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Generation of dendritic cells from human peripheral blood monocytes - comparison of different culture media

Nataliya Tkachenko, Kamila Wojas, Jacek Tabarkiewicz and Jacek Rolinski

Department of Clinical Immunology, Medical University, Lublin, Poland

Abstract: Culture medium or medium supplement is one of the factors responsible for dendritic cell (DC) generation, but little is known about the influence of various media on DC culture. In our study we generated DC from adherent monocytes of human peripheral blood in the presence of GM-CSF, IL-4 and TNF-α. The following culture media were used: RPMI 1640 supplemented with 2% human serum albumin; RPMI 1640 supplemented with 2% TCH serum replacement; X-VIVO 15 and Panserin 501. Flow cytometry analysis revealed that in all media cells were CD83+ and lost CD14. Interestingly, the use of Panserin and RPMI with albumin preferentially gave rise to CD1a+ DC, whereas in X-VIVO and RPMI with TCH we observed both CD1a+ and CD1a-. Our results showed that RPMI with TCH yielded the highest percentage of cells expressing both CD80 and CD86 molecules and, in contrast to other media, the higher percentage of CD86+ cells in comparison to CD80+ cells.

Key words: Dendritic cells - Serum-free medium - Cell culture

Introduction

Dendritic cells (DC) are the most potent antigen presenting cells that play a pivotal role in the immune response by processing and presenting antigens to T-cells [2, 9]. Following the discovery of methods to generate large numbers of DC *ex vivo*, the use of DC as effective adjuvants for cancer treatment was demonstrated in clinical studies [8, 13, 15]. The interest of recent investigations is focused on the development of DC-based immunotherapy of human malignant diseases [22, 28]. So, there is a demand to establish reproducible and efficient methods to generate adequate number of fully functional DC for clinical applications. For the first time, human DC were cultured from CD34+ haematopoietic progenitors in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α) [5]. Then it was reported that DC can also be generated from nonproliferating CD14+ monocytes of human peripheral blood by cultivation with GM-CSF, IL-4 and maturation stimuli such as monocyte-conditioned medium (MCM), TNF-α, CD40L and PGE_2 [3, 17-19, 20, 26, 27, 29]. Some studies demonstrated DC culturing from cord blood CD34+ cells and monocytes [12, 30].

Correspondence: N. Tkachenko, Dept. Clinical Immunology, Medical University, Jaczewskiego 8, 20-090 Lublin, Poland; e-mail: natkaukr@yahoo.com

The method of DC generation from peripheral blood monocytes is widespread in experimental and clinical studies. It allows to obtain large numbers of monocyte-derived DC without pretreatment of donors with any cytokines to mobilize CD34+ stem cells from the bone marrow into the peripheral blood [18]. The DC obtained by this approach are mature and stable which is preferable for the purpose of immunotherapy [18, 26]. Moreover, this method gives a possibility to generate DC without involving xenologous proteins such as fetal calf serum (FCS), which can be potentially infective and immunogenic [3, 18]. Although the protocols for DC culturing have been developed, they are still not standardized and need further improvements for clinical use. It has been shown that generation of monocyte-derived DC is not only dependent on certain cytokines [24], but also on culture media and supplements [1, 7, 14, 16, 26]. However, little is known about the role of culture media in DC cultivation. The aim of the present study was to investigate the influence of different culture media on generation of DC from peripheral blood mononuclear cells (PBMC).

Materials and methods

Isolation of peripheral blood mononuclear cells (PBMC). Peripheral blood (50-100 ml) was obtained from 10 healthy volunteers by venous puncture and collected in sterile heparinized tubes. The blood was diluted 1:1 with phosphate buffered saline (PBS) without Ca^{2+}

