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Allostimulatory activity as a criterion of the functional phenotype of human macrophages

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ABSTRACT

The functional phenotype of macrophages (Mφ) is determined by both differentiation factors and polarization stimuli. In mouse Mφ could be easily divided into the distinct Mφ subtypes. However, the identification of human M1 and M2 cells is much more difficult due to the lack of M1- or M2-specific markers. We assumed that the Mφ capacity to induce T cell proliferation in mixed leukocyte culture, or allostimulatory activity, may be a marker of Mφ functional phenotype. We compared the allostimulatory activity of Mφ differentiated with GM-CSF or M-CSF and polarized into M1, M2a, M2c subtypes using appropriate stimuli. GM-CSF-differentiated M1 Mφ showed pronounced allostimulatory activity whereas the polarization into M2a and M2c of GM-CSF-differentiated Mφ was associated with decreased allostimulatory activity. M-CSF-differentiated M1 Mφ demonstrated the moderate increasing of allostimulatory activity but its level has never reached that of GM-CSF-activated M1. The level of allostimulatory activity of M2a and M2c M-CSF-induced Mφ was comparable to that of GM-CSF-induced M2a and M2c Mφ. Thus, low allostimulatory activity is a common property of human M2a and M2c macrophages regardless of the differentiating factor and a polarizing stimulus and can be used to distinguish between M1 and M2 phenotypes.

1. Introduction

Macrophages (Mφ) are the key cells of innate immunity with a wide range of biological effects due to their great plasticity [1,2]. The functional phenotype of Mφ is largely determined by the activation conditions. Mφ with pro-inflammatory (pro-M1) and anti-inflammatory (pro-M2) activities is induced by differentiation factors, namely, GM-CSF and M-CSF. It is important to note that pro-M1 and pro-M2 macrophages are able to polarize further in both M1 and M2 phenotypes, and the type of differentiation factor predetermines the functional features of polarized Mφ. Thus, activation by LPS and IFN-γ induces pro-inflammatory, M1 phenotype. In turn, M2 phenotype is induced by IL-4/IL-13 (M2a), immune complexes (M2b), IL-10, dexamethasone, vitamin D3 or by apoptotic cell engulfment (M2c) (Table 1) [3–6]. Thereby, Mφ heterogeneity is determined by both activating stimuli and differentiation factors.

In contrast to murine Mφ that could be quite easily divided into the distinct Mφ subtypes by expression IL-10, NO, arginase-1, FIZZ-1, Ym-1 [7], the identification of human M1 and M2 cells is much more difficult due to the lack of M1- and M2-specific markers [8]. For example, human M2 macrophages express arginase-1 weakly, and M2-associated markers are present in M1 cells [9]. In addition, IL-10 is not necessarily increased in cultures of human M2 macrophages and, moreover, is also produced by M1 macrophages [10,11].

One of the functional parameters allowed discrimination of M1 and M2 Mφ, in our opinion, may be allostimulatory activity, i.e. capacity of Mφ to stimulate allogeneic T cell proliferation. Allostimulatory activity is an integral parameter determined by surface expression of HLA-DR as well as co-stimulatory/co-inhibitory molecules, cytokine/chemokine profile, expression of different enzymes, etc. Previously, we have shown GM-CSF-differentiated (pro-M1) Mφ demonstrated the significantly higher allostimulatory activity as compared to M-CSF-activated (pro-M2) Mφ [12]. Nevertheless, the allostimulatory activity of polarized M1 and M2 Mφ was not evaluated in these studies. It also remains an open question whether the allostimulatory activity of M1 and M2 cells can be determined by the type of differentiation factors (GM-CSF *vs* M-

Abbreviations: Mφ, macrophage(s); IFN-γ, interferon gamma; LPS, lipopolysaccharide; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cells; Dex, dexamethasone; TGF-β, transforming growth factor beta; MLC, mixed leukocyte culture; SI, stimulation index

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Table 1
Stimuli, functions and markers of different macrophages phenotypes.

	M1	M2a	M2b	M2c
Polarized stimuli	LPS; IFN-γ; TNF-α	IL-4; IL-13	Immune complexes and LPS	IL-10; GCs; TGF-β
Functions	Th-1 activation; inflammation; antiviral response; antitumor activity; host defense	Allergy; parasite destruction and encapsulation; Th-2 activation; anti-inflammatory activity; profibrotic	Immune regulation; Th-2 activation	Immune regulation; anti-inflammatory function; immunosuppression; matrix remodeling; tissue repair
Markers	CD68; CD86; CD80; MHC II; IL-1R; TLR2; TLR4; CD14; CD25; CD127; CD163; iNOS	CD163; D200R; CD209; MHC II; CD206; PPAR; IL-1R II; mouse only: Ym-1; Fizz-1; Arg-1	CD86; MHC II	Arg-1; CD163; CD206; CD150; SRA-1; TLR1; TLR8

CSF). Based on the above, the aim of the present study is comparative characteristic of allostimulatory activity of macrophages differentiated with GM-CSF or M-CSF and polarized into M1, M2a, M2c subtypes using appropriate stimuli.

