



Universiteit  
Leiden  
The Netherlands

## **Apoptotic cell clearance by macrophages and dendritic cells : immunoregulation in the context of innate immunity**

Xu, W.

### **Citation**

Xu, W. (2007, September 26). *Apoptotic cell clearance by macrophages and dendritic cells : immunoregulation in the context of innate immunity*. Retrieved from <https://hdl.handle.net/1887/12354>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/12354>

**Note:** To cite this publication please use the final published version (if applicable).

# 5

## Reversible differentiation of pro- and anti-inflammatory macrophages

Wei Xu, Xiwen Zhao, Mohamed R. Daha, and Cees van Kooten

Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands

### Summary

Macrophages ( $M\phi$ ) represent dynamic cell populations that develop according to the nature of environmental signals. We and others have recently shown that  $M\phi$  can be polarized *in vitro* into pro-inflammatory ( $M\phi1$ ) and anti-inflammatory cells ( $M\phi2$ ) by the lineage-determining factors GM-CSF and M-CSF, respectively. Here we show that polarized  $M\phi1$  and  $M\phi2$  are not an end stage of differentiation and are able to reversibly undergo functional re-differentiation into anti-inflammatory and pro-inflammatory  $M\phi$ . GM-CSF-driven  $M\phi1$  exposed to M-CSF for 6 days obtained a  $M\phi2$ -like phenotype, inhibited the production of pro-inflammatory cytokine IL-6 and TNF- $\alpha$ , and exhibited a reduced T cell stimulatory capacity. *Vice versa*,  $M\phi2$  exposed to GM-CSF exhibited a  $M\phi1$ -like phenotype with significant lower production of anti-inflammatory cytokine IL-10 and a higher T cell stimulatory activity, and a decreased capacity for phagocytosis of early apoptotic cells. Our data suggest that polarized macrophages are flexible in modulating their immune functions upon environmental changes, i.e., steady-state versus inflammatory conditions. These observations are important for our understanding of the regulatory role of macrophages in tissue homeostasis and disease pathogenesis.

----- submitted -----

## Introduction

Macrophages (M $\phi$ ), as one of the professional antigen presenting cells with phagocytic capacity, play an essential role in homeostasis as well as in innate and acquired immunity, and as such may be implicated in autoimmunity, inflammation, and immunopathology<sup>1</sup>. In a normal adult, resident tissue M $\phi$  are derived from circulating bone marrow-derived monocytes, and are largely heterogeneous<sup>1,2</sup>. Classically, granulocyte/macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are the primary growth factors for the differentiation of macrophages from monocytes<sup>3</sup>. Among those two, M-CSF is the only primary M $\phi$  growth factor that is detectable in peripheral blood under steady-state conditions<sup>4</sup>. In contrast, GM-CSF is a pro-inflammatory cytokine, mostly generated during inflammation and hardly detectable in circulation<sup>5</sup>. Compatible with this reasoning, it has been shown that *op/op* mice lacking M-CSF develop a profound macrophage deficiency<sup>6</sup>, whereas GM-CSF knockout mice do not show major deficiency of M $\phi$ , except that the M $\phi$  are smaller than normal<sup>7,8</sup>, confirming that M-CSF is a crucial growth factor in M $\phi$  differentiation. Thus under steady-state conditions, M-CSF could be the default cytokine in driving M $\phi$  differentiation.

We and others have recently shown that M $\phi$  can be polarized *in vitro* into pro-inflammatory (M $\phi$ 1) and anti-inflammatory cells (M $\phi$ 2) by GM-CSF and M-CSF (also termed CSF-1), respectively<sup>9-11</sup>. Importantly, several resident tissue M $\phi$ , including alveolar, intestinal and peritoneal M $\phi$ , display anti-inflammatory properties<sup>12-15</sup>. It is tempting to hypothesize that under inflammatory conditions, anti-inflammatory M $\phi$  may undergo functional adaptation when GM-CSF is produced locally. Therefore, in the current study we investigated whether polarized M $\phi$ 1 and M $\phi$ 2 can be re-differentiated into M $\phi$ 2- and M $\phi$ 1-like cells when exposed to M-CSF and GM-CSF, respectively. We found that both M $\phi$ 1 and M $\phi$ 2 can undergo reversibly functional changes, i.e., exposure of M $\phi$ 1 to M-CSF resulted in re-differentiation of these cells into M $\phi$ 2-like cells, and *vice versa*. Our data reveal the importance of the local cytokine environment in driving M $\phi$  polarization and provide additional options for the modulation of M $\phi$  and therapeutic targeting.

## Materials and Methods

### Generation of monocyte-derived M $\phi$ 1 and M $\phi$ 2.

Human mononuclear cells were isolated from buffy-coats obtained from healthy donors using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO), followed by anti-CD14 microbeads magnetic cell sorting, according to the manufacturer's instruction (Miltenybiotec/CLB, Amsterdam, the Netherlands). Two types of macrophages, namely M $\phi$ 1 and M $\phi$ 2, were generated in 6-well culture plates (Costar, Cambridge, MA) in RPMI culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 90 U/ml

penicillin and 90 µg/ml streptomycin) (all from Gibco/Life technologies, Breda, the Netherlands) for 6 days supplemented with 5 ng/ml GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively, as previously described<sup>16</sup>. In all experiments, these two types of M $\phi$  were generated in parallel from monocytes of the same donor. For re-differentiation, day-6 M $\phi$ 1 were cultured for another 6 days in the presence of GM-CSF (named as M $\phi$ 1<sub>GM</sub>) or M-CSF (named as M $\phi$ 1<sub>M</sub>). Similarly, day-6 M $\phi$ 2 were cultured for another 6 days in the presence of GM-CSF (named as M $\phi$ 2<sub>GM</sub>) or M-CSF (named as M $\phi$ 2<sub>M</sub>).

