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

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Reversible differentiation of pro- and anti-inflammatory macrophages

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Summary

Macrophages (Mφ) represent dynamic cell populations that develop according to the nature of environmental signals. We and others have recently shown that Mφ can be polarized *in vitro* into pro-inflammatory (Mφ1) and anti-inflammatory cells (Mφ2) by the lineage-determining factors GM-CSF and M-CSF, respectively. Here we show that polarized Mφ1 and Mφ2 are not an end stage of differentiation and are able to reversibly undergo functional re-differentiation into anti-inflammatory and pro-inflammatory Mφ. GM-CSF-driven Mφ1 exposed to M-CSF for 6 days obtained a Mφ2-like phenotype, inhibited the production of pro-inflammatory cytokine IL-6 and TNF-α, and exhibited a reduced T cell stimulatory capacity. *Vice versa*, Mφ2 exposed to GM-CSF exhibited a Mφ1-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.

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Introduction

Macrophages (Mφ), 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 [1]. In a normal adult, resident tissue Mφ are derived from circulating bone marrow–derived monocytes, and are largely heterogeneous [1,2]. 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 [3]. Among those two, M-CSF is the only primary Mφ growth factor that is detectable in peripheral blood under steady-state conditions [4]. In contrast, GM-CSF is a pro-inflammatory cytokine, mostly generated during inflammation and hardly detectable in circulation [5]. Compatible with this reasoning, t has been shown that *op/op* mice lacking M-CSF develop a profound macrophage deficiency [6], whereas GM-CSF knockout mice do not show major deficiency of Mφ, except that the Mφ are smaller than normal [7,8], confirming that M-CSF is a crucial growth factor in Mφ differentiation. Thus under steady-state conditions, M-CSF could be the default cytokine in driving Mφ differentiation.

We and others have recently shown that Mφ can be polarized *in vitro* into pro-inflammatory (Mφ1) and anti-inflammatory cells (Mφ2) by GM-CSF and M-CSF (also termed CSF-1), respectively [9-11]. Importantly, several resident tissue Mφ, including alveolar, intestinal and peritoneal Mφ, display anti-inflammatory properties [12-15]. It is tempting to hypothesize that under inflammatory conditions, anti-inflammatory Mφ may undergo functional adaptation when GM-CSF is produced locally. Therefore, in the current study we investigated whether polarized Mφ1 and Mφ2 can be re-differentiated into Mφ2- and Mφ1-like cells when exposed to M-CSF and GM-CSF, respectively. We found that both Mφ1 and Mφ2 can undergo reversibly functional changes, i.e., exposure of Mφ1 to M-CSF resulted in re-differentiation of these cells into Mφ2-like cells, and *vice versa*. Our data reveal the importance of the local cytokine environment in driving Mφ polarization and provide additional options for the modulation of Mφ and therapeutic targeting.

Materials and Methods

Generation of monocyte-derived Mφ1 and Mφ2.

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 Mφ1 and Mφ2, 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 [16]. In all experiments, these two types of Mφ were generated in parallel from monocytes of the same donor. For re-differentiation, day-6 Mφ1 were cultured for another 6 days in the presence of GM-CSF (named as $M\varphi1_{GM}$) or M-CSF (named as $M\varphi1_{M}$). Similarly, day-6 Mφ2 were cultured for another 6 days in the presence of GM-CSF (named as $M\varphi2_{GM}$) or M-CSF (named as $M\varphi2_{M}$).

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_3. 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 Mφ1 and Mφ2 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, Mφ1 and Mφ2 were cultured with GM-CSF or M-CSF for only one additional day and supernatants were collected. The measurements of IL-6, TNF-α and IL-10 were performed as described before [16,17].

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^2, 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 succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands), according to a previously described method [18]. 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 (1×10^5) were co-cultured with different Mφ 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 Mφ 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 [15]. Briefly, responder T cells were isolated by sheep erythrocyte rosetting of mononuclear cells that were obtained from healthy donors. Stimulator cells, i.e. different Mφ were first irradiated (50 Gy) and then added in graded doses to 1.5×10^5 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 µCi (37 kBq) of [methyl-^{3}H]thymidine (NEN, Boston, MA) during the last 8 h of the 6-day cultures. Results are presented as the mean cpm ± 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 Mφ1 and Mφ2.

