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Research paper

Phenotypic and functional changes of GM-CSF differentiated human macrophages following exposure to apoptotic neutrophils

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ABSTRACT

The engulfment of apoptotic cells by monocytes and unprimed macrophages results in M2 polarization. In the current study, we investigated whether apoptotic cells influence the phenotypic and functional characteristics of GM-CSF-differentiated human macrophages (GM-Mφ). Our results demonstrate that GM-Mφ preincubated with apoptotic neutrophils (GM-Mφ_{Neu}) show significantly increased expression of CD206 and FasL and decreased capacity to stimulate allogeneic *T*-cell proliferation thus adopting M2 features. The 27-plex analysis demonstrates the down-regulation of 24 cytokines (including IL-10) in GM-Mφ_{Neu} cultures. In contrast, apoptotic neutrophils enhance PGE2 synthesis by GM-Mφ, and blocking PGE2 production with indomethacin restores an allostimulatory activity of GM-MφNeu. These data provide evidence that GM-Mφ following exposure to apoptotic cells acquire features of M2 cells. Given the global suppression of cytokine secretion, GM-Mφ_{Neu} resemble deactivated (M2c) macrophages, and their capacity to inhibit allogeneic *T*-cell proliferation appears to be mediated by an enhanced synthesis of PGE2 but not IL-10.

1. Introduction

Macrophages (Mφ) are a heterogeneous cell population which according to their microenvironment can differentiate into either a pro-inflammatory (M1) cells, also known as classically activated macrophages, or an anti-inflammatory alternatively activated (M2) cells [1]. Polarization of monocytes/macrophages into M2 cells may be induced by various stimuli: IL-4 and/or IL-13 (M2a); immune complexes and LPS (M2b); IL-10, TGFβ or glucocorticoids (M2c); adenosine, IL-6 (M2d), etc [2–4].

The uptake of apoptotic cells (efferocytosis) is another factor which plays an important role in M2 polarization [3,5,6]. Macrophage recognition of apoptotic cells induces an anti-inflammatory state, which is characterized by the release of immunosuppressive factors (IL-10, TGFβ, platelet activating factor (PAF), and prostaglandin E2 (PGE2) with concurrent decrease of proinflammatory cytokines (TNFα, IL-12, IL-1β and IL-8) [7–9]. Initially, the reduction of pro-inflammatory cytokine synthesis was considered to be mediated through the release of soluble suppressor factors [7,8,10]. However, later it had been shown that the decreased transcription of TNFα and IL-6 genes was observed early after the exposure to apoptotic cells, did not relate to the levels of suppressive factors and was associated with the inhibition of NF-κB [11,12]. Thus, an immediate activation of anti-inflammatory signaling pathways mediated NF-κB inhibition is another anti-inflammatory mechanism triggered by recognition of apoptotic cell molecules via phagocyte receptors. Emerging evidence indicates that miR-21 may be implicated in this process, since efferocytosis-induced miR-21 tempers LPS-induced NF-κB transcriptional activity and inflammatory response [13].

Despite the interest to the effect of efferocytosis on cytokine production, studies in the field are limited by a rather narrow spectrum of evaluated cytokines. A serious shortcoming of some studies is the evaluation of cytokines in macrophage cultures containing apoptotic cells, which can themselves release TGFβ and IL-10 [14,15]. Fairly conflicting data have also obtained concerning the impact of efferocytosis on IL-10 production which have been considered as a typical M2-associated marker [3,5,16,17]. Some studies demonstrate stimulatory effect of apoptotic cells on the production of IL-10 [8,12], whereas the others show the decreased IL-10 secretion following exposure to apoptotic cells [7,18]. In most studies polarizing effect of efferocytosis have been investigated using undifferentiated (naive) macrophages or monocytes. Meanwhile, in human, monocyte-to-macrophage

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differentiation is largely controlled by colony-stimulating factors [19]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) drives the generation of macrophages with a "proinflammatory" cytokine profile and some features of M1 cells (termed Mφ-1), whereas M-CSF stimulates differentiation of macrophages with an "anti-inflammatory" cytokine repertoire and some features of M2 macrophages (termed Mφ-2) [20,21]. Although GM- and M-CSF-differentiated macrophages have been considered as pro-M1 and pro-M2 cells, respectively [22], both macrophage subsets may polarize toward M2 cells. However, the type of differentiation growth factors (GM-CSF *vs* M-CSF) determinates functional profiles of polarized cells [16]. Elevated levels of GM-CSF in inflammatory environment favor expansion of GM-CSF-differentiated macrophages. In this case the uptake of apoptotic cells by pro-inflammatory macrophages is considered as a key mechanism governing resolution of inflammation [6,23]. However, GM-CSF-driven macrophages have significantly lower capacity to bind to and ingest apoptotic cells than M-CSF [24]. So, the important question to be investigated is, whether interaction with apoptotic cells induces an anti-inflammatory state of GM-CSF-differentiated macrophages, and if these cells share features of M2 cells.

The present work was aimed at the investigation of the influence of apoptotic cells on the functional phenotype of GM-CSF-differentiated macrophages (GM-Mφ). Our previous studies have shown that hallmark of human M-CSF-derived macrophages and M2-like cells (generated under low serum conditions) is a reduced capacity to stimulate proliferation of allogeneic T cells in mixed lymphocyte culture (allo-MLC). Besides, M2-like cells differ from conventional GM-CSF-treated macrophages by higher expression of CD206, B7-H1, TRIAL and FasL [25,26]. Therefore, to test changes in functional phenotype of GM-Mφ following contact with apoptotic cells, we measured the expression of M2-associated and pro-apoptotic molecules, allostimulatory activity, cytokine profile (using multiplex analysis) and release of PGE2 following short-term exposure of GM-Mφ to apoptotic neutrophils.