### **Analysis of cell surface markers by flow cytometry.**

Cells were harvested and washed in buffer containing 1% BSA, 1% heat-inactivated normal human serum, and 0.02% NaN<sub>3</sub>. The following mAbs were used for flow cytometric analysis to detect expression of certain surface molecules: PE-conjugated anti-CD14 (Leu-M3), mAb of anti-mannose receptor (MR) /CD206 (D547.3, a gift of F. Koning, LUMC, Leiden, the Netherlands) and anti-CD163 (EDhu1, a gift of Dr. T.K. van den Berg, Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam). Expression was visualized by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark) using appropriate isotype controls. Cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences). Dead cells, identified by propidium iodide (PI) uptake, were excluded from analysis.

### **Detection of cytokines.**

Day-6 M $\phi$ 1 and M $\phi$ 2 cultured with either GM-CSF or M-CSF for another 6 days were stimulated with 200 ng/ml lipopolysaccharide (LPS, Salmonella Typhosa, Sigma-Aldrich) for 24 h and the supernatants were harvested for quantification of cytokine release by ELISA. In some experiments, M $\phi$ 1 and M $\phi$ 2 were cultured with GM-CSF or M-CSF for only one additional day and supernatants were collected. The measurements of IL-6, TNF- $\alpha$  and IL-10 were performed as described before<sup>16,17</sup>.

### **Phagocytosis assay.**

Early apoptosis of Jurkat T cells was induced by ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m<sup>2</sup>, and followed by culture for 3 hours in serum-free RPMI medium. Prior to the induction of apoptosis, Jurkat cells were fluorescently labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands), according to a previously described method<sup>18</sup>. Early apoptosis was established by two-color flow cytometry as positive for annexin V but negative for PI. Routinely, about 60% of early apoptotic cells were obtained. For the phagocytosis assay, CFSE-labeled apoptotic cells ( $1 \times 10^5$ ) were co-cultured with different M $\phi$  at 1:1 ratio 0.5 h at 37°C or 4°C in 100 µl RPMI culture medium in round-bottom glass tubes followed by staining with a PE-conjugated mAb against CD11b. The uptake was analyzed by flow cytometry. The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of M $\phi$  that had ingested (37°C) and/or bound (4°C) apoptotic cells.

### **Allogeneic mixed lymphocyte reaction.**

An allogeneic mixed lymphocyte reaction (MLR) assay was performed as described previously<sup>15</sup>. Briefly, responder T cells were isolated by sheep erythrocyte rosetting of mononuclear cells that were obtained from healthy donors. Stimulator cells, i.e. different M $\phi$  were first irradiated (50 Gy) and then added in graded doses to  $1.5 \times 10^5$  allogeneic T cells in 96-well round-bottom tissue culture plates in RPMI culture medium. T cell proliferation was quantified by incubation of the cells with 1  $\mu$ Ci (37 kBq) of [methyl-<sup>3</sup>H]thymidine (NEN, Boston, MA) during the last 8 h of the 6-day cultures. Results are presented as the mean cpm  $\pm$  SD obtained from triplicate cultures.

### **Statistical analysis.**

Statistical analysis was performed by one sample *t* test or Mann-Whitney U using GraphPad Prism (GraphPad software, San Diego, CA). Differences were considered statistically significant when *p* values were less than 0.05.

