CHAPTER 3

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 markers 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.¹ ² 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), M2b (immune complexes in combination with IL-1β or LPS) and M2c (IL-10, transforming growth factor (TGF)-β or glucocorticoids).³

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.⁴ ⁵ These different macrophage populations, M1 and M2, play different roles in various processes such as wound healing, tumor metastasis, and neuroinflammation.⁶ ⁷ ⁸

In wound healing, ablation of macrophages results in delayed reepithelialization, reduced collagen deposition, impaired angiogenesis, and delay of fibroblast migration.⁹ ¹¹ 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.¹² 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.¹³ ¹⁴ 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. M1 macrophages are able to kill tumor cells in vitro. In contrast, M2 macrophages facilitate tumor progression and invasion. M2 macrophages outnumber M1 macrophages in lung tumors and breast carcinoma. In amongst others, breast carcinoma the presence of M2 macrophages is correlated with poor prognosis and disease progression. Whereas, M1 macrophages are the dominant phenotype in colon carcinomas, which is associated with diminished metastasis and increased patient survival.

In neuroinflammation macrophages have a neuroprotective or neurodamaging role depending on their activation status. In multiple sclerosis (MS), a neuroinflammatory disease, macrophages are the dominant cells in active lesions. 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.

Many maturation methods and activation protocols are available to induce macrophage polarization in vitro. Read outs for macrophage activation are morphology, marker expression and cytokine production. The morphology of macrophages is highly variable in culture. 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. 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. 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 x 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% CO₂. 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 two days in the presence of 1 x 10^3 U/ml recombinant human IFN-γ (U-Cytech, Utrecht, The Netherlands). For the last 24h, 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 DFC420C, 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 two 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 markers (CD40 and MR) of the adherent and non-adherent cells was analyzed by flow cytometry combined with Cellquest Pro software (Becton Dickinson) and FlowJo software version 9.4.0 for Microsoft (Tree Star, San Carlos, CA, USA). Data obtained using FACS analysis were presented as mean fluorescent intensity (MFI). The data from three separate experiments performed in duplicate were averaged and expressed as mean ± SEM.

**Macrophage marker expression**

After activation of the macrophages for 48h supernatant was collected, and adherent cells were harvested by scraping after incubation with lidocaine. Cells were fixed with PBS containing 4% formalin. Subsequently the macrophages were labelled for 1 h at room temperature with primary antibody (CD40, CD64, CXCL11, CCR7 as M1 markers, MR, stabilin-1, CD180, CD163, and TREM2 as M2 markers, Table 1) diluted in PBS containing 0.1% bovine serum albumin (BSA)/0.1% saponine. Cells were washed twice in PBS and incubated for 1 h (room temperature) with fluorescent labelled secondary antibody diluted in PBS 1% BSA, washed twice and resuspended in FACS buffer prior to analysis. Marker expression was analyzed by flow cytometry as described above.

**Cytokine production**

Cytokine production was measured in macrophage supernatants after 48h activation. TGF-β1 (Promega, Leiden, The Netherlands) was determined by ELISA.
according to manufacturer’s description. The levels of TNF-α, IL-1β, IL-6, IL-10, and IL-12p40 were analyzed with a Human Inflammatory 5-Plex Panel according to the users manual (Invitrogen) and measured by Luminex® 200™ (Bio-Rad, Hercules, CA, USA). Analysis was performed using Bio-plex Manager™ 6.0 software.

**Statistical analysis**
Statistical evaluation was performed by GraphPad Prism (GraphPad Software Inc., San Diego, USA), using a one-way ANOVA with Bonferroni correction or unpaired t-test. *P* values < 0.05 were defined as statistically significant.

**RESULTS**

**Macrophage morphology**
Morphology of adherent macrophages that matured and activated by different protocols was assessed after 7 days of culture. Striking differences were observed between the maturation by NHS, M-CSF or GM-CSF. Macrophages matured in the presence of NHS (untreated macrophages, M0) showed a mixture of round and spindle shaped cells (Fig. 1). Addition of M-CSF to the culture medium resulted in an increased amount of elongated macrophages, and maturation in the presence of GM-CSF resulted in mostly elongated macrophages.