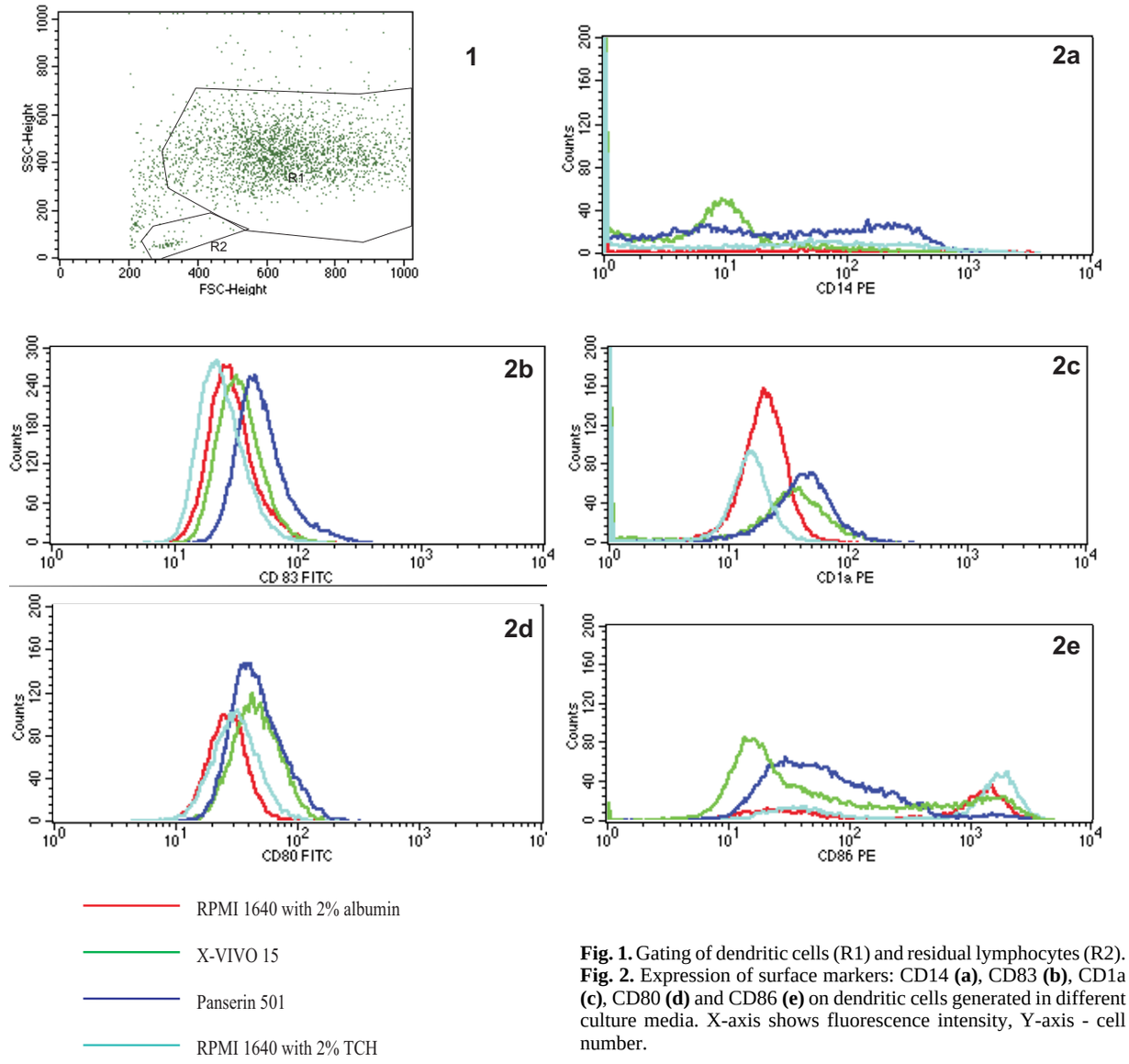

Fig. 1. Gating of dendritic cells (R1) and residual lymphocytes (R2).
Fig. 2. Expression of surface markers: CD14 **(a)**, CD83 **(b)**, CD1a **(c)**, CD80 **(d)** and CD86 **(e)** on dendritic cells generated in different culture media. X-axis shows fluorescence intensity, Y-axis - cell number.

and Mg^{2+} (used in all following experiments). PBMC were isolated by centrifugation in density gradient (Gradisol L, Aqua Medica, Poland) and washed twice in PBS before being resuspended in the appropriate assay medium.