## **Results**

### **Morphology of re-differentiated M $\phi$ 1 and M $\phi$ 2.**

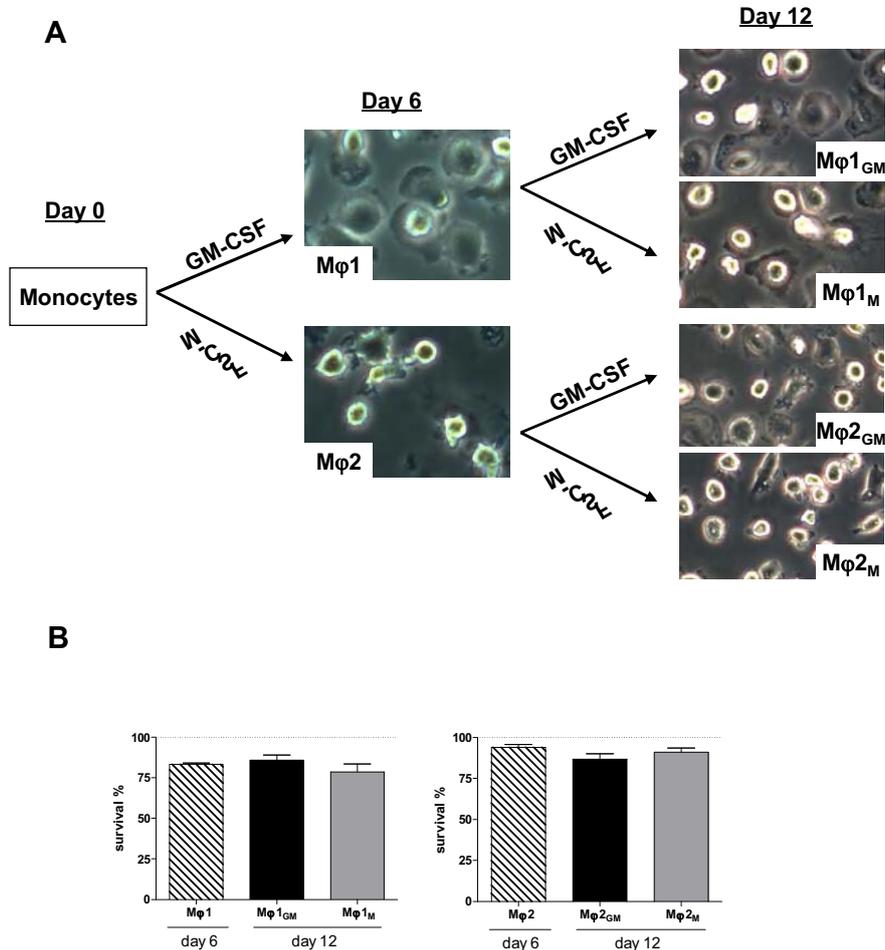
M $\phi$ 1 and M $\phi$ 2 were polarized in parallel from peripheral blood monocytes derived from the same donor by GM-CSF and M-CSF, respectively. After 6 days of differentiation, M $\phi$ 1 became adherent and mostly showed a “fried-egg” morphology, whereas M $\phi$ 2 were less-adherent with irregular shapes as compared to M $\phi$ 1 (Fig. 1A). Day-6 M $\phi$ 1 that were cultured further in GM-CSF for another 6 days (M $\phi$ 1<sub>GM</sub>) retained their “fried-egg” morphology. Similarly, M $\phi$ 2 cultured in M-CSF for additional 6 days (M $\phi$ 2<sub>M</sub>) retained their M $\phi$ 2 morphology (Fig. 1A). By exposure of M $\phi$ 1 to M-CSF for 6 days, M $\phi$ 1<sub>M</sub> did not show obvious morphological changes. However, culturing of M $\phi$ 2 in GM-CSF for another 6 days (M $\phi$ 2<sub>GM</sub>) completely rendered them into M $\phi$ 1-like cells, i.e. most of the cells became adherent and showed “fried-egg” morphology (Fig 1A).

To exclude that the observed changes were caused by differential survival of different M $\phi$  subsets during the prolonged cultures, we determined the viable cell counts. Counts of viable cells by exclusion of PI staining by flow cytometric analysis were related to total numbers of monocyte /M $\phi$  harvested. We observed that under all conditions, cell survival was between 79% and 94% (Fig 1B).

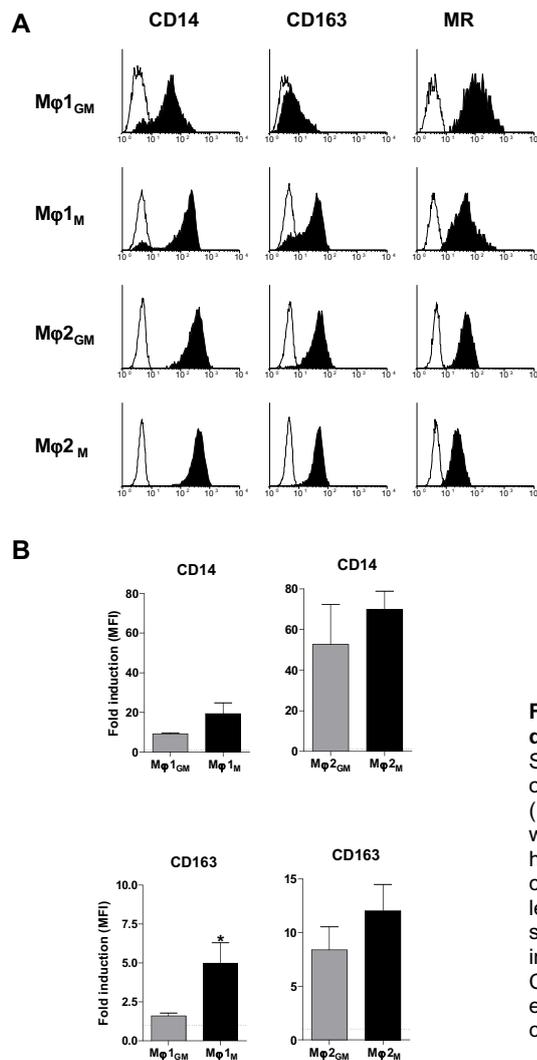
### **Phenotypes of re-differentiated M $\phi$ 1 and M $\phi$ 2.**

Polarized M $\phi$ 1 were previously shown to express low levels of CD14 as compared with M $\phi$ 2, and had no detectable expression of CD163, whereas M $\phi$ 2 expressed

CD163 and high level of CD14<sup>11,15</sup>. M $\phi$ 1<sub>GM</sub> conserved their phenotype, i.e. CD14<sup>low</sup>CD163<sup>-</sup>, whereas M $\phi$ 1<sub>M</sub> showed significantly increased expression of CD14 and CD163 ( $p=0.01$ , Mann-Whitney U) (Fig 2A, B). In contrast, M $\phi$ 2<sub>GM</sub> showed no major phenotypical changes as compared with M $\phi$ 2<sub>M</sub> (Fig 2A, B). MR expression on both cells was not significantly influenced by additional cultures (Fig. 2A).