Mφ1 and Mφ2 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, Mφ1 became adherent and mostly showed a “fried-egg” morphology, whereas Mφ2 were less-adherent with irregular shapes as compared to Mφ1 (Fig. 1A). Day-6 Mφ1 that were cultured further in GM-CSF for another 6 days ($M\varphi1_{GM}$) retained their “fried-egg” morphology. Similarly, Mφ2 cultured in M-CSF for additional 6 days ($M\varphi2_M$) retained their Mφ2 morphology (Fig. 1A). By exposure of Mφ1 to M-CSF for 6 days, $M\varphi1_M$ did not show obvious morphological changes. However, culturing of Mφ2 in GM-CSF for another 6 days ($M\varphi2_{GM}$) completely rendered them into Mφ1-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 Mφ 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 /Mφ harvested. We observed that under all conditions, cell survival was between 79% and 94% (Fig 1B).

Phenotypes of re-differentiated Mφ1 and Mφ2.

Polarized Mφ1 were previously shown to express low levels of CD14 as compared with Mφ2, and had no detectable expression of CD163, whereas Mφ2 expressed

CD163 and high level of CD14 [11,15]. $M\varphi1_{GM}$ conserved their phenotype, i.e. $CD14^{low}CD163^{-}$, whereas $M\varphi1_{M}$ showed significantly increased expression of CD14 and CD163 (p=0.01, Mann-Whitney U) (Fig 2A, B). In contrast, $M\varphi2_{GM}$ showed no major phenotypical changes as compared with $M\varphi2_{M}$ (Fig 2A, B). MR expression on both cells was not significantly influenced by additional cultures (Fig. 2A).

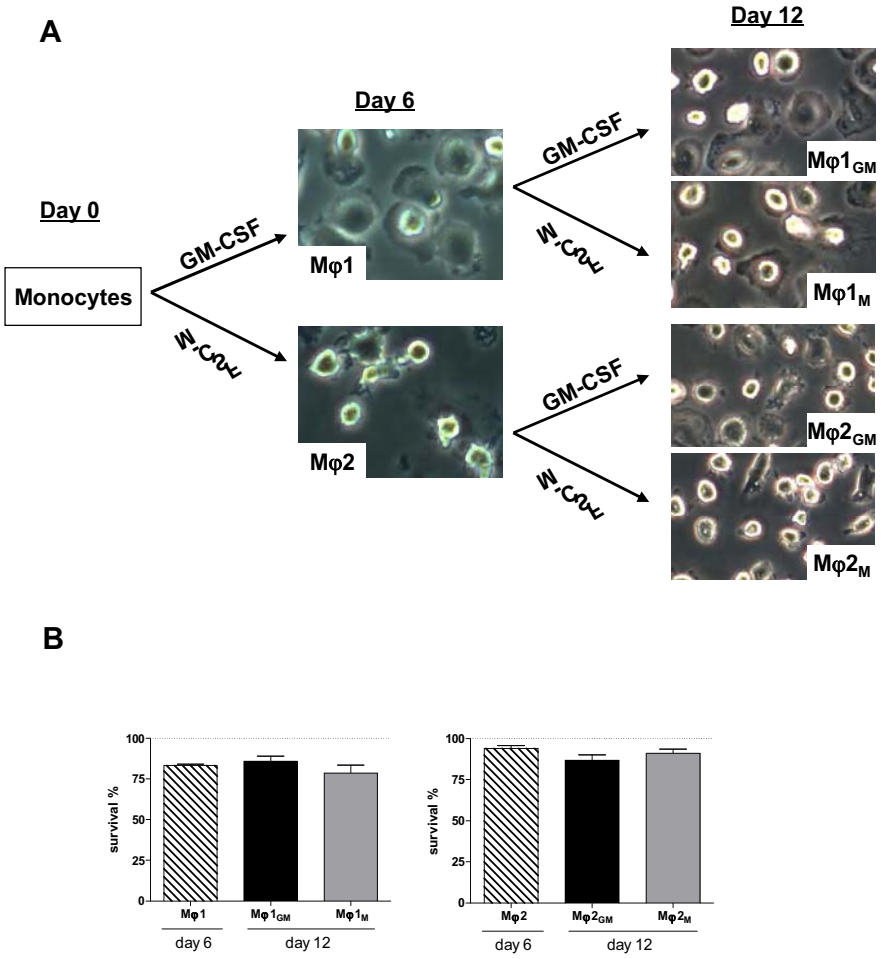

Figure 1. Morphology of Mφ1, Mφ2. Mφ1 and Mφ2 were generated in parallel from the same healthy donor following culture of monocytes for 6 days, and then cultured with GM-CSF ($M\varphi1_{GM}$ or $M\varphi2_{GM}$) or M-CSF ($M\varphi1_{M}$ or $M\varphi2_{M}$) for additional 6 days. (A) Pictures show the 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.