2. Materials and methods

2.1. Generation of M0, M1, M2a, and M2c subtypes of macrophages

Twelve healthy donors aged 22–48 were included in the study. Informed consent was obtained in accordance with the Declaration of Helsinki. All the experiments using human samples were performed according to a protocol approved by the Institutional Review Board of the Research Institute of Fundamental and Clinical Immunology. Human peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich, St. Louis, MO, USA) of heparinized blood samples. PBMCs (4×10^6/ml) were plated into 12-well tissue culture plates. The monocytes were allowed to adhere for 1 h and cultured for 7 days in RPMI-1640 (BioloT, St. Petersburg, Russia) supplemented with 10% FBS (BioWest, France), 0.05 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.3 mg/ml L-glutamine, 1% nonessential amino acids, 100 μg/ml gentamycin and 50 ng/ml recombinant human GM-CSF (R&D Systems, Minneapolis, MN, USA) or recombinant human M-CSF (Sigma-Aldrich, USA) at 37 °C with 5% CO_2. On day 5, appropriate stimuli were added: LPS (10 μg/ml) (E. coli 0114:B4, Sigma-Aldrich) for M1 macrophages, IL-4 (20 ng/ml) (Sigma-Aldrich) for M2a, and dexamethasone (Dex, 50 ng/ml) (KRKA, Novo mesto, Slovenia) for M2c. Non-polarized Mφ (cultured without any additional stimuli) were used as a control (M0). On day 7, the macrophages were gently scraped off with a cell scraper followed by centrifugation and cell counting (Fig. 1, the scheme with the experimental procedure). Less than 2% cell death was observed in all conditions (by excluding trypan blue).

2.2. Phenotypic characterization of M1/M2 Mφ

For evaluation of the phenotype, macrophages were stained with CD14-FITC, CD206-PE, and CD163-PerCP (all antibodies from BD Biosciences, USA). The relative amounts of CD206+ and CD163+ cells were assessed in the gate of CD14+ cells by FACS (FACSCalibur, Becton-Dickinson, USA). To quantitatively determine the percentage of cells that are actively undergoing apoptosis PE Annexin V Apoptosis Detection kit was used (BD Biosciences, USA).

2.3. Allostimulatory activity of macrophages

Allostimulatory activity of polarized Mφ was determined by measuring allogeneic T cell proliferation in the mixed leukocyte culture (MLC). For this, PBMCs (1×10^5/well) were plated in 96-well tissue culture plates in RPMI-1640 (BioloT, St. Petersburg, Russia) with 10% inactivated donor serum (AB (IV) group) in the absence or presence of different Mφ subtypes (in the ratio PBMCs:Mφ 10:1). Cells were incubated for 4 days, followed by pulse-labelling with 1 μCi/well of [3H] thymidine for additional 18 h. The allostimulatory activity of Mφ was expressed by a stimulation index (SI) calculated as PBMC proliferation in the presence of macrophages/spontaneous PBMC proliferative responses.

2.4. Statistical analysis

Statistical analysis was performed using the STATISTICA software version 6.0 (StatSoft. Inc., USA). All data were displayed as median and inter-quartile range (IQR, 25–75% quartiles) or mean and standard error of mean (M ± SEM). The significance of data was determined using the Mann-Whitney nonparametric *U* test and Wilcoxon matched pairs test. Values of $p < 0.05$ were considered statistically significant.