**Figure 1. Morphology of M $\phi$ 1, M $\phi$ 2.** M $\phi$ 1 and M $\phi$ 2 were generated in parallel from the same healthy donor following culture of monocytes for 6 days, and then cultured with GM-CSF (M $\phi$ 1<sub>GM</sub> or M $\phi$ 2<sub>GM</sub>) or M-CSF (M $\phi$ 1<sub>M</sub> or M $\phi$ 2<sub>M</sub>) for additional 6 days. (A) Pictures show the morphology of cells at day 6 and day 12. Images were obtained using an Axiovert 25 inverted microscope (Carl Zeiss, Sliedrecht, The Netherlands) with a 20 x /0.3 NA objective and Zeiss Axiovision software version 3.1. Magnification, x 200. (B) After harvesting from day 6 or day 12 cultures, cells were stained with PI for the measurement of cell survival by flow cytometry.

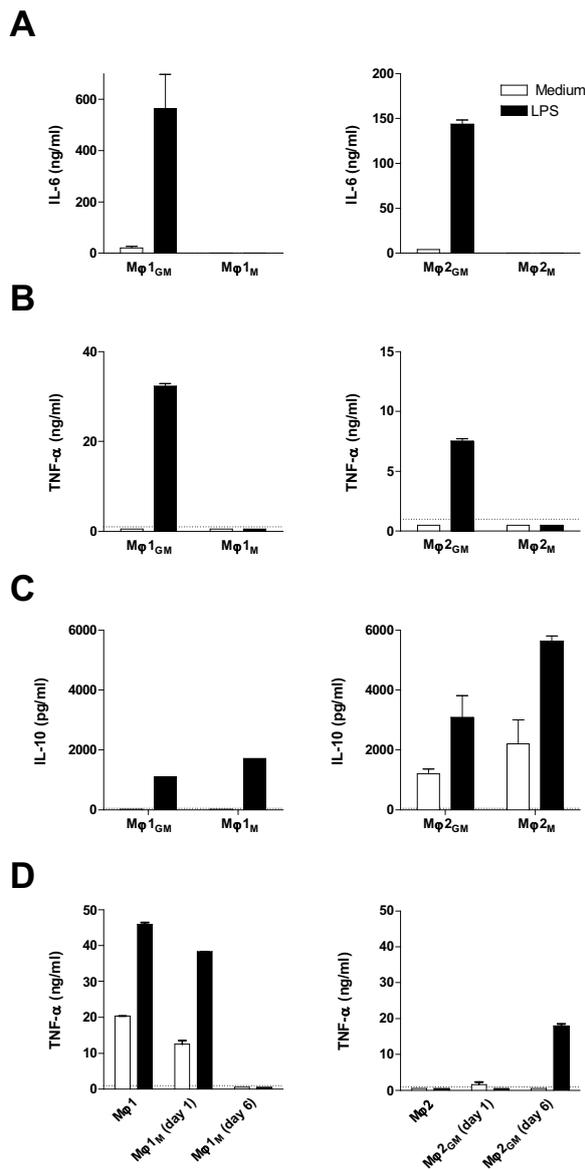


**Figure 2. Phenotypes of re-differentiated Mφ1 and Mφ2.** (A) Surface expression (closed histograms) of CD14, CD163 and mannose receptor (MR) on Mφ1<sub>GM</sub>, Mφ1<sub>M</sub>, Mφ2<sub>GM</sub> and Mφ2<sub>M</sub> was determined by flow cytometry. Open histograms represent matched isotype controls. Data are representative of at least 3 independent experiments using separate unrelated donors. (B) Fold induction of expression of CD14 and CD163 were calculated MFI of expression divided by MFI of isotype control. \*, p<0.01, Mann-Whitney U.

### Functional reversal in cytokine production of re-differentiated Mφ1 and Mφ2.

We and others have previously shown that Mφ1 are pro-inflammatory cells that produce pro-inflammatory cytokines such as IL-6, IL-23 and TNF- $\alpha$ , whereas Mφ2 have an anti-inflammatory profile as documented by a large production of IL-10 but complete absence of IL-6 and TNF- $\alpha$ <sup>10,11,16</sup>. In the absence of LPS activation, none of the Mφ populations produced IL-6 (Fig. 3A) or TNF- $\alpha$  (Fig. 3B). Upon LPS activation, Mφ1<sub>GM</sub> retained their capacity to produce IL-6 and TNF- $\alpha$ . Importantly, under the same conditions, Mφ1<sub>M</sub> completely lost their capacity to produce IL-6 and TNF- $\alpha$ . Like Mφ2, Mφ2<sub>M</sub> are unable to produce IL-6 or TNF- $\alpha$  upon LPS activation.

However, M $\phi$ 2<sub>GM</sub> gained the capacity to produce IL-6 and TNF- $\alpha$  although this production was lower than production by M $\phi$ 1<sub>GM</sub>.