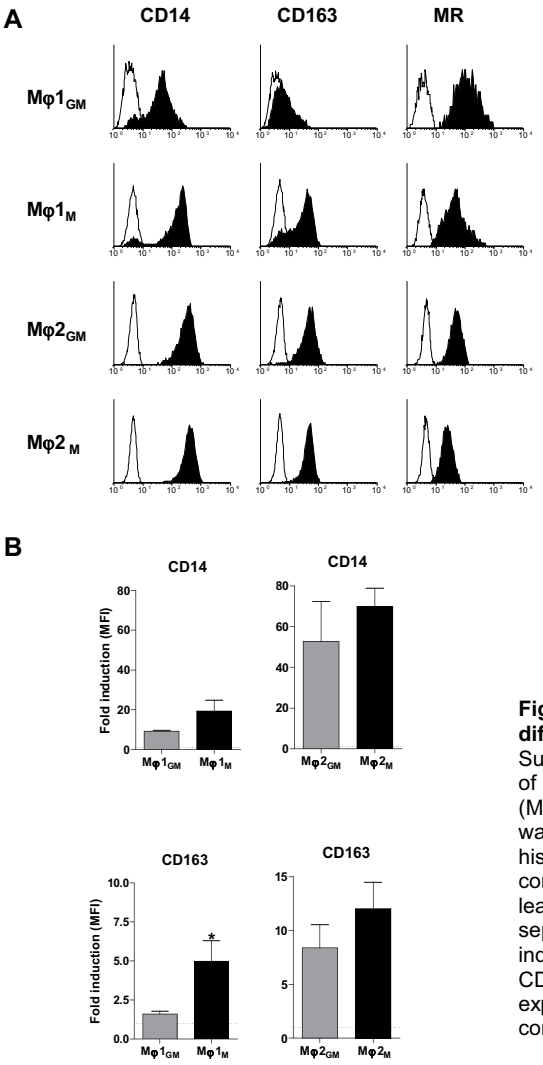

Figure 2. Phenotypes of re-differentiated Mφ1 and Mφ2. (A) Surface expression (closed histograms) of CD14, CD163 and mannose receptor (MR) on $M\varphi1_{GM}$, $M\varphi1_{M}$, $M\varphi2_{GM}$ and $M\varphi2_{M}$ was determined by flow cytometry. Open histograms represent matched isotype controls. Data 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-α, whereas Mφ2 have an anti-inflammatory profile as documented by a large production of IL-10 but complete absence of IL-6 and TNF-α [10,11,16]. In the absence of LPS activation, non of the Mφ populations produced IL-6 (Fig. 3A) or TNF-α (Fig. 3B). Upon LPS activation, $M\varphi1_{GM}$ retained their capacity to produce IL-6 and TNF-α. Importantly, under the same conditions, $M\varphi1_{M}$ completely lost their capacity to produce IL-6 and TNF-α. Like Mφ2, $M\varphi2_{M}$ are unable to produce IL-6 or TNF-α upon LPS activation.

However, $M\varphi 2_{GM}$ gained the capacity to produce IL-6 and TNF-α although this production was lower than production by $M\varphi 1_{GM}$.

