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Human macrophage polarization *in vitro*: Maturation and activation methods compared

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ABSTRACT

Macrophages form a heterogeneous cell population displaying multiple functions, and can be polarized into pro- (M1) or anti-inflammatory (M2) macrophages, by environmental factors. Their activation status reflects a beneficial or detrimental role in various diseases. Currently several *in vitro* maturation and activation protocols are used to induce an M1 or M2 phenotype. Here, the impact of different maturation factors (NHS, M-CSF, or GM-CSF) and activation methods (IFN-γ/LPS, IL-4, dexamethason, IL-10) on the macrophage phenotype was determined. Regarding macrophage morphology, pro-inflammatory (M1) activation stimulated cell elongation, and anti-inflammatory (M2) activation induced a circular appearance. Activation with pro-inflammatory mediators led to increased CD40 and CD64 expression, whereas activation with anti-inflammatory factors resulted in increased levels of MR and CD163. Production of pro-inflammatory cytokines was induced by activation with IFN-γ/LPS, and TGF-β production was enhanced by the maturation factors M-CSF and GM-CSF. Our data demonstrate that macrophage marker expression and cytokine production *in vitro* is highly dependent on both maturation and activation methods. *In vivo* macrophage activation is far more complex, since a plethora of stimuli are present. Hence, defining the macrophage activation status *ex vivo* on a limited number of markers could be indecisive. From this study we conclude that maturation with M-CSF or GM-CSF induces a moderate anti- or pro-inflammatory state respectively, compared to maturation with NHS. CD40 and CD64 are the most distinctive makers for human M1 and CD163 and MR for M2 macrophage activation and therefore can be helpful in determining the activation status of human macrophages *ex vivo*.

Introduction

Macrophages are highly plastic cells that respond to a variety of environmental cues by changing their phenotype and function. Circulating monocytes, the precursors of macrophages, pass the vascular endothelium to mature into macrophages in the peripheral tissues. In these tissues, macrophages can then be activated in various ways by endogenous or exogenous factors. To study macrophage activation *in vitro*, various stimuli are used to induce a particular macrophage activation phenotype (Ambarus et al. 2012a; Verreck et al. 2004). In general, macrophages can be 'classically' activated by interferon (IFN)-γ and lipopolysaccharide (LPS) resulting in a pro-inflammatory (M1) phenotype and with interleukin (IL)-4/IL-13, immune complexes or glucocorticoids to induce an 'alternatively' activated (M2) phenotype. The M2 phenotypes are further subdivided in M2a (after exposure to IL-4 or IL-13),

Abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; IFN(-γ), interferon(-γ); IL, interleukin; LPS(-EB), lipopolysaccharide; M0, unactivated matured macrophage; M1, classical activated macrophage; M2, alternatively activated macrophage; M-CSF, macrophage colony-stimulating factor; MFI, mean fluorescence intensity; MS, multiple sclerosis; MR, mannose receptor; NHS, normal human serum; TGF(-β), transforming growth factor (-β); TNF(-α), tumor necrosis factor (-α).

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M2b (immune complexes in combination with IL-1β or LPS) and M2c (IL-10, transforming growth factor [TGF]-β or glucocorticoids) (Martinez et al. 2008).

For studies on human M1 and M2 phenotypes, many maturation and activation regimens have been applied. For instance granulocyte macrophage colony-stimulating factor (GM-CSF) (as priming or maturation factor), and activation factors IFN-γ, LPS, or a combination of these stimuli are used for M1 activation. Whereas macrophage colony-stimulating factor (M-CSF) (as priming or maturation factor), and activation factors IL-4, IL-10, IL-13, or a mixture of these mediators are used for M2 induction (Durafourt et al. 2012; Mantovani et al. 2004). These different macrophage populations, M1 and M2, play different roles in various processes such as wound healing, tumor metastasis, and neuroinflammation (Bogels et al. 2012; Lucas et al. 2010; Vogel et al. 2013).

In wound healing, ablation of macrophages results in delayed re-epithelialization, reduced collagen deposition, impaired angiogenesis, and delay of fibroblast migration (Goren et al. 2009; Leibovich and Ross 1975; Mirza et al. 2009). M1 macrophages produce pro-inflammatory cytokines and phagocytose microorganisms and matrix debris, features important in the early phases of healing. On the other hand, M2 macrophages contribute to repair by promoting angiogenesis, tissue remodeling and repair, due to the release of molecules such as vascular endothelial growth factor, TGF-β or fibroblast growth factor (Mantovani et al. 2003). Compared to healthy controls, M2 macrophages are more prominent in patients with for example kidney fibrosis, pulmonary fibrosis and sclerotic skin lesions. Additionally, there is accumulating evidence that M2 macrophages are involved in peritoneal fibrosis caused by peritoneal dialysis, a renal replacement therapy (Bellon et al. 2011; Wang et al. 2013). These findings suggest a prominent role for M2 macrophages in repair and pathogenesis.