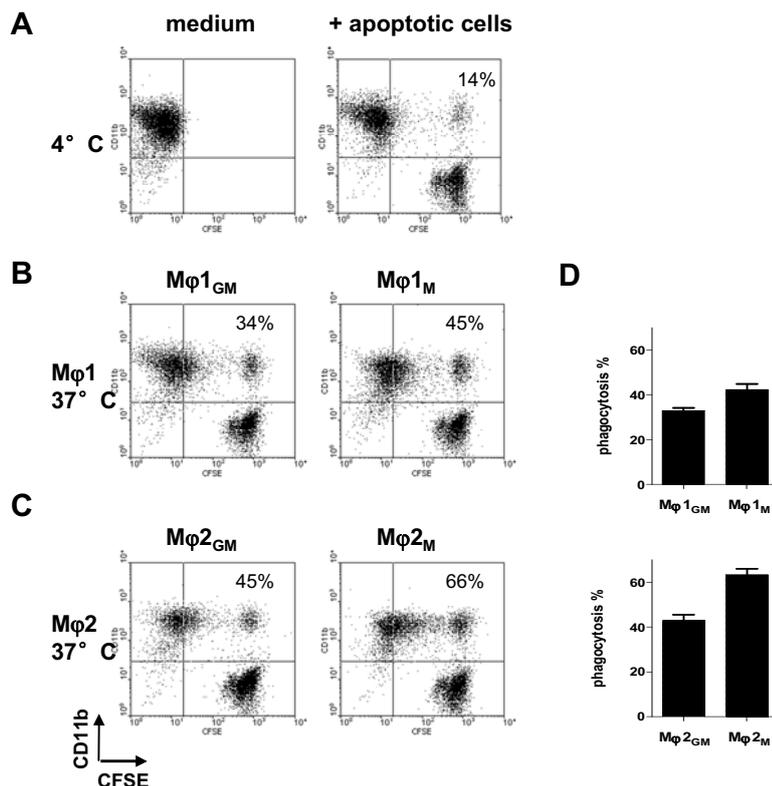


**Figure 3. Cytokine production by re-differentiated M $\phi$ 1 and M $\phi$ 2.** M $\phi$ 1 and M $\phi$ 2 were cultured with GM-CSF or M-CSF for another 6 days. After harvesting, cells were stimulated with or without LPS (200 ng/ml) for 24 h in 48-well plate. Supernatants were harvested and measured by ELISA for IL-6 (A), TNF- $\alpha$  (B) and IL-10 (C). (D) TNF- $\alpha$  production of M $\phi$ 1 and M $\phi$ 2 cultured with GM-CSF or M-CSF for additional 1 or 6 days. Data shown are mean  $\pm$  SD of duplicate cultures and represent 4 independent experiments.

We next measured the production of IL-10. Compatible with M $\phi$ 1 and M $\phi$ 2 data<sup>10,16</sup>, M $\phi$ 2<sub>M</sub> produced high levels of IL-10 compared to M $\phi$ 1<sub>GM</sub>. In all five independent experiments, M $\phi$ 2<sub>GM</sub> produced significantly lower levels of IL-10 as

compared with  $M\phi 2_{GM}$  ( $p < 0.001$ , two way ANOVA). However, we found only minor effects when  $M\phi 1$  were exposed to M-CSF as compared with GM-CSF ( $p = 0.28$ ).

To rule out the possibility that difference in cytokine production were a direct consequence of the presence of GM-CSF or M-CSF, we performed kinetic experiments.  $M\phi 1$  cultured with M-CSF for one day did not reverse TNF- $\alpha$  production, whereas an inhibition of TNF- $\alpha$  production was observed in 6-days culture of  $M\phi 1_M$  (Fig. 3D). Similarly, TNF- $\alpha$  production by  $M\phi 2$  was only induced when GM-CSF was given for 6 days but not 1 day (Fig. 3D). Together, these data suggest that upon changes of growth factors, pro-inflammatory  $M\phi 1$  and anti-inflammatory  $M\phi 2$  can be re-differentiated into anti-inflammatory and pro-inflammatory  $M\phi$ , respectively.

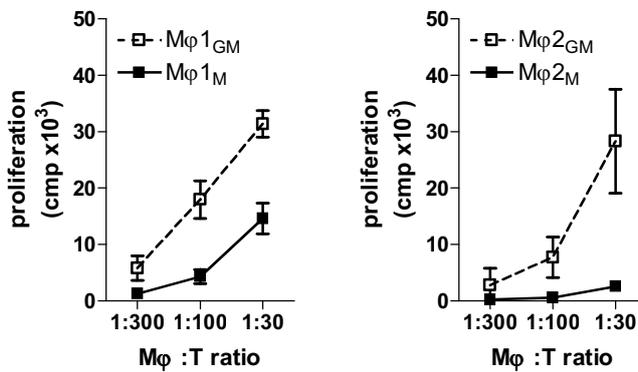


**Figure 4. Phagocytosis of early apoptotic cells by re-differentiated  $M\phi 1$  and  $M\phi 2$ .** CFSE-labeled Jurkat T cells were induced into early apoptosis by treating cells with UV-C light at a dose of  $50 \text{ J/m}^2$  and cultured in serum-free RPMI medium for another 3 h. Early apoptotic cells ( $1 \times 10^5$  cells) were co-incubated with  $M\phi 1_{GM}$ ,  $M\phi 1_M$ ,  $M\phi 2_{GM}$  or  $M\phi 2_M$  at 1:1 ratio for 0.5 h at  $37^\circ\text{C}$  or  $4^\circ\text{C}$ . Prior to flow cytometric analysis, cells were stained with PE-conjugated mAb against CD11b. (A) shows binding of early apoptotic cells to  $M\phi$  at  $4^\circ\text{C}$ . CD11b $^+$ CFSE $^+$  cells were  $M\phi 1$  (B) or  $M\phi 2$  (C) that have taken up early apoptotic cells at  $37^\circ\text{C}$ . (D) Quantification of uptake (at  $37^\circ\text{C}$ ) was calculated as  $100\% \times ((\text{CD11b}^+\text{CFSE}^+)/\text{CD11b}^+)$ . Data indicate the mean  $\pm$  SEM of 2 independent experiments where duplicated cultures were performed.