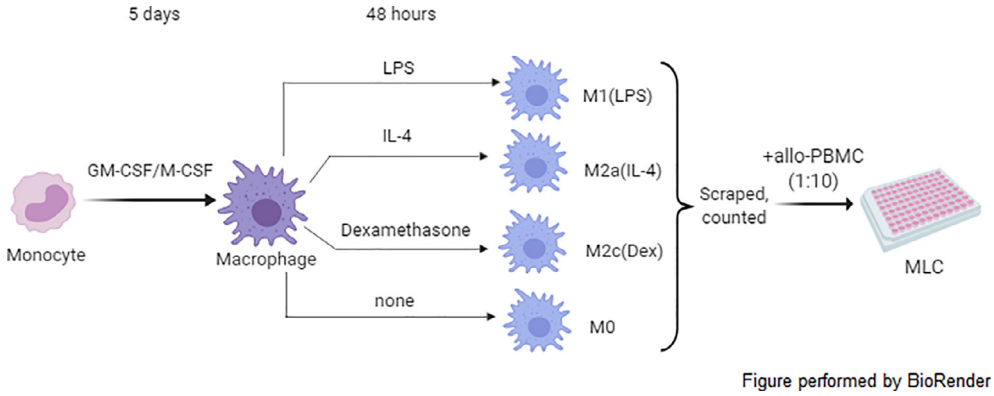

Fig. 1. The scheme with the experimental procedure.

The criterion of significance was *p < 0.05, **p < 0.01 ***p < 0.001.

3. Results

3.1. Characterization of GM-CSF-differentiated Mφ

To evaluate the morphology and the yield of different GM-CSF-induced macrophage subtypes, all macrophage subtypes (M0, M1(LPS), M2a(IL-4) and M2c(Dex)) were obtained from the same donor (n = 8). As shown in Fig. 2A, M0, M1, M2a and M2c macrophage cultures contained predominantly rounded shape cells and a small number of elongated fibroblast-like cells and did not differ from each other. Similar tendency was observed in eight donors.

At the same time, the analyzed cell cultures varied considerably in the yield of cells. Maximum number of Mφ was noted in M0 and M1(LPS) Mφ (Fig. 2B). However, adding IL-4 and Dex led to a 1.6-fold decrease in M2a(IL-4) (p = 0.08) and M2c(Dex) (p = 0.028) cell number. Analyzing possible reasons for reducing cell number, we suggested dexamethasone might have proapoptogenic effect on Mφ. Actually (Fig. 2C), there was increasing of relative numbers of cells with both AnnV+/7AAD− (early apoptosis; from 5 to 8%), and AnnV+/7AAD+ (late apoptosis; from 6 to 11%) phenotype in M2c(Dex) cultures.

For evaluating phenotypic features of Mφ subtypes, we analyzed relative amounts of CD206+ (as M2 marker) and CD163+ (as M2c marker) macrophages in CD14-positive cell population. As shown in Fig. 2D, M2c cells were characterized by a high CD206 and CD163 expression, while M2a macrophages had a lower one. In contrast, CD206 and CD163 expression by M1 macrophages was the lowest.

3.2. Characterization of M-CSF-differentiated Mφ

Next, we studied different M-CSF-induced Mφ and revealed a number of features. Firstly, stimulation with M-CSF revealed a different morphology that was manifested by an increase in the number of cells with fibroblast-like morphology (Fig. 3). A similar pattern was observed in M0, M1(LPS), M2a(IL-4) and M2c(Dex) cell cultures. Secondly, the number of non-polarized M-CSF-induced M0 macrophages was two times lower (3×10^5) than in GM-CSF-M0 cultures (6×10^5). Besides, the addition of polarizing stimuli (IL-4 or Dex) did not lead to a further decrease in the number of cells, as was shown for GM-CSF-differentiated macrophages (Fig. 3B). Another feature of M-CSF-differentiated Mφ is a high expression of M2-associated markers by LPS-stimulated M1 cells comparable to that showed in M2a(IL-4) macrophages (Fig. 3C).

3.3. Allostimulatory activity of GM-CSF- and M-CSF-differentiated Mφ

Investigation of allostimulatory activity of GM-CSF-induced Mφ showed that all Mφ subtypes could stimulate T cell proliferation in MLC but differed in magnitude of the stimulating effect (Fig. 4A). For example, LPS-stimulated M1 macrophages had the most pronounced allostimulatory activity with stimulation indexes (SI) ranged from 4.7 to 57.5 and significantly exceeded SI of non-polarized M0 cells (p = 0.008). Stimulation by IL-4 (M2a) and dexamethasone (M2c) led to generation of Mφ with significantly lower allostimulatory activity compared to M0 and M1(LPS).

When studying allostimulatory activity of various subtypes of M-CSF-differentiated Mφ, it was shown that M1(LPS) Mφ were found to have the highest allostimulatory activity, while M2a(IL-4) and M2c (Dex) enhanced allogeneic T cell proliferation only 1.58 and 1.65 times, respectively (Fig. 4B). Noteworthy, M2a(IL-4) and M2c(Dex) did not differ significantly from non-polarized M0 Mφ in allostimulatory activity. When compared to GM-CSF-induced cells, M-CSF-differentiated M0 and M1(LPS) showed significantly lower allostimulatory activity. At the same time, M2a and M2c cells were characterized by equally low allostimulatory activity, regardless of the type of differentiation factor.