2. Materials and methods

2.1. Monocyte isolation and generation of human macrophages

This study was conducted in accordance with the principles of 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. Informed consent was obtained from all subjects who were peripheral blood donors.

Twenty eight healthy donors aged 23–38 years have been enrolled into the present study. Macrophages were generated from mononuclear cells (MNCs) obtained from donor's peripheral blood. MNCs were incubated in 6-well tissue culture plates (TPP, Switzerland) for 60 min, followed by the removal of non-adherent cells. The adherent fraction of MNCs was then cultured in RPMI-1640 medium supplemented with 0.05 mm 2-mercaptoethanol, 2 mm sodium pyruvate, 0.3 mg/ml L-glutamine, 1% non-essential amino acids (all reagents from Sigma-Aldrich), 100 μg/ml gentamycin, 5% autologous plasma and 2% fetal bovine serum (FBS, BioWest, France) in the presence of recombinant human GM-CSF (rhGM-CSF, 50 ng/ml, R&D Systems). In 7 days the GM-CSF-differentiated macrophages (GM-Mφ) were washed twice with ice-cold PBS and then gently scraped off with a cell scraper followed by centrifugation and cell counting.

2.2. Flow cytometry

For phenotypic evaluation, GM-Mφ were incubated with fluorescein isothiocyanate- (FITC-) or phycoerythrin (PE)-conjugated antibodies specific for human CD14, CD86, HLA-DR and isotype control (BD Biosciences, USA), and then were analyzed with a FACSCalibur using CellQuest software (BD Biosciences, USA). Cell staining for M2-associated and pro-apoptotic/inhibitory molecules was performed in the $CD14^{\pm}$ fraction using FITC-labeled anti-CD206 and PE-labeled anti-B7-H1, anti-CD200R, anti-FasL, and anti-TRAIL monoclonal antibodies (BD Biosciences).

2.3. Isolation of human neutrophils

Neutrophils were isolated by centrifugation on a stepwise gradient ficoll/urografin (density solution 1.119; 1.078 g/ml). A $CD66b^{+}$ cell count in the obtained population was 90% (IQR 73–95%). The isolated neutrophils ($3x10^6$/ml) were labeled with carboxyfluorescein succinimidyl ester (CFSE, 4 μM, Sigma) for 15 min. Next, they were washed and resuspended in RPMI-1640 with 10% FBS and incubated at 37° with 5% CO_2 for 24 h. The level of spontaneous neutrophil apoptosis was evaluated by flow cytometry (FACSCalibur, Becton Dickinson, USA) using Annexin V (AnnV) and Propidium Iodide (PI) staining (Apoptosis Detection Kit, Becton Dickinson, USA). An AnnV-positive and PI-negative early apoptotic cell count constituted 61% (IQR 40–66%), which was consistent with the literature data [27]. An $AnnV^{\pm}PI^{+}$ late apoptotic cell count along with the number of $AnnV^{-}PI^{+}$ necrotic neutrophils accounted for 12% (IQR 4–13%) and 1.0% (IQR 0.5–1.3%), respectively. Subsequently, the mentioned above fraction of neutrophils was used as a source of apoptotic cells.

2.4. Phagocytosis assay

To assess phagocytosis, CFSE-labeled neutrophils were exposed to spontaneous apoptosis (1–2 × 10^6 cells/ml at a 10:1 neutrophil to Mφ ratio). They were then added to the monolayer of generated GM-Mφ and incubated at 37° with 5% CO_2 for 60 min. At the following stage, the culture medium was removed; the cells were washed twice with ice-cold PBS and detached by gently scraping. The macrophages obtained in this way (GM-$M\varphi_{Neu}$) were tested for phenotype, allostimulatory activity and cytokine production. A two-colour flow cytometry (FACSCalibur) was used to evaluate the quantity of $CFSE^{+}$ GM-$M\varphi_{Neu}$ among $CD14^{+}$ cells. The relative amount of $CFSE^{\pm}CD14^{\pm}$ Mφ which ingested apoptotic neutrophils comprised 15% (IQR 12–21%).

2.5. Evaluation of allostimulatory activity of Mφ

The allostimulatory activity of Mφ was assessed in the mixed lymphocyte culture (MLC) by culturing Mφ (GM-Mφ or GM-$M\varphi_{Neu}$) with allogeneic donor MNCs (0.1 × 10^6/well) at a 1:10 Mφ to MNCs ratio. If necessary, GM-$M\varphi_{Neu}$ were pre-treated with indomethacin (Sigma-Aldrich) for 45 min, followed by three washes with RPMI-1640, and then added to allogeneic MNCs. Cell proliferation was measured radiometrically by [^{3}H] thymidine incorporation after 5 days. The stimulation index (SI) of Mφ in the MLC was estimated as a ratio of MNC proliferative response in the presence of Mφ to the level of spontaneous proliferation (without Mφ).

2.6. Quantification of cytokine levels

To evaluate the production of cytokines, either apoptotic cells (as it was described earlier) or the medium (as control) were added to the monolayer of generated GM-Mφ and incubated for 60 min. Neutrophils were then removed, macrophages were washed and cultivated in RPMI-1640 with 5% FBS for additional 24 h. Then supernatants from GM-Mφ and GM-$M\varphi_{Neu}$ cultures were collected and stored at −80 °C. A dual-laser flow cytometry system (Bio-Plex Protein Assay System, Bio-Rad, USA) was utilized for the evaluation of 27 cytokines according to the manufacturer's instructions. The percentage of suppression was calculated according to the formula: % = (1 − X/Y) × 100%, where X is cytokine concentration in GM-$M\varphi_{Neu}$ culture supernatants, and Y is cytokine concentration in the culture supernatants of intact GM-Mφ macrophages.