After maturation with NHS, GM-CSF or M-CSF, and activation with IFN-γ/LPS (M1), macrophages were predominantly spindle-like shaped (Fig. 1). Macrophages matured with NHS, GM-CSF or M-CSF, and activated with IL-4 or dexamethason (M2) changed their morphology into a predominantly circular shape although in M-CSF or GM-CSF matured macrophage cultures some elongated macrophages were still present (Fig. 1). After maturation with NHS, and activation with IL-10, macrophages showed a circular morphology, while macrophages cultured in the presence of M-CSF or GM-CSF and subsequently activated with IL-10, showed spindle and circular morphology.

**Floating versus adherent macrophages: marker expression**
No papers describe in the methods section whether floating or adherent macrophages are used for further studies. To investigate whether there is a difference in marker expression between floating and adherent cells, monocytes matured to macrophages in the presence of NHS for five days and subsequently
Figure 1: Macrophage morphology upon different maturation and activation methods. Pictures of macrophages cultured for 7 days by different maturation and activation methods. Overall, macrophages matured in the presence of NHS have a circular morphology, excluding macrophages activated with IFN-γ/LPS, these show an elongated morphology. Macrophages matured in the presence of M-CSF or GM-CSF appear more elongated, however after activation with dexamethason the cells change to a more circular appearance. Data are representative for three separate experiments. Scale bar: 25 µm.
activated with IFN-γ/LPS (M1) or IL-4 (M2) for two days. The expression of both MR and CD40 was higher on adherent cells compared to the floating cells (Fig. 2). These differences in marker expression between floating and adherent cells could be an indication of a moderately different activation status of floating macrophages in comparison to adherent macrophages. Therefore, further experiments were conducted with only adherent macrophages.

Figure 2: Macrophage characteristics of adherent and non-adherent cells.
Monocytes matured to macrophages for 7 days in the presence of NHS and activated with IFN-γ/LPS or IL-4 or left unactivated (-). Both non-adherent (floating) and adherent cells were collected, and analyzed by flow cytometry. The plots represent the forward sideward scatter of the floating and adherent cells (A). Representative histograms of the MFI for MR and CD40 (B, C) are depicted. MR shows comparable levels for floating and adherent cells whereas CD40 shows a clear higher expression of CD40 on adherent cells compared to floating cells. Data are expressed as mean ± SD of three independent experiments. *= P<0.05, **= P<0.01

Marker expression on differently activated macrophages
Next, the effect of different maturation factors (NHS, M-CSF, or GM-CSF) and activation stimuli (IFN-γ/LPS, IL-4, dexamethason, or IL-10) on macrophage marker expression was investigated. CD40, CD64, CXCL-11 and CCR7 expression were determined since these were described to be enhanced in M1 macrophages. 5, 28, 30 In addition, MR, stabilin-1, CD180, CD163, and TREM2 were analyzed because they were reported to have an increased expression on M2 macrophages. 5, 27, 30-33
The expression of all tested markers was higher when macrophages matured in the presence of M-CSF compared to NHS. GM-CSF resulted in a significantly increased expression of CD40 and CD180, and to a lesser extent MR, stabilin-1 and CXCL11 (not significant; Fig. 3, S1).

Of the M1 markers, CD40 expression was increased by activation with IFN-γ/LPS, regardless of the maturation method (Fig. 3, S1). CD64 expression was significantly increased by activation with IFN-γ/LPS when cells matured by NHS. No differences in expression of CXCL11 between the various activation procedures were found. Macrophages matured with NHS or M-CSF had increased expression of CCR7 when activated by all activation methods but this increase was not significant compared to unactivated macrophages (Fig. 3, S1).

The M2 marker MR was significantly increased by maturation with NHS and subsequent IL-4 activation (Fig. 3, S1). Expression of stabilin-1 and CD180 was not significantly different after activation when compared to the unactivated condition. Finally, upregulation of CD163 was only observed when cells were exposed to dexamethasone after NHS, M-CSF, or GM-CSF maturation (Fig. 3, S1). TREM2, however, was not expressed on macrophages, nor enhanced by any maturation or activation method (data not shown).

**Cytokine production by differently activated macrophages**

Finally, IL-12p40, TNF-α, IL-6, IL-1β (for M1), IL-10 and TGF-β (for M2), were determined in macrophage supernatants. IL-12p40 production was not induced by one of the maturation factors (Fig. 4A). Compared to unstimulated, activation with IFN-γ/LPS resulted in enhanced IL-12p40 production, which was significant when cells matured with M-CSF and GM-CSF.