Thus, different GM-CSF- and M-CSF-differentiated Mφ subtypes differ in their phenotype and allostimulatory activity. Indeed, M1(LPS) cells are characterized by low M2-associated marker expression and high allostimulatory activity while M2c(Dex) Mφ have minimal allostimulatory activity and high CD206 and, especially, CD163 expression levels. M2a macrophages have low allostimulatory activity, and intermediate expression of M2-associated antigens. Interesting, the number of CD206+ and CD163+ cells varied significantly among M2 Mφ (M2a+M2c), but their capacity to stimulate T cell proliferation in MCL was definitely low in all cases.

Based on these results, we analyzed the operational characteristics of the test to identify the Mφ phenotype, based on the evaluation of the allostimulatory activity of Mφ (SI) in the MLC. For this, a characteristic curve was constructed (receiver-operator curve, ROC). Fig. 5 demonstrated ROC of the stimulation index of M1 and M2 (M2a+M2c) GM-CSF-differentiated Mφ in the MLC. It shows that the area under the curve was 0.925 (95% CI 0.87–0.98, p = 0.0001) indicating the highest diagnostic accuracy of method. The next step was to determine the sensitivity and specificity for the various cut-off points. For the stimulation index of M1 and M2 (M2a+M2c) GM-CSF-differentiated Mφ, the separation point corresponding to the maximum sensitivity (86.96%) and specificity (81.48%) was a cut-off value of SI < 4.6 calc. units (likelihood ratio 4.70). For M-CSF-differentiated Mφ (Fig. 6) the area under the curve was 0.833 (95% CI 0.79–0.99, p = 0.0001). The separation point corresponding to the maximum sensitivity (81.67%) and specificity (88.89%) was a cut-off value of SI < 2.95 calc. units (likelihood ratio 7.30). Thus, the findings suggest that the allostimulatory activity of Mφ allows differentiating M1 and M2 macrophages with

