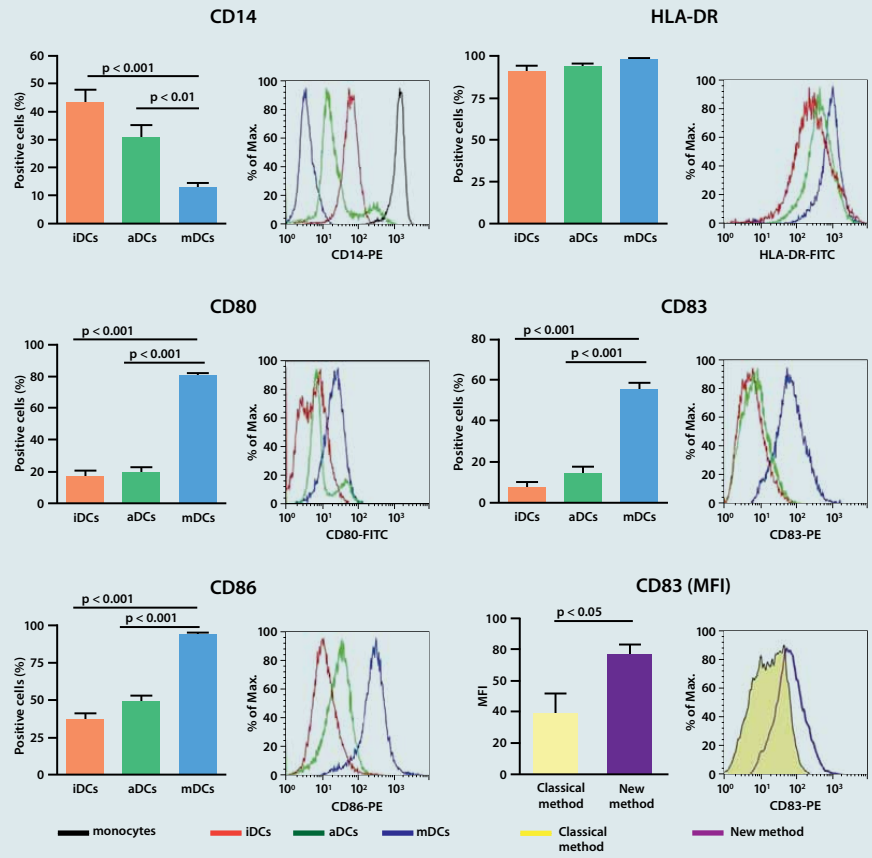

Figure 2 Phenotypic analysis of DCs. Cells were cultured as described in the materials and methods section. iDCs (day 5), aDCs (day 6), and mDCs (day 7) were stained with fluorochrome-conjugated antibodies and analyzed by flow cytometry. **(A)** The bar charts show percentages of the cell types that were positive for the respective markers. Data represent means±sd from 31 independent experiments. The histograms show the results from one representative experiment. **(B)** CD83 expression in mDCs that were prepared by using tumor lysates from either the classical or the new method. The bar chart indicates the MFI normalized to isotype controls (left bar: 12 experiments, right bar: 19 experiments). The histogram shows the results from one representative experiment. Data were adapted from reference 11.

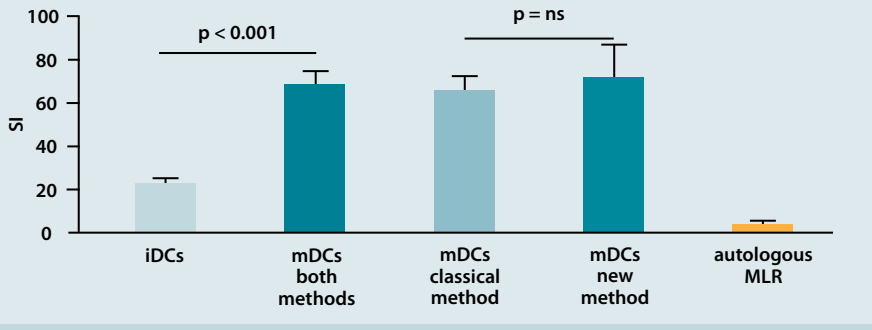

Figure 3 Functional analysis of DCs. The capacity of iDCs and mDCs to induce MLR was assessed as described in the materials and methods section. The results indicate means±sd from the following numbers of experiments: 18 (iDCs), 31 (mDCs both methods), 12 (mDCs classical method), 19 (mDCs new method), and 18 (autologous MLR). Data are expressed as stimulation index (SI) and represent means±sd. ns: not significant. Data were adapted from reference 11.