Macrophages matured in the presence of GM-CSF produced significantly more TNF-α compared to NHS or M-CSF matured macrophages (Fig. 4B). Upon activation with IFN-γ/LPS, macrophages produced increased levels of TNF-α in all three maturation conditions, compared to unactivated macrophages. Significantly higher levels of TNF-α upon activation with IFN-γ/LPS were produced by macrophages matured with GM-CSF or M-CSF compared to macrophages matured with NHS. Finally, only GM-CSF matured macrophages showed increased levels of TNF-α upon IL-4 activation (Fig. 4B).

Maturation with GM-CSF and M-CSF resulted in a slight but not significant IL-6 production compared to NHS (Fig. 4C). Increased levels of IL-6 were measured after activation with IFN-γ/LPS in all three maturation conditions; the highest
Figure 3: Marker expression on differently matured and activated macrophages.

Macrophages were cultured upon maturation by NHS (open bar), M-CSF (black bar), or GM-CSF (grey bar), and further activated with IFN-γ/LPS, IL-4, dexamethason (Dex), IL-10, or left untreated (-) for two days. Markers reported for M1 (left graphs) and M2 (right graphs) macrophages were determined by FACS. MFI was measured and depicted as relative expression as compared to NHS unactivated condition. Represented is the mean of three donors ± SD. Significance was determined as compared to control maturation (NHS, M-CSF, or GM-CSF only). *= P<0.05, **= P<0.01, ***= P<0.001. Significance in marker expression between NHS, M-CSF, or GM-CSF maturation was also calculated and represented by the following symbols: ≠= P<0.05, ≠≠≠= P<0.001.
Figure 4: Cytokine secretion by differently matured and activated macrophages.

Macrophages matured in the presence of NHS, M-CSF, or GM-CSF, with additional activation of IFN-γ/LPS, IL-4, dexamethasone (Dex), IL-10, or left untreated (-) for two days. Supernatant was harvested and the following cytokines were determined: IL-12p40 (A), TNF-α (B), IL-6 (C), TGF-β1 (D). Represented is the mean of three donors ± SD. Significance was assessed as compared to NHS maturation (*), and activations were compared to unactivated in all separate graphs (#). #/#/#/# P<0.05, ##/##/#/#/# P<0.01, ###/###/#/# P<0.001.
concentration was found when cells matured in NHS. Maturation with GM-CSF and activation with IL-4 resulted in significantly higher levels of IL-6 production than unactivated macrophages matured with NHS.

Macrophages showed increased levels of TGF-β1 production when matured with M-CSF or GM-CSF, compared to maturation with NHS (Fig. 4D). Macrophages matured with NHS, did not show significant changes upon activation with IFN-γ/LPS, IL-4, dexamethason, or IL-10. Macrophages matured with M-CSF and GM-CSF and activated with all activation factors (IFN-γ/LPS, IL-4, dexamethason, IL-10) showed increased levels of TGF-β1 compared to NHS matured and activated macrophages (Fig. 4D).

Levels of IL-1β and IL-10 were neglectable and consequently, results are not shown.

DISCUSSION

Here, we have performed a comparative study to examine the impact of the different maturation and activation stimuli on morphology, macrophage marker expression and cytokine secretion. Our data show a complex pattern of phenotypical changes in human macrophages after different maturation and activation protocols. This study should aid translational studies on macrophage profiling in human tissues in health and disease, but also shows the complexity of the use of activation markers.

Maturation and activation protocols have clear effects on morphology, most likely by affecting cytoskeletal arrangements. Maturation in the presence of M-CSF resulted in more spindle-like shaped macrophages as compared to maturation with GM-CSF which led to a mixture of circular and elongated macrophages as described previously.25, 35, 36 Macrophages showed a more spheroid morphology after activation with IL-4 or dexamethason, whereas activation by IFN-γ/LPS resulted in increased numbers of in elongated macrophages. This is in line with reports that show that IFN-γ/LPS acts on the cytoskeleton of the macrophage, stimulating macrophages to stretch.26, 37 Remarkably, opposite effects were found when studying mouse macrophages; pro-inflammatory macrophages appeared more circular while the anti-inflammatory cells were elongated.38