In solid tumors, macrophages are the predominant immune cells and are correlated with high vessel density and tumor progression (Bingle et al. 2002; Mantovani et al. 2003). M1 macrophages are able to kill tumor cells *in vitro* (Allavena et al. 2008; Braster et al. 2013). In contrast, M2 macrophages facilitate tumor progression and invasion. M2 macrophages outnumber M1 macrophages in lung tumors (Zhang et al. 2011) and breast carcinoma (Lewis and Pollard 2006). In, amongst others, breast carcinoma the presence of M2 macrophages is correlated with poor prognosis and disease progression (Lewis and Pollard 2006). Whereas, M1 macrophages are the dominant phenotype in colon carcinomas, which is associated with diminished metastasis and increased patient survival (Bogels et al. 2012).

In neuroinflammation macrophages have a neuroprotective or neurodamaging role depending on their activation status (Huitinga et al. 1990; Kotter et al. 2005; Larsen et al. 2003; Shechter et al. 2013). In multiple sclerosis (MS), a neuroinflammatory disease, macrophages are the dominant cells in active lesions (de Groot et al. 1997).

The activation status of macrophages varies in different types of lesions. In active lesions, macrophages contain myelin and express both M1 and M2 markers, whereas in chronic active lesion, macrophages express M1 markers only (Vogel et al. 2013). Many maturation methods and activation protocols are available to induce macrophage polarization *in vitro*. Read outs for macrophage activation are morphology (Jaguin et al. 2013; Porcheray et al. 2005), marker expression (Czimmerer et al. 2012; Glim et al. 2013; Mantovani et al. 2004) and cytokine production (Durafourt et al. 2012; Gordon and Martinez 2010; Mantovani et al. 2003; Martinez et al. 2008). The morphology of macrophages is highly variable in culture (Jaguin et al. 2013; Porcheray et al. 2005). Common markers used to identify M1 or M2 macrophages in humans, are CD40, CD64, CXCL11, CCR7 and MR, stabilin-1, CD180, CD163, and TREM2 respectively (Czimmerer et al. 2012; Glim et al. 2013; Kzhyshkowska et al. 2004; Mantovani et al. 2004; Martinez et al. 2006; Takahashi et al. 2005; Varin and Gordon 2009). However, none of these markers is completely distinctive or specific for M1 or M2. The activation status of macrophages can also be determined by cytokine production, IL-12p40, tumor necrosis factor (TNF)-α, IL-6 for M1 and TGF-β and IL-10 for M2 (Durafourt et al. 2012; Mantovani et al. 2002). Here, we investigated and compared several well-known maturation and activation methods and studied the effect on macrophage morphology, marker expression, and cytokine secretion.

Materials and methods

Monocyte isolation

Blood monocytes were isolated from healthy donor buffy coats (Sanquin Blood Bank, Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll (Lymphoprep™, Axis-Shield, Oslo, Norway) density gradient, and subsequently monocytes were isolated from PBMCs using anti-CD14 magnetic beads (Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer's protocol.

For macrophage maturation, monocytes were cultured in 100 mm Ø plastic Petri dishes (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) at a concentration of 1×10^6 cells/ml in the presence of DMEM (Invitrogen, Breda, The Netherlands), supplemented 1% (v/v) penicillin–streptomycin–glutamine (PSG; Invitrogen), containing normal human serum (NHS 5%; Bio Whittaker, East Rutherford, NJ, USA), M-CSF (25 ng/ml; ImmunoTools, Friesoythe, Germany), or GM-CSF (10 ng/ml; ImmunoTools), at 37 °C, 5% CO_2. Fetal bovine serum (FBS 10%; Lonza Cologne GmbH, Walkersville, United States), was added to medium containing M-CSF or GM-CSF. After 5 days, macrophage viability and purity was determined by flow cytometry (FACSCalibur™, Becton Dickinson, Erembodegem, Belgium). Macrophage viability was assessed by staining the death cells with 7-aminoactinomycin D (7AAD; Molecular Probes Invitrogen, Eugen, USA) (data not shown). The cell population negative for 7AAD was analyzed further for CD68 expression (>99%).

Macrophage differentiation and morphology

The M1 phenotype was induced by culturing matured macrophages in 6-wells plates (Greiner Bio-One) for 2 days in the presence of 1×10^3 U/ml recombinant human IFN-γ (U-Cytech, Utrecht, The Netherlands). For the last 24 h, 10 ng/ml LPS-EB Ultrapure (InvivoGen, San Diego, USA) was added to induce an M1 phenotype. M2 macrophages were generated using 10 ng/ml human IL-4 (ImmunoTools), 10 ng/ml IL-10 (ImmunoTools), or 10 μM dexamethason (Sigma–Aldrich, St. Louis, MO, USA). Unactivated macrophages were cultured in medium and left untreated (M0 phenotype).

Macrophage morphology

For examination of the macrophage morphology, adherent cells were photographed (Leica DMIL and DFC420 C, Leica, Rijswijk, The Netherlands).

Floating versus adherent cells

To investigate the differences in marker expression between adherent and non-adherent macrophages, cells matured in NHS and activated by IFN-γ and LPS, IL-4 or left untreated as described above. After activation for 2 days, the non-adherent (floating) cells were collected with the supernatant. Then the adherent cells were harvested following treatment with lidocaine (4 mg/ml; Sigma–Aldrich) and subsequently scraped. The expression of