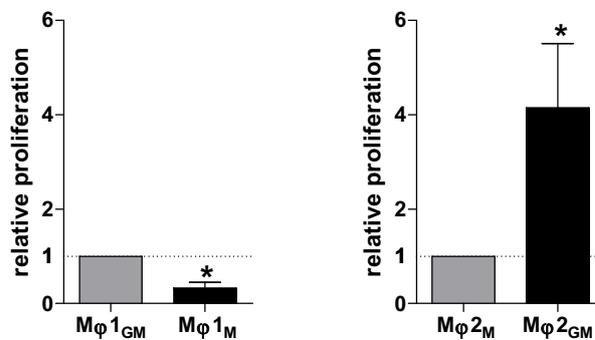
**Phagocytosis of early apoptotic cells by re-differentiated M $\phi$ 1 and M $\phi$ 2.**

One important functional difference between M $\phi$ 1 and M $\phi$ 2 is that M $\phi$ 2 is superior in phagocytosis of early apoptotic cells, as compared with M $\phi$ 1<sup>16</sup>. We therefore examined whether by switching growth factors, M $\phi$ 1 and M $\phi$ 2 can change their capacity to phagocytose early apoptotic cells. As a control, co-incubation was performed at 4°C to measure the binding of apoptotic cells to M $\phi$  (Fig. 4A), whereas the phagocytosis assay was performed at 37°C (Fig. 4B, C) to allow active ingestion. M $\phi$ 1<sub>M</sub> slightly increased their capacity to take up early apoptotic cells, as compared with M $\phi$ 1<sub>GM</sub> (Fig. 4B, D). In contrast, M $\phi$ 2<sub>GM</sub> decreased their capacity to take up early apoptotic cells, as compared with M $\phi$ 2<sub>M</sub> (Fig. 4C, D).

**A**



**B**



**Figure 5. T cell stimulatory capacity of re-differentiated M $\phi$ 1 and M $\phi$ 2.** (A) Irradiated M $\phi$ 1<sub>GM</sub>, M $\phi$ 1<sub>M</sub>, M $\phi$ 2<sub>GM</sub> or M $\phi$ 2<sub>M</sub> were added in graded dose to  $1.5 \times 10^5$  allogeneic T cells. T cell proliferation was quantified by incubating cells during the last 8 h of 6-day cultures with [methyl-<sup>3</sup>H]thymidine. Data show mean  $\pm$  SD of triplicate cultures, and represent 5 independent experiments. (B) Relative proliferation was calculated as cmp (M $\phi$ :T ratio at 1:100) of M $\phi$ 1<sub>M</sub> against those cultured with M $\phi$ 1<sub>GM</sub>, and M $\phi$ 2<sub>GM</sub> against that M $\phi$ 2<sub>M</sub>. Dashed line indicates the relative proliferation as 1. Data are mean  $\pm$  SEM of 5 independent experiments where triplicate cultures were obtained. \*,  $p < 0.01$ , one sample  $t$  test.

**T cell stimulatory capacity of re-differentiated M $\phi$ 1 and M $\phi$ 2.**

We have recently shown that M $\phi$ 2 exhibit a lower capacity to stimulate allogeneic T cell proliferation, as compared with M $\phi$ 1<sup>15</sup>. Therefore we investigated whether re-differentiated M $\phi$ 1 and M $\phi$ 2 undergo a conversion in their T cell stimulatory capacity in an allogeneic mixed lymphocyte reaction. M $\phi$ 1<sub>M</sub> showed a significantly reduced capacity to stimulate T cell proliferation, as compared with M $\phi$ 1<sub>GM</sub> (Fig. 5A, B). *Vice versa*, M $\phi$ 2<sub>GM</sub> significantly increased their capacity to stimulate T cell proliferation, as compared to M $\phi$ 2<sub>M</sub>, which reached the same level of M $\phi$ 1<sub>M</sub> to induce T cell proliferation (Fig. 5A, B).

**Discussion**

We demonstrate in the current study that polarized pro-inflammatory M $\phi$ 1 and anti-inflammatory M $\phi$ 2 are able to reversibly undergo functional re-differentiation into anti-inflammatory and pro-inflammatory M $\phi$  when growth factors, i.e. GM-CSF and M-CSF are switched, respectively. This reversal was demonstrated at the level of phenotype, cytokine release, phagocytic capacity and T cells stimulatory capacity. Our data reveal the importance of growth factors in modulating M $\phi$  plasticity, and provide important implications for therapeutic targeting of M $\phi$ .

M $\phi$  represent heterogeneous populations<sup>2,19,20</sup>. Mirroring the Th1/Th2 nomenclature, many researchers refer to classically activated M $\phi$  by IFN- $\gamma$  as M $\phi$ 1 and alternatively activated M $\phi$  by IL-4 and/or IL-13 as M $\phi$ 2<sup>20</sup>. Functional plasticity of M $\phi$  has been documented for those classically and alternatively activated M $\phi$  in human<sup>21,22</sup> and mice<sup>23</sup>, showing that polarized M $\phi$  were able to respond to an “opposing” stimulus. In our study, we have used GM-CSF and M-CSF for the generation of M $\phi$ 1 and M $\phi$ 2, resulting pro-inflammatory and anti-inflammatory M $\phi$  respectively, according to a recent publication of Verreck *et al.*<sup>10</sup>. We realize that the M-CSF-driven M $\phi$ 2 might have some resemblance with alternatively activated macrophages by IL-4 and IL-13<sup>2</sup> or type 2-activated macrophages in the mouse<sup>24</sup>. However alternatively activated macrophages express low CD14 and high MR<sup>2</sup>, and mouse type 2-activated macrophages secrete high level of TNF- $\alpha$  after stimulation<sup>25</sup>. These characteristics are different from human M-CSF polarized M $\phi$ 2, therefore we think that GM-CSF- and M-CSF-driven M $\phi$  are distinct from previously clarified macrophage subsets. Indeed, when GM-CSF-driven M $\phi$ 1 or M-CSF-driven M $\phi$ 2 were stimulated with IFN- $\gamma$  or IL-4 for 24 till 48 h, pro-inflammatory cytokine pattern such as IL-6 and TNF- $\alpha$  were not changed (data not shown).