[^{3}H]-thymidine (Amersham Biosciences™) was added for further 18 hours. The radioactivity incorporated into DNA was measured in a β-scintillation counter. Results were expressed as stimulation index (SI) allowing the comparison of results from different donors. SI was calculated as follows: mean counts per minute (cpm) from stimulated cells divided by mean cpm from non-stimulated cells. MLR responses were considered positive when SI was ≥3 for PBMC-induced stimulation and SI ≥ 6 for DC-induced stimulation.[11]

Statistical analysis

One-way ANOVA and two-tailed test were utilized for all statistical analyses.

Results

Isolation of monocytes and preparation of mature DCs

DCs can be obtained through differentiation of CD14$^+$ monocytes into iDCs in the presence of GM-CSF and IL-4[13], and subsequent maturation to mDCs with a pro-inflammatory cocktail (TNF-α, IL-1β, IL-6, and PGE_2). As the first step, we isolated CD14$^+$ monocytes from leukapheresis products using the closed, automated CliniMACS System. In 31 preparations we achieved CD14$^+$ cell purities of 98.7±2.3% (mean±sd). A starting population of at least 5×10^9 WBCs was used for the isolation of CD14$^+$ monocytes to end up with a reasonable amount of DC vaccine, a final number of at least 55×10^6 mDCs. In 31 preparations, we used $(11.52\pm4.0)\times10^9$ WBCs (mean±sd), which resulted in $(1.3\pm0.5)\times10^9$ CD14$^+$ monocytes. Following differentiation and maturation, we obtained $(117.1\pm47.0)\times10^6$ mDCs, which corresponds to a yield of 1.2±0.7% and 9.1±3.1%, in relation to the numbers of WBCs and CD14$^+$ monocytes, respectively. Cell viability after one freeze-thaw cycle amounted to 94.3±3.9% (mean±sd).[11]

Novel method for the preparation of tumor lysates

DC-based vaccines can be generated by pulsing DCs with synthetic peptides or recombinant proteins representing certain tumor-associated antigens (TAA). However, expression of TAA in GB is heterogeneous, and selected peptides or proteins might not reflect the actual expression profile of relevant (and possibly unknown) TAA in a particular tumor. To tailor a DC-based vaccine to a specific glioblastoma, it is therefore crucial to pulse the DCs with autologous whole tumor lysates containing all potential TAA. Both soluble and membrane-bound proteins are extracted in our new method for the preparation of tumor lysates.[11] The procedure is highly standardized through automation, and takes place in a closed system. The process is highly efficient as reflected by a 130% increase in the protein yield compared to a traditional standard method (fig. 1). The high amount of protein in the tumor lysate allowed us to load all the iDCs obtained after monocyte differentiation.[11]

Phenotypic analysis of iDCs, aDCs, and mDCs

To evaluate the differentiation and maturation status of the DCs, we used the monocyte marker CD14 as well as the maturation markers CD80, CD83, and CD86. HLA-DR was used as a marker for antigen-presenting cells. Flow cytometric analysis showed that the maturation markers were significantly up-regulated in mDCs compared to iDCs and aDCs. CD14 was down-regulated accordingly. Expression of HLA-DR persisted indicating that the mDCs were functional antigen-presenting cells (fig. 2A).

CD83 is currently the most specific marker for mature DCs and plays a key role in the initiation of anti-tumor immune responses[14]. Therefore, we compared expression of CD83 in mDCs prepared with tumor lysates from either the classical method or the new method. The mean fluorescence index (MFI) of CD83$^+$ cells prepared with the new method was significantly higher (fig. 2B; 75.3±8.1 vs. 38.9±13.4; $p < 0.05$). The histogram in figure 2B shows that CD83 fluorescence was higher and more uniform in cells prepared with the new method, indicating that CD83 expression in these cells occurred at a higher level and more consistently than in cells prepared with the classical method. Thus, the lysate prepared with the new method resulted in a more efficient maturation of DCs.[11]

Functional analysis of DCs

To study DC functionality we used a one-way MLR with iDCs and mDCs as stimulating cells and PBMCs from unrelated healthy volunteers as responder cells. Overall, the mDCs induced a higher allogeneic MLR than iDCs (fig. 3), regardless of whether mDCs were prepared with lysates from the new method or the classical method. As a negative control, we also tested antigen-loaded mDCs in combination with PBMCs from the same patient prior to vaccination. We found that the mDCs did not induce autologous MLR.[11]

Conclusions

- The CliniMACS System enables isolation of CD14$^+$ monocytes with high purity.
- We developed a new procedure for the efficient preparation of tumor lysates in an automated, closed system using the gentleMACS Dissociator.
- mDCs pulsed with tumor lysates from the new method showed a higher and more consistent CD83 expression than mDCs pulsed with the classical method, indicating a more effective and uniform maturation of DCs by lysates from the new method.
- The mDCS are functional antigen-presenting cells as assessed by MLR.
- The high yields of isolated monocytes and tumor lysates enable the manufacture of mDCs in numbers that are sufficient for a clinical trial.

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