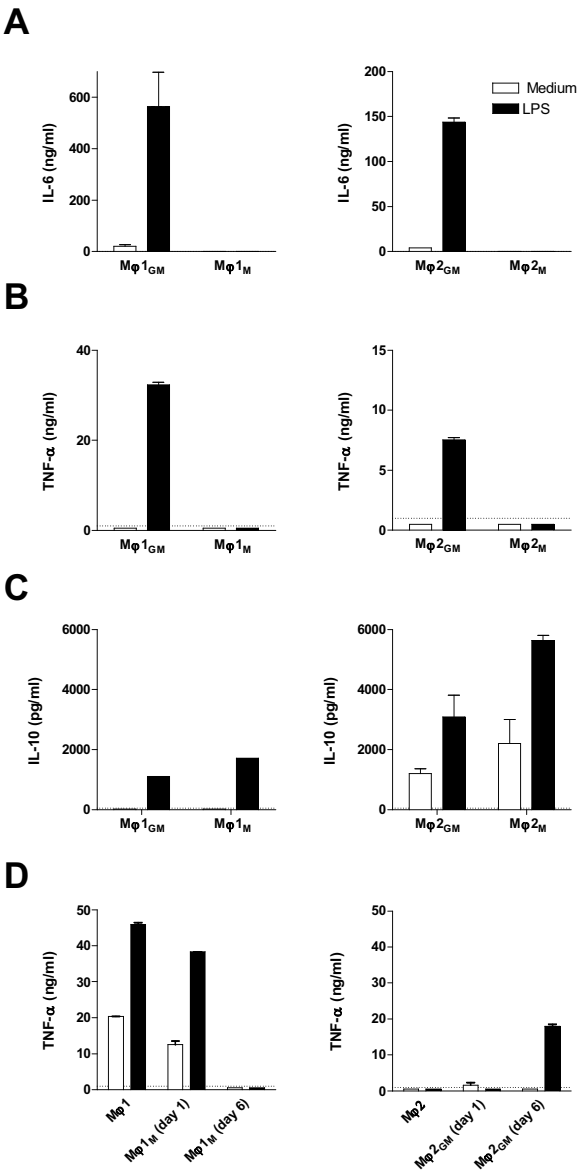

Figure 3. Cytokine production by re-differentiated Mφ1 and Mφ2. Mφ1 and Mφ2 were cultured with GM-CSF or M-CSF for another 6 days. After harvesting, cells were stimulated with or without LPS (200 ng/ml) for 24 h in 48-well plate. Supernatants were harvested and measured by ELISA for IL-6 (A), TNF-α (B) and IL-10 (C). (D) TNF-α production of Mφ1 and Mφ2 cultured with GM-CSF or M-CSF for additional 1 or 6 days. Data shown are mean ± SD of duplicate cultures and represent 4 independent experiments.

We next measured the production of IL-10. Compatible with Mφ1 and Mφ2 data [10,16], $M\varphi 2_M$ produced high levels of IL-10 compared to $M\varphi 1_{GM}$. In all five independent experiments, $M\varphi 2_{GM}$ produced significantly lower levels of IL-10 as

compared with $M\varphi 2_{GM}$ ($p<0.001$, two way ANOVA).However, we found only minor effects when $M\varphi 1$ 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. $M\varphi 1$ cultured with M-CSF for one day did not reverse TNF-α production, whereas an inhibition of TNF-α production was observed in 6-days culture of $M\varphi 1_M$ (Fig. 3D). Similarly, TNF-α production by $M\varphi 2$ 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 $M\varphi 1$ and anti-inflammatory $M\varphi 2$ can be re-differentiated into anti-inflammatory and pro-inflammatory $M\varphi$, respectively.