GM-CSF and M-CSF (or CSF-1) are two growth factors which drive M $\phi$  differentiation from monocytes<sup>3,26</sup>. From these, M-CSF is the only primary M $\phi$  growth factor which is detectable in peripheral blood under steady-state conditions<sup>4</sup>. As a pro-inflammatory cytokine, GM-CSF is hardly detectable in circulation, and

much of the production and action of GM-CSF occurs locally at sites of inflammation<sup>5</sup>. For example, allergic patients with late-phase cutaneous reactions show markedly increased levels of GM-CSF mRNA in the skin<sup>27</sup>. Enhancement of GM-CSF levels in circulating are observed in response to endotoxin (LPS)<sup>28</sup>. Interestingly, GM-CSF can induce M-CSF production by monocytes<sup>29,30</sup>. Therefore, it is likely that under inflammatory conditions, both GM-CSF and M-CSF will be present and that this balance might impact the functional differentiation of M $\phi$ . Our data provide insights on how these two growth factors interplay and modify the plasticity of M $\phi$  for a desired immune reaction.

One of the characteristic functions of M $\phi$ 2 is that they preferentially recognize and ingest early apoptotic cells, leading to a non-inflammatory removal<sup>16</sup>. We have recently suggested that most resident M $\phi$  such as peritoneal M $\phi$  are anti-inflammatory cells that are the major phagocytes who clear early apoptotic cells<sup>15</sup>. Data in the current paper showed that the pro-inflammatory M $\phi$ 1 could acquire higher phagocytic capacity for early apoptotic cells once exposure to M-CSF, supporting the role of M-CSF in modulating phagocytosis of M $\phi$  subsets. We hypothesize that *in vivo* recruitment of M $\phi$ 1-like cells locally to modify them into M $\phi$ 2-like cells is essential to ensure a silent clearance of apoptotic cells when overloaded apoptosis occurs.

We showed that exposure of pro-inflammatory M $\phi$ 1 to GM-CSF completely inhibited their production of IL-6 and TNF- $\alpha$ , and strongly enhanced their capacity to stimulate T cell proliferation, whereas treatment of anti-inflammatory M $\phi$ 2 with M-CSF induced their production of IL-6 and TNF- $\alpha$ , and reduced their capacity to stimulate T cell proliferation. In the case of acute inflammation, the early phase is dominated by pro-inflammatory and/or cytotoxic cells, whereas the terminal phase is dominated by anti-inflammatory /tissue regenerative cells<sup>31</sup>. Therefore, our data support the notion that during the due course of an inflammatory reaction, M $\phi$ 1 that sustain and stimulate inflammation may convert their function to participate in the healing phase of the reaction<sup>32</sup>.

Emerging evidence show that tissue resident M $\phi$  including alveolar<sup>12,13</sup>, intestinal<sup>14</sup> and peritoneal M $\phi$ <sup>15</sup> have anti-inflammatory properties. In chronic diseases such as tumors, M $\phi$  have been suggested to have a dual role either in killing tumor or in promoting tumor survival<sup>33</sup>. Tumor associated M $\phi$  acquire CD163 expression and have anti-inflammatory properties<sup>34-36</sup>, which potentially promotes immune escape of tumor cells. Our data indicated that GM-CSF treatment on M $\phi$ 2 strongly increased their capacity to stimulate T cell proliferation.

In conclusion, *in vitro*-polarized GM-CSF-driven pro-inflammatory M $\phi$ 1 and M-CSF-driven pro-inflammatory M $\phi$ 2 can undergo functional re-differentiation upon exposure the opposing growth factor. Such plasticity of myeloid cells has been observed in other experimental models including a skewing from dendritic cells

(DCs) to M $\phi$  upon exposure to interferon-gamma (IFN- $\gamma$ )<sup>37</sup>, or IL-6 and M-CSF<sup>29,38</sup>, and a transdifferentiation of monocyte-derived DCs into osteoclasts upon culture in M-CSF combined with receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)<sup>39</sup>. Together with the current data, this clearly indicates that myeloid cells have a high plasticity and the local microenvironment will be a determining factor for the functional differentiation of these cells. However, it also indicates that these cells can adapt and functional re-differentiate when there are changes in the environment. These observations are important for our understanding of the regulatory role of M $\phi$  and other myeloid cells in tissue homeostasis and disease pathogenesis.

### Acknowledgements

We thank Nicole Schlagwein for the excellent technical assistance. This study is supported in part by a grant (C02.2015) from the Dutch Kidney Foundation.