Generation of monocyte-derived DC. PBMC were plated in 6-well tissue culture dishes (Greiner, Germany) at a density of 2×10^6 cells/ml. Four culture media were used: RPMI 1640 (PAN Biotech GmbH, Germany) supplemented with 2% human serum albumin (Bioplasma AG, Switzerland), which we used in the standard procedure; RPMI 1640 containing 2% TCH serum replacement (ICN Biomedicals, USA); X-VIVO 15 serum-free medium (Biowittaker, Walkersville, MD, USA); Panserin 501 with L-glutamine (PAN Biotech GmbH, Germany). All media were supplemented with penicillin (50 IU/ml), streptomycin (0.05 mg/ml) and neomycin (0.1 mg/ml) (Sigma, Germany). Mononuclear cells were incubated at 37˚C and 5% CO_2 for 90 min for monocyte adherence. After the incubation period, the non-adherent cells were removed by washing twice with the respective medium; the cell culture dishes were filled with cold PBS for 20 min at 4˚C and then finally washed with cold PBS. The adherent cells were cultured in the respective medium containing GM-CSF (1000 IU/ml, Leukomax Novartis, Switzerland) and IL-4 (500 IU/ml, Strathmann Biotec AG, Germany). Cytokines in the same dosages were added again on days 3 and 5; culture medium was not changed. The maturation of DC was induced by addition of TNF-α (50 ng/ml, Strathmann Biotec AG, Germany) on day 6. On day 8 cells were harvested and used for subsequent experiments.

Cell staining. Immunofluorescent staining was performed according to manufacturer's protocols. Cells were stained with the following directly conjugated monoclonal antibodies (MoAbs) in the following combinations: mouse isotype control IgG1/IgG2A/IgG2A FITC/PE/TC, CD45/CD14 FITC/PE, CD83/CD1a/HLA-DR FITC/PE/CyChrome, CD80/CD86/HLA-DR FITC/PE/CyChrome (HLA-DR from Pharmingen, USA; other MoAbs from Caltag, Bur-

Table 1. Immunophenotypes of cells cultured in different culture media

Cell surface immunophenotype (CD)	Percentages of cells in the studied culture media			
	RPMI 1640 with 2% albumin	RPMI 1640 with 2% TCH	X-VIVO 15	Panserin 501
45+14-	87.64 77.45-95.34	80.65 71.89-86.18	84.58 67.76-94.37	83.44 79.60-94.39
83+	37.29 26.95-47.56	55.41 16.28-68.45	48.10 35.53-66.07	61.31 45.11-64.47
1a+	28.63 13.96-55.19	23.52 8.98-37.59	38.43[b] 29.54-48.37	43.17[c] 33.31-64.07
83+1a+	22.34 11.20-32.70	17.96 8.55-34.88	28.22 20.33-37.47	40.37[c] 31.33-57.47
83+1a-	7.04[a] 2.12-14.86	13.70 8.19-40.15	15.78[d] 3.85-37.38	9.06 5.36-30.96
80+	70.40 44.09-83.94	80.05 60.34-91.58	56.21[b] 33.88-72.11	59.99[c] 44.28-61.16
86+	73.63[a] 45.51-83.75	87.12 78.37-93.67	55.88[b] 37.61-71.94	58.67[c] 44.03-77.56
80+86+	67.73 42.97-82.23	79.50 59.42-90.97	49.75[b] 33.58-71.86	57.23[c] 43.58-60.93

Results are expressed as medians and 25th-75th percentiles of 10 experiments. Significant difference ($p \leq 0.05$) between: [a]RPMI with albumin and RPMI with TCH, [b]X-VIVO 15 and RPMI with TCH, [c]Panserin and RPMI with TCH, [d]X-VIVO 15 and Panserin.

lingame, CA, USA). In brief, after culturing cells were collected, washed with PBS and incubated with MoAbs for 30 min at 4˚C. To minimize FcR-mediated MoAb binding, cells were stained in the presence of FcR-blocking reagent (Miltenyi Biotec, Germany).