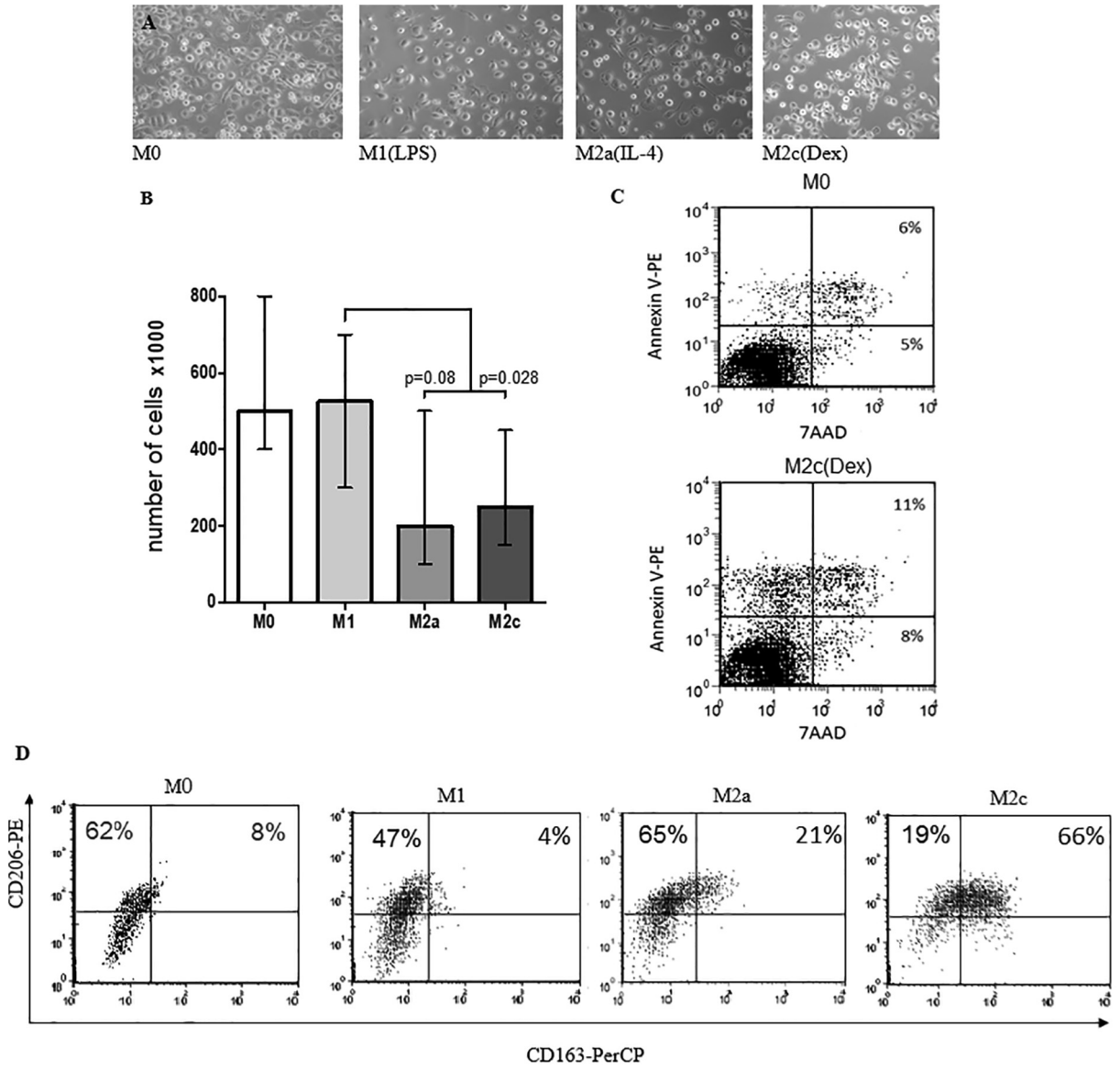

Fig. 2. Characteristic of different GM-CSF-differentiated Mφ subtypes A – 7-day cultures M0, M1(LPS), M2a(IL-4) and M2c(Dex) Mφ (native preparations, ×250 magnification). B – The yield of cells (a number of Mφ after 7 day of generation). Data are presented as median, n = 8. C – The relative number of AnnV + /7AAD − (early apoptosis) and AnnV + /7AAD + (late apoptosis) Mφ in presence/absence Dex. D – The relative number of CD206 + and CD163 + Mφ in the gate of CD14 + cells.

high specificity and sensitivity.

4. Discussion

The present study was aimed to a comparative evaluation of the morphology, expression of M2-associated markers and allostimulatory activity of various GM-CSF- and M-CSF-induced Mφ subtypes, and the rationale for the hypothesis that allostimulatory activity can be used to discriminate M1 and M2a/M2c cells is presented. It was shown M-CSF-differentiated Mφ differed by an increased number of fibroblast-like cells, but there was no difference between M0, M1, M2a(IL-4), M2c (Dex) obtained from GM-CSF- or M-CSF-induced macrophages. Polarized M2c(Dex) derived from both GM-CSF- and M-CSF-differentiated macrophages are characterized by highest expression of CD206 and CD163, while M2a macrophages differed from M1 in the expression of these antigens to a lesser extent.

In the most of studies GM-CSF is used for M1 generation and M-CSF – to obtain M2 cells [4]. Later it was shown that M1 and M2 polarized macrophages could be obtained from both GM-CSF- and M-CSF-induced Mφ [10], because not only differentiating factors, but also polarizing stimuli determine the Mφ final phenotype. Indeed, INF-γ, LPS or their combination can induce M1 Mφ; IL-4 or IL-13 (alone or in combination) can model M2a polarization; IL-10, TGF-β, or glucocorticoids are used for M2c polarization [13]. In the present study, we characterized GM-CSF- and M-CSF-differentiated macrophages polarized by LPS, IL-4 and dexamethasone for obtaining M1, M2a and M2c phenotypes, respectively.

Morphological features of macrophage subtypes are described quite rarely, which apparently is due to the lack of attributes unique to a particular Mφ phenotype. Our findings revealed the morphological differences depending on the differentiating factor used, i.e. prevalence of fibroblast-like cells in M-CSF-stimulated Mφ cultures. However, we did not see any influence of polarization stimuli (LPS, IL-4, or Dex) on the cell morphology.