### Reference List

- 1 Taylor PR, Martinez-Pomares L, Stacey M et al. Macrophage receptors and immune recognition. *Annu Rev Immunol.* 2005;23:901-944.
- 2 Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* 2003;3:23-35.
- 3 Wiktor-Jedrzejczak W, Gordon S. Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiol Rev.* 1996;76:927-947.
- 4 Bartocci A, Mastrogiannis DS, Migliorati G et al. Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc Natl Acad Sci U S A.* 1987;84:6179-6183.
- 5 Hamilton JA. GM-CSF in inflammation and autoimmunity. *Trends Immunol.* 2002;23:403-408.
- 6 Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Jr. et al. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A.* 1990;87:4828-4832.
- 7 Stanley E, Lieschke GJ, Grail D et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A.* 1994;91:5592-5596.
- 8 Cook AD, Braine EL, Hamilton JA. Stimulus-dependent requirement for granulocyte-macrophage colony-stimulating factor in inflammation. *J Immunol.* 2004;173:4643-4651.
- 9 Smith W, Feldmann M, Londei M. Human macrophages induced in vitro by macrophage colony-stimulating factor are deficient in IL-12 production. *Eur J Immunol.* 1998;28:2498-2507.
- 10 Verreck FA, de Boer T, Langenberg DM et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A.* 2004;101:4560-4565.

- 11 Verreck FA, de Boer T, Langenberg DM, van der ZL, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN- $\gamma$ - and CD40L-mediated costimulation. *J Leukoc Biol.* 2005;79:285-293.
- 12 Bilyk N, Holt PG. Inhibition of the immunosuppressive activity of resident pulmonary alveolar macrophages by granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1993;177:1773-1777.
- 13 Holt PG, Oliver J, Bilyk N et al. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med.* 1993;177:397-407.
- 14 Smythies LE, Sellers M, Clements RH et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest.* 2005;115:66-75.
- 15 Xu W, Schlagwein N, Roos A et al. Human peritoneal macrophages show functional characteristics of M-CSF-driven anti-inflammatory type 2 macrophages. *Eur J Immunol.* 2007;37:1594-1599.
- 16 Xu W, Roos A, Schlagwein N et al. IL-10-producing macrophages preferentially clear early apoptotic cells. *Blood.* 2006;107:4930-4937.
- 17 de Fijter JW, Daha MR, Schroeijers WE, Van Es LA, van Kooten C. Increased IL-10 production by stimulated whole blood cultures in primary IgA nephropathy. *Clin Exp Immunol.* 1998;111:429-434.
- 18 Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods.* 1994;171:131-137.
- 19 Hume DA. The mononuclear phagocyte system. *Curr Opin Immunol.* 2006;18:49-53.
- 20 Mantovani A, Sica A, Sozzani S et al. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004;25:677-686.
- 21 Porcheray F, Viaud S, Rimaniol AC et al. Macrophage activation switching: an asset for the resolution of inflammation. *Clin Exp Immunol.* 2005;142:481-489.
- 22 Gratchev A, Kzhyshkowska J, Kothe K et al. Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology.* 2006;211:473-486.
- 23 Stout RD, Jiang C, Matta B et al. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol.* 2005;175:342-349.
- 24 Mosser DM. The many faces of macrophage activation. *J Leukoc Biol.* 2003;73:209-212.
- 25 Gerber JS, Mosser DM. Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol.* 2001;166:6861-6868.
- 26 Becker S, Warren MK, Haskill S. Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *J Immunol.* 1987;139:3703-3709.
- 27 Kay AB, Ying S, Varney V et al. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med.* 1991;173:775-778.
- 28 Sheridan JW, Metcalf D. Studies on the bone marrow colony stimulating factor (CSF): relation of tissue CSF to serum CSF. *J Cell Physiol.* 1972;80:129-140.
- 29 Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol.* 2000;1:510-514.

- 30 Rieser C, Ramoner R, Bock G et al. Human monocyte-derived dendritic cells produce macrophage colony-stimulating factor: enhancement of c-fms expression by interleukin-10. *Eur J Immunol.* 1998;28:2283-2288.
- 31 Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol.* 2004;76:509-513.
- 32 Hume DA, Ross IL, Himes SR et al. The mononuclear phagocyte system revisited. *J Leukoc Biol.* 2002;72:621-627.
- 33 Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol.* 2002;196:254-265.
- 34 Nguyen TT, Schwartz EJ, West RB et al. Expression of CD163 (hemoglobin scavenger receptor) in normal tissues, lymphomas, carcinomas, and sarcomas is largely restricted to the monocyte/macrophage lineage. *Am J Surg Pathol.* 2005;29:617-624.
- 35 Avila-Moreno F, Lopez-Gonzalez JS, Galindo-Rodriguez G et al. Lung squamous cell carcinoma and adenocarcinoma cell lines use different mediators to induce comparable phenotypic and functional changes in human monocyte-derived dendritic cells. *Cancer Immunol Immunother.* 2006;55:598-611.
- 36 Bachli EB, Schaer DJ, Walter RB, Fehr J, Schoedon G. Functional expression of the CD163 scavenger receptor on acute myeloid leukemia cells of monocytic lineage. *J Leukoc Biol.* 2006;79:312-318.
- 37 Delneste Y, Charbonnier P, Herbault N et al. Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. *Blood.* 2003;101:143-150.
- 38 Menetrier-Caux C, Montmain G, Dieu MC et al. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood.* 1998;92:4778-4791.
- 39 Rivollier A, Mazzorana M, Tebib J et al. Immature dendritic cell transdifferentiation into osteoclasts: a novel pathway sustained by the rheumatoid arthritis microenvironment. *Blood.* 2004;104:4029-4037.