Flow cytometry. Cells were analysed by three-color flow cytometry technique using Becton Dickinson FACSCalibur, equipped with 488 nm argon laser. A minimum of 30 000 events were acquired and analysed by CellQuest Software.

Statistical analysis. The nonparametric Wilcoxon matched pair signed rank test was applied. Differences were considered as statistically significant at $p \leq 0.05$.

Results

After culturing, all media gave comparable DC number (79.44%±10.98 in RPMI with albumin, 70.74%±12.04 in RPMI with TCH, 77.56%±14.08 in X-VIVO, 72.74%±22.56 in Panserin), with a significant difference between albumin- and TCH- containing media. The percentage of residual lymphocytes did not differ significantly between these media (7.61%±5.21 in RPMI supplemented with albumin, 10.19%±4.58 in RPMI with TCH, 11.49%±9.16 in X-VIVO 15 and 7.34%±5.17 in Panserin).

Analysis of surface markers expression of cultured cells is shown in a representative experiment (Fig. 1). The immunophenotypic characteristics of generated cells is presented in Table 1.

We found that all media led to the loss of monocyte surface marker CD14. There was also no significant difference in the percentage of cells expressing CD83 molecule, a marker of mature DC. The percentage of cells expressing CD1a, a marker of immature DC, was the lowest in RPMI with TCH, and it significantly differed from X-VIVO and Panserin.

We noticed a significant difference in the percentage of cells with fully mature DC phenotype (83+1a-) between RPMI with albumin and RPMI with TCH as well as between X-VIVO and Panserin. The percentage of cells with partially mature DC phenotype (83+1a+) was the highest in Panserin and the lowest in TCH-containing RPMI, with a significant difference between these media. Comparison of fully mature and partially mature DC showed that their percentages did not differ significantly in RPMI with TCH as well as in X-VIVO. The percentage of 83+1a+ cells was significantly higher in comparison to 83+1a- cells in albumin-containing medium and Panserin.

Our findings showed that RPMI with TCH yielded the highest percentage of cells expressing both CD80 and CD86 costimulatory molecules, and it significantly differed from X-VIVO and Panserin. Comparison of CD80+ and CD86+ cells demonstrated that the percentage of CD86+ cells was significantly higher than percentage of CD80+ cells in RPMI with TCH, while there was no significant difference between these cells in other media.

Discussion

Growth and maturation of DC in culture can be influenced by a variety of factors. One of them has been shown to be culture medium or medium supplement.

The generation of DC was described in media supplemented with FCS, human plasma or serum [1, 7, 16, 18]. For immunotheraupetic purposes it is desirable to avoid FCS because its potential immunogenicity. Serum or plasma contain growth factors or other substances, which may influence the generation of DC, so it is necessary to determine the appropriate concentration of autologous plasma or serum in DC generation for each patient. For serum-free cultures, X-VIVO, AIM-V and RPMI 1640 were used [7, 14, 18, 26, 27], also human albumin as protein component [1, 25]. However, the literature on the effects of different media on monocyte-derived DC generation is scarce.

In our study we investigated the influence of RPMI 1640 supplemented with 2% albumin, RPMI 1640 supplemented with 2% TCH, X-VIVO 15, and Panserin 501 on differentiation of DC from monocytes, with respect to their phenotype. All these media are serum-free and can be used for mammalian cell cultivation. The use of medium supplemented with TCH and Panserin for DC generation has not yet been described.

The number of cultured DC is one of important characteristics of DC generation. Such medium supplements as autologous plasma or serum, human serum and fetal calf serum had no influence on DC yields [7, 14]. Also it has been shown that there is no difference between RPMI 1640 and X-VIVO media in DC yields [7, 26]. In our experiment, all media gave comparable DC number. Nevertheless, the number of DC obtained in RPMI 1640 with albumin was significantly higher than in RPMI with TCH. Araki *et al.* observed that DC number in culture with 2% albumin was consistently higher than in cultures with autologous plasma or serum [1].