Analyzing influence of differentiation/polarization factors on the cell yield, we revealed two-fold reduction in number of M-CSF-

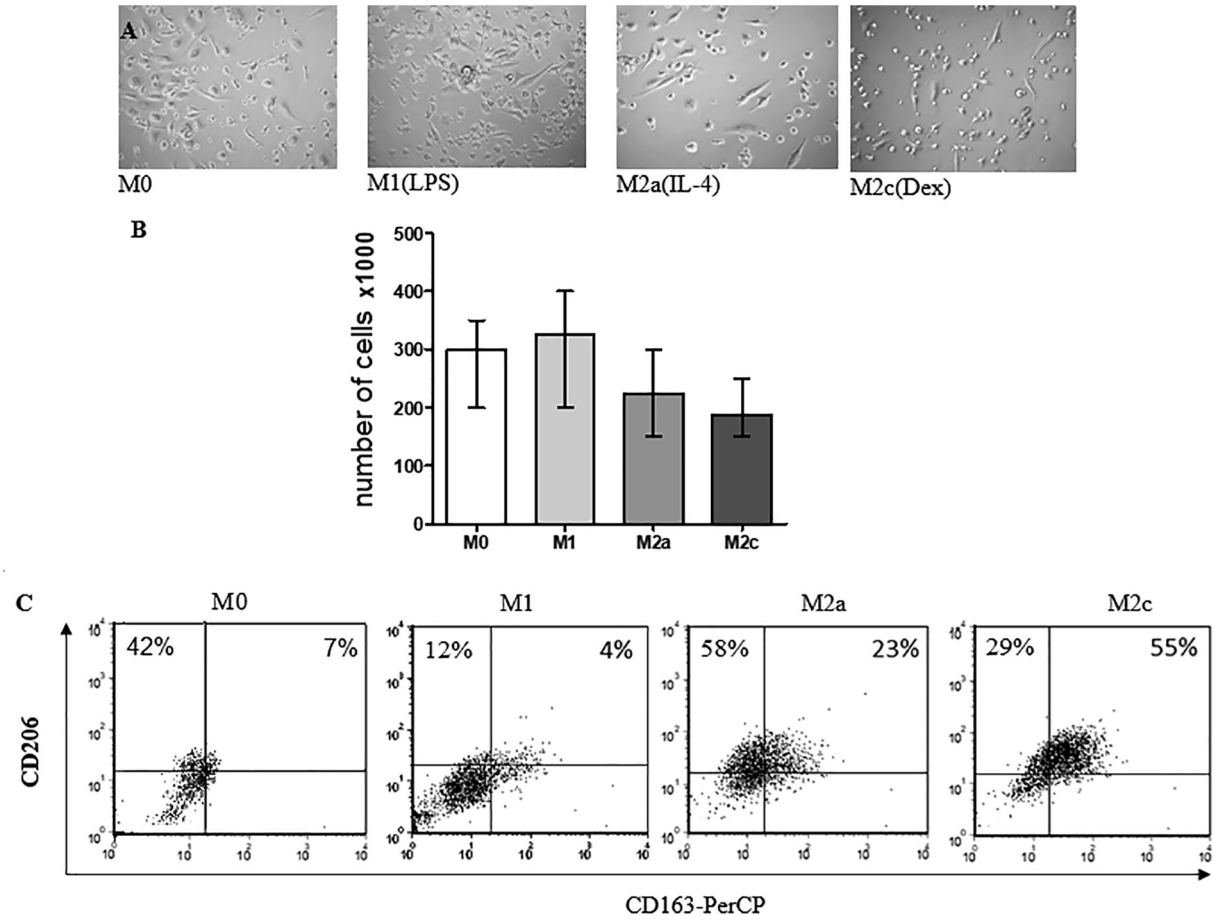

Fig. 3. Characteristic of different M-CSF-differentiated Mφ subtypes A – 7-day cultures M0, M1(LPS), M2a(IL-4) and M2c(Dex) Mφ (native preparations, ×250 magnification). B – The yield of cells (a number of Mφ after 7 day of generation). Data are presented as median, n = 4. C – The relative number of CD206+ and CD163+ Mφ in the gate of CD14+ cells.