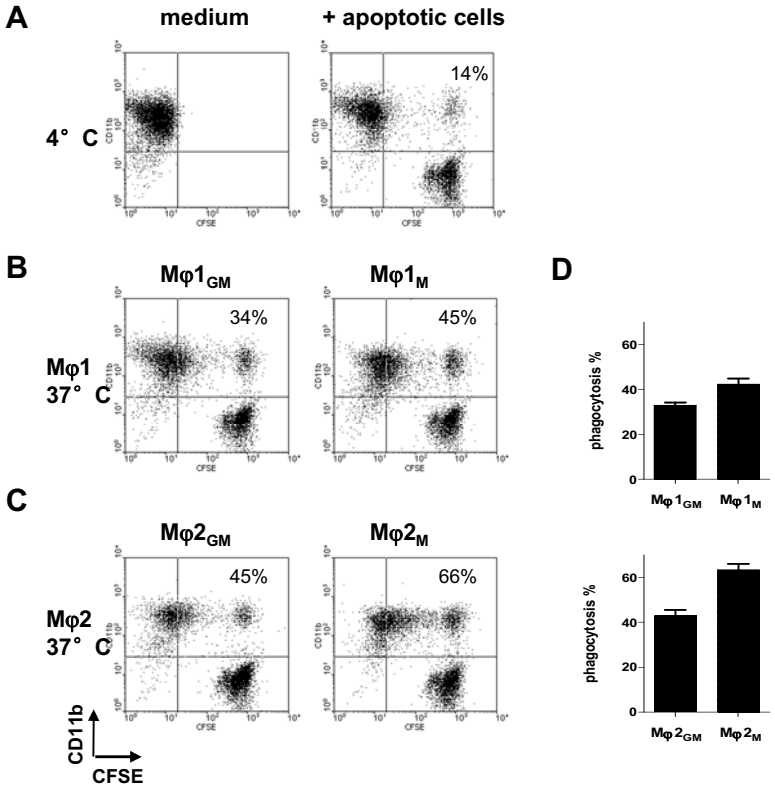

Figure 4. Phagocytosis of early apoptotic cells by re-differentiated Mφ1 and Mφ2. CFSE-labeled Jurkat T cells were induced into early apoptosis by treating cells with UV-C light at a dose of 50 J/m^2 and cultured in serum-free RPMI medium for another 3 h. Early apoptotic cells (1×10^5 cells) were co-incubated with $M\varphi 1_{GM}$, $M\varphi 1_M$, $M\varphi 2_{GM}$ or $M\varphi 2_M$ at 1:1 ratio for 0.5 h at 37°C or 4°C. Prior to flow cytometric analysis, cells were stained with PE-conjugated mAb against CD11b. (A) shows binding of early apoptotic cells to Mφ at 4°C. CD11b+CFSE$^+$ cells were Mφ1 (B) or Mφ2 (C) that have taken up early apoptotic cells at 37°C. (D) Quantification of uptake (at 37°C) was calculated as 100% × ((CD11b$^+$CFSE$^+$)/CD11b$^+$). Data indicate the mean ± SEM of 2 independent experiments where duplicated cultures were performed.

Phagocytosis of early apoptotic cells by re-differentiated Mφ1 and Mφ2.

One important functional difference between Mφ1 and Mφ2 is that Mφ2 is superior in phagocytosis of early apoptotic cells, as compared with Mφ1 [16]. We therefore examined whether by switching growth factors, Mφ1 and Mφ2 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 Mφ (Fig. 4A), whereas the phagocytosis assay was performed at 37°C (Fig. 4B, C) to allow active ingestion. $M\varphi1_M$ slightly increased their capacity to take up early apoptotic cells, as compared with $M\varphi1_{GM}$ (Fig. 4B, D). In contrast, $M\varphi2_{GM}$ decreased their capacity to take up early apoptotic cells, as compared with $M\varphi2_M$ (Fig. 4C, D).

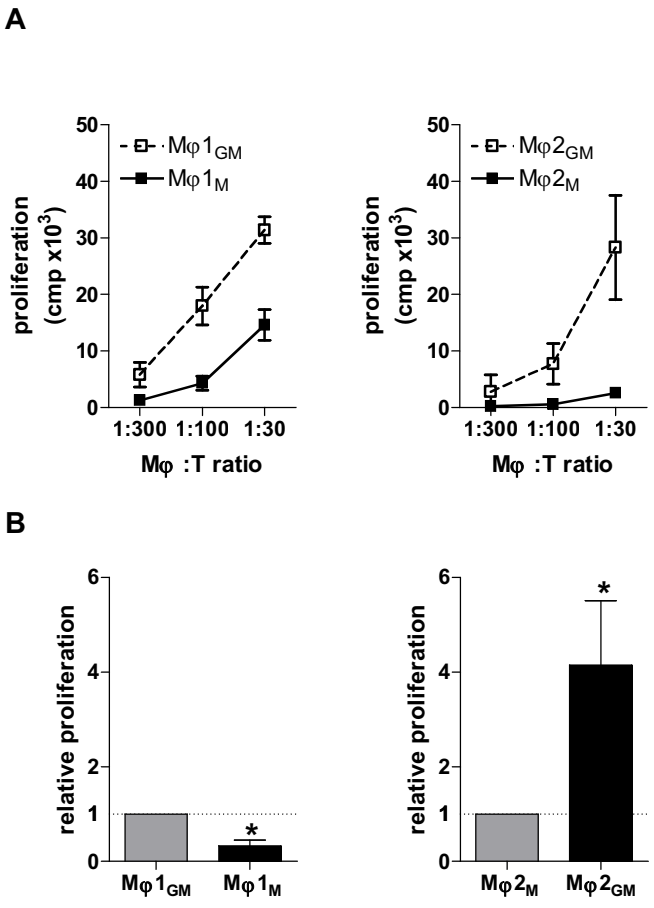

Figure 5. T cell stimulatory capacity of re-differentiated Mφ1 and Mφ2. (A) Irradiated $M\varphi1_{GM}$, $M\varphi1_M$, $M\varphi2_{GM}$ or $M\varphi2_M$ were added in graded dose to 1.5×10^5 allogeneic T cells. T cell proliferation was quantified by incubating cells during the last 8 h of 6-day cultures with [methyl-^{3}H]thymidine. Data show mean ± SD of triplicate cultures, and represent 5 independent experiments. (B) Relative proliferation was calculated as cmp (Mφ:T ratio at 1:100) of $M\varphi1_M$ against those cultured with $M\varphi1_{GM}$, and $M\varphi2_{GM}$ against that $M\varphi2_M$. Dashed line indicates the relative proliferation as 1. Data are mean ± SEM of 5 independent experiments where triplicate cultures were obtained. *, $p<0.01$, one sample *t* test.