Maturation in the presence of MCSF or GM-CSF led to increased expression of most markers tested in this study. CD64 and CD40, which are known to be M1 markers28, 8 were higher expressed after activation with IFN-γ/LPS. Of the
markers related to M2 macrophages, only MR was upregulated when matured by NHS and then activated with IL-4. As detected by Gratchev et al., CD163 was significantly induced by dexamethasone, in particular when macrophages matured in the presence of M-CSF. The scavenger receptor CD163 is regulated by anti-inflammatory stimuli, but based on our data only by dexamethasone and not by the anti-inflammatory cytokines IL-4 and IL-10. Activation by dexamethasone has also been shown to be required for stabilin-1 expression on macrophages, but our present data show no induction of stabilin-1 expression after activation with dexamethasone. We conclude that no single marker is typical for M1 or M2 activation, although CD40 and CD64 are the most distinctive markers for human M1 and CD163 and MR for M2 macrophage activation. Therefore, identification of these markers can be helpful in determining the activation status of human macrophages ex vivo.

M-CSF and GM-CSF used as maturation factors already prime or induce activation of macrophages, as indicated by an increased cytokine production, compared to NHS. We showed significant more IL-12p40, IL-6 and TNF-α production by macrophages activated with IFN-γ and LPS, independently of the maturation method (NHS/M-CSF/GM-CSF). We are the first to show that maturation with M-CSF which is considered to be an inducer of the M2 profile, results in an increased production of TNF-α compared to naïve macrophages. Macrophages matured in the presence of GM-CSF and activated with IL-4 are referred to as pro-inflammatory monocyte-derived dendritic cells, which produce TNF-α. We used different concentrations for the maturation of macrophages, compared to monocyte-derived dendritic cells and found similar results for monocytes derived macrophages.

In contrast to M1 macrophages, M2 polarized macrophages are characterized by the production of anti-inflammatory mediators as IL-4, IL-10, and TGF-β. IL-10, a widely studied cytokine produced by anti-inflammatory macrophages, was under detection levels in our hands. Verreck et al. showed an upregulation of IL-10 production on M-CSF activated macrophages compared to GM-CSF activated macrophages. However, our activation method is slightly different; we used smaller concentrations of M-CSF and GM-CSF as maturation factors, which could be an explanation for our results. In addition, other studies detected increased levels of human IL-10 by LPS stimulation and absence of IL-10 production by IL-4 stimulation. Based on that, we suggested IL-10 production not relevant as marker for human M2 macrophage identification. TGF-β is important in regulation
Figure 5: Schematic overview at a glance.

*In vitro* cultured monocytes matured to macrophages by adding NHS, M-CSF or GM-CSF as represented in this figure. Maturation with M-CSF polarizes macrophages towards a partial anti-inflammatory phenotype (light green), whereas GM-CSF maturation activates a macrophage to a moderate pro-inflammatory phenotype (vague red). Furthermore, activation of the differently matured macrophages with IL-4, dexamethason, or IL-10 leads towards an anti-inflammatory activation status (bright green cells). Addition of IFN-γ/LPS polarizes the macrophages to a pro-inflammatory status (bright red cells). The most discriminating markers between the phenotypes are MR (green), CD163 (blue), CD40 (red) and CD64 (yellow), the intensity of the markers is depicted by the quantity (1 low expression, 2 medium expression, 3 high expression) of the receptors on the macrophage.
and differentiation of T-cells and blocking the activation of lymphocytes and monocyte derived phagocytes. We show that TGF-β production of macrophages is increased after maturation in the presence of M-CSF or GM-CSF and further increased after activation with IFN-γ/LPS, IL-4 or dexamethason, while macrophages matured in the presence of NHS, produce no TGF-β. Overall, 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.

In summary, our data fully supports the notion that human macrophages are highly heterogeneous (Fig. 6). 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 macrophage maturation in the presence of 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.
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Figure S1: FACS-plots of macrophage markers.
Expression of CD40, CD64, CXCL11, CCR7, MR, stabilin-1, CD180, and CD163 was determined on differently matured and activated macrophages by FACS analysis. Macrophages matured in the presence of NHS, M-CSF or GM-CSF for 5 days, and subsequently activated for two days with IFN-γ/LPS (blue line), IL-4 (green line), dexamethason (red line), IL-10 (pink line), or left untreated (-, black line). Data are representative for three separate experiments.