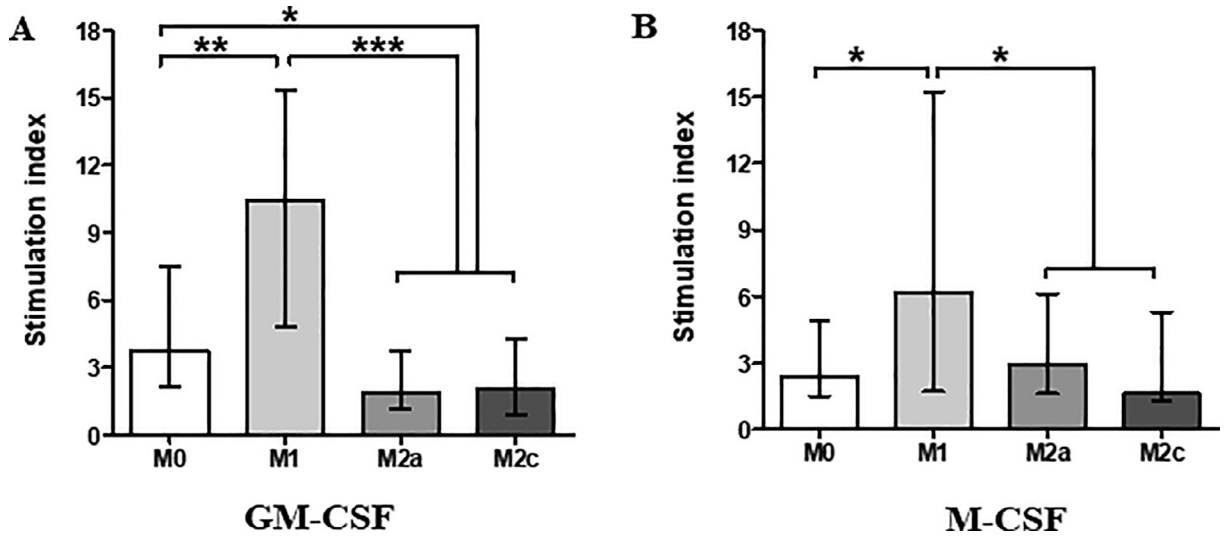

Fig. 4. Allostimulatory activity of GM-CSF- and M-CSF-differentiated Mφ subtypes A – Allostimulatory activity of different GM-CSF-differentiated Mφ subtypes. Data are displayed as median and inter-quartile range (IQR, 25–75% quartiles), n = 8. B – Allostimulatory activity of different M-CSF-differentiated Mφ subtypes. Data are displayed as median and inter-quartile range (IQR, 25–75% quartiles), n = 4. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

differentiated non-polarized M0 macrophages compared to GM-CSF-stimulated Mφ. Since M-CSF is "homeostatic" factor providing Mφ functions in «steady-state» conditions, these data seem quite logical. During inflammation, the production of GM-CSF increases [3] followed by an enhancement of macrophage proliferation [14], which is also confirmed by our data on the cell yield enhancement (6×10^5 GM-CSF-M0 versus 3×10^5 M-CSF-M0 after 7 days of culture).

It is noteworthy that adding of IL-4 and Dex led to decreased of M2a and M2c cell yields, regardless of differentiation factor used. The addition of IL-4 was accompanied by a significant increase in the number

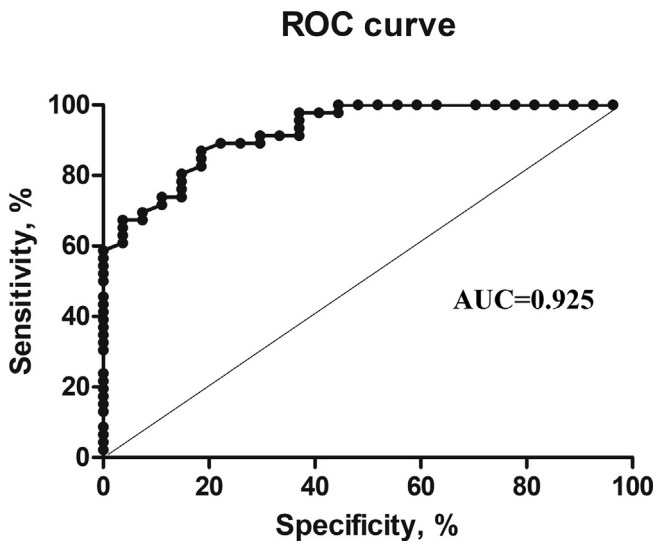

Fig. 5. ROC-curve that illustrates the ratio between sensitivity and specificity for the various cut-off points of stimulation index GM-CSF-induced M1 and M2 Mφ.

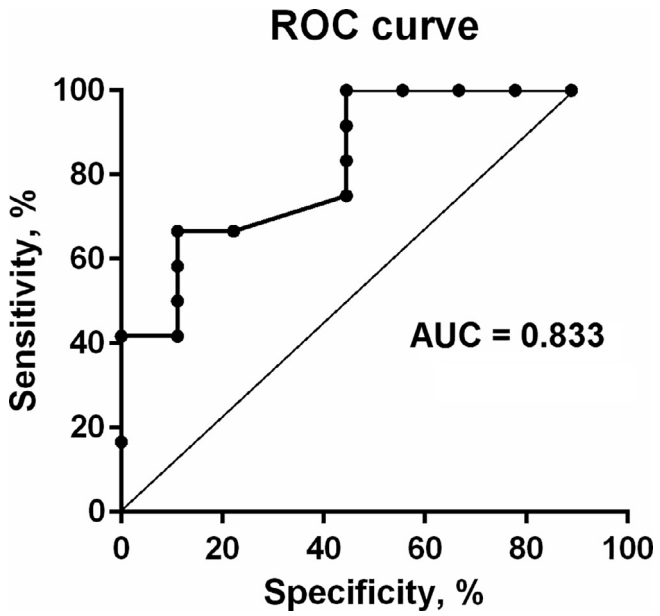

Fig. 6. ROC-curve that illustrates the ratio between sensitivity and specificity for the various cut-off points of stimulation index M-CSF-induced M1 and M2 Mφ.