The morphological appearance of DC generated in X-VIVO was similar to that of cells cultured in RPMI supplemented with albumin: during the culture cells detached from plastic dishes and formed aggregates consisting of dendritic-like cells. However, X-VIVO contained smaller DC aggregates and occasional fibroblastic cells. In Panserin and RPMI with TCH we observed less cells with dendritic morphology, accompanied by fibroblast-like cells adherent to the culture plates. TCH-containing medium was found to have the strongest tendency to yield fibroblastic cells (data not shown).

Our experiments showed that cells obtained in different media displayed characteristic features of DC: they lost CD14, monocyte surface marker, and expressed CD83 molecule. CD83 is a typical marker of maturation on human DC [18, 31].

CD1a is expressed on Langerhans cells but peripheral blood DC never express it [9, 21]. It was also shown that during culture with GM-CSF and IL-4, monocytes differentiate into CD1a+ DC with further decreased CD1a expression after maturation [18, 32] . We noticed that DC cultured in X-VIVO 15 and RPMI with TCH consisted of quantitatively almost equal populations of CD1a+ and CD1a- cells, whereas RPMI supplemented with albumin and Panserin preferentially led to the generation of CD1a+ DC. In contrast to our data, some studies demonstrated that X-VIVO resulted in rather homogenous populations of mature DC, but these results were controversial. Thurner *et al.* reported that the use of X-VIVO 15 gave rise to CD1a- DC [26]. Other authors observed CD1a-positive DC generated in X-VIVO [7, 14, 30]. Interestingly, it was noticed that culture media with such supplements as autologous plasma or serum resulted in CD1a- and CD1a+ DC, whereas DC obtained in serum-free media mostly had high up-regulation of that marker [1, 7, 14, 16].

Our observation concerning albumin-supplemented RPMI is in agreement with results of Araki *et al.* who showed that monocytes differentiated into CD1a+ population in 2% albumin-supplemented culture [1]. The authors also noticed that DC obtained in albumin-containing medium were effective in phagocytic activity, similarly to DC generated in cultures with autologous plasma or serum. Although CD1a molecule is considered to be a specific marker of immature DC [9, 21], Thurner *et al.* found that fully mature DC generated in RPMI 1640 still expressed CD1a molecules [26]. Similarly, another investigation demonstrated that during DC generation from peripheral and cord blood monocytes in X-VIVO 15 medium even after addition of TNF-α, CD1a+ was further up-regulated in mature DC [30].

CD1a belongs to CD1 family of antigen presenting molecules that are responsible for presenting lipid and glycolipid antigens to T cells [4, 10, 23]. Thus, expression of CD1a+ on DC may be significant in creating non-peptide vaccines.

CD80 and CD86 are characteristic of mature DC. These costimulatory molecules initiate and stabilize DC interaction with T-cells through the corresponding ligands [2, 9]. In our study, the number of cells expressing both CD80 and CD86 was the highest in RPMI supplemented with TCH.

The expression of CD80 and CD86 molecules and their role are likely dependent on DC precursors [6, 30]. The data on the influence of medium or culture supplements on expression of CD80 or CD86 are controversial. Vries *et al.* reported that DC generated from monocytes of melanoma patients in AIM-V, X-VIVO 15 and RPMI 1640 media supplemented with human serum had comparable expression levels of costimulatory molecules [27]. In contrast to these findings, Duperrier *et al.* found that generation in human serum or autologous plasma resulted in higher number of DC with up-regulated CD86 expression in comparison with X-VIVO or FCS-supplemented media [7]. Describing DC generation in human plasma and FCS, Pietschmann *et al.* showed that FCS-derived DC had higher CD80 expression, while in

human plasma DC exhibited more mature profile and higher expression of CD86 [16]. Interestingly, we observed higher percentage of CD86-positive cells in RPMI with TCH, while there was no difference between cells expressing costimulatory molecules in other media. Different expression of CD80 and CD86 molecules in various culture media may be connected with the compensation of each other in costimulatory functions, which is supported by the fact that CD80 and CD86 provide similar costimulatory signals in T-cell response [11], but further investigations are needed for understanding of such influences.

We believe that our findings demonstrate the significance of culture medium role in DC generation from peripheral blood monocytes and will facilitate to work out a reproducible method that will be the most suitable for experimental and clinical applications.

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