of non-adherent cells in M2a(IL-4) cultures which may be due to the loss of adhesive properties of macrophages. This phenomenon requires further study. As for M2c macrophages, the low cell yield in these cultures may be due to dexamethasone-induced apoptosis.

Currently, there is no any specific marker which would allow uniquely identify a particular subtype of human macrophages [15]. The useful surface markers characterized M1 phenotype are CD68, CD86, CD80, MHC II; human M2a macrophages could be described by surface expression of CD163, MHC II, CD206, CD200R, CD209; useful human M2c markers are CD163, CD206 and CD150 [16]. According to our findings, M2a(Il-4) and M2c(Dex) are characterized by a high level of CD206 expression, while M2c(Dex) also express CD163, which is in line with other authors [17,18]. Treatment with dexamethasone to induce M2c phenotype up-regulates CD163 expression in both GM-CSF- and M-CSF-derived macrophages. Interestingly, non-polarized M0 and M1 macrophages also expressed CD206 although to a lesser extent than M2c(Dex) cells. These findings do not contradict the data describing M2-associated antigens on human M1 macrophages [9], but do not allow for a specific phenotypic profile (if any exist) for each Mφ subtype.

One of the premises of this study was the assumption that various types of Mφ can be determined using allostimulatory activity. Actually, allostimulatory activity reflects a number of Mφ characteristic, namely, HLA-DR expression, co-inhibitory and co-stimulatory molecules expression, cytokine production, capacity to induce regulatory T cells, etc. The data obtained showed LPS-stimulated M1 macrophages have the highest allostimulatory activity, and the stimulation index of GM-CSF-differentiated M1(LPS) exceeds that of the M-CSF – M1(LPS) (14.2 *vs* 7.7). Allostimulatory activity of M-CSF-induced M1(LPS) Mφ is comparable to that of GM-CSF-induced non-polarized M0 (7.73 and 6.2, respectively). In contrast, M2a(IL-4) and M2c(Dex) macrophages display a poor capacity to stimulate T cell proliferation, and the low level of the stimulating effect does not depend on the differentiation signal used (GM-CSF or M-CSF). Interestingly, M-CSF does not enhance the M2 phenotype of polarized macrophages, since the stimulation index of M-CSF-differentiated M2a and M2c macrophages in MLC (2.49 and 2.87) do not differ from those of GM-CSF-differentiated M2a and M2c (2.23 and 3.4).

The ROC-analysis showed that allostimulatory activity may be an effective method for distinguish M1 and M2a/M2c macrophages, since the area under the ROC curve is more 0.7, according to Swets [19], indicates a high diagnostic value of the test and the area under the ROC curve above 0.9 indicates highest diagnostic accuracy of method.

The data obtained demonstrate the potential possibility of generating the M1 phenotype under stimulation with M-CSF and M2 phenotype – with GM-CSF, thus expanding our knowledge on macrophages plasticity. Taken together, our data indicate an ambiguous effect of the differentiation factor on the functional activity of macrophages.

5. Conclusion

1. M1 Mφ have pronounced allostimulatory activity, and its level is determined by differentiation factors. Thus, GM-CSF-differentiated M1 Mφ have higher allostimulatory activity than M-CSF-differentiated ones.
2. M2a(IL-4) and M2c(Dex) Mφ displayed a lower capacity to induce T cell proliferation irrespective of differentiation signal, and M2 cells polarized by appropriate stimuli from GM-CSF- and M-CSF-differentiated Mφ have equally low allostimulatory activity.
3. Allostimulatory activity as a marker of the human Mφ functional phenotype may be used to distinguish between M1 and M2a/M2c Mφ.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contributions

EC and ES conceived and designed the experiments. AY and ES were principal investigators and take primary responsibility for the paper; AY, LS, AD and MT performed the experiments for this study; AY, ES and AO participated in statistical analysis; AY, ES and EC wrote the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2019.08.003.

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