T cell stimulatory capacity of re-differentiated Mφ1 and Mφ2.

We have recently shown that Mφ2 exhibit a lower capacity to stimulate allogeneic T cell proliferation, as compared with Mφ1 [15]. Therefore we investigated whether re-differentiated Mφ1 and Mφ2 undergo a conversion in their T cell stimulatory capacity in an allogeneic mixed lymphocyte reaction. $M\varphi1_M$ showed a significantly reduced capacity to stimulate T cell proliferation, as compared with $M\varphi1_{GM}$ (Fig. 5A, B). *Vice versa,* $M\varphi2_{GM}$ significantly increased their capacity to stimulate T cell proliferation, as compared to $M\varphi2_M$, which reached the same level of $M\varphi1_M$ to induce T cell proliferation (Fig. 5A, B).

Discussion

We demonstrate in the current study that polarized pro-inflammatory Mφ1 and anti-inflammatory Mφ2 are able to reversibly undergo functional re-differentiation into anti-inflammatory and pro-inflammatory Mφ 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 Mφ plasticity, and provide important implications for therapeutic targeting of Mφ.

Mφ represent heterogeneous populations [2,19,20]. Mirroring the Th1/Th2 nomenclature, many researchers refer to classically activated Mφ by IFN-γ as Mφ1 and alternatively activated Mφ by IL-4 and/or IL-13 as Mφ2 [20]. Functional plasticity of Mφ has been documented for those classically and alternatively activated Mφ in human [21,22] and mice [23], showing that polarized Mφ were able to respond to an "opposing" stimulus. In our study, we have used GM-CSF and M-CSF for the generation of Mφ1 and Mφ2, resulting pro-inflammatory and anti-inflammatory Mφ respectively, according to a recent publication of Verreck *et al.* [10]. We realize that the M-CSF-driven Mφ2 might have some resemblance with alternatively activated macrophages by IL-4 and IL-13 [2] or type 2-activated macrophages in the mouse [24]. However alternatively activated macrophages express low CD14 and high MR [2], and mouse type 2-activated macrophages secret high level of TNF-α after stimulation [25]. These characteristics are different from human M-CSF polarized Mφ2, therefore we think that GM-CSF- and M-CSF-driven Mφ are distinct from previously clarified macrophage subsets. Indeed, when GM-CSF-driven Mφ1 or M-CSF-driven Mφ2 were stimulated with IFN-γ or IL-4 for 24 till 48 h, pro-inflammatory cytokine pattern such as IL-6 and TNF-α were not changed (data not shown).

GM-CSF and M-CSF (or CSF-1) are two growth factors which drive Mφ differentiation from monocytes [3,26]. From these, M-CSF is the only primary Mφ growth factor which is detectable in peripheral blood under steady-state conditions [4]. 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 [5]. For example, allergic patients with late-phase cutaneous reactions show markedly increased levels of GM-CSF mRNA in the skin [27]. Enhancement of GM-CSF levels in circulating are observed in response to endotoxin (LPS) [28]. Interestingly, GM-CSF can induce M-CSF production by monocytes [29,30]. 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 Mφ. Our data provide insights on how these two growth factors interplay and modify the plasticity of Mφ for a desired immune reaction.

One of the characteristic functions of Mφ2 is that they preferentially recognize and ingest early apoptotic cells, leading to a non-inflammatory removal [16]. We have recently suggested that most resident Mφ such as peritoneal Mφ are anti-inflammatory cells that are the major phagocytes who clear early apoptotic cells [15]. Data in the current paper showed that the pro-inflammatory Mφ1 could acquire higher phagocytic capacity for early apoptotic cells once exposure to M-CSF, supporting the role of M-CSF in modulating phagocytosis of Mφ subsets. We hypothesize that *in vivo* recruitment of Mφ1-like cells locally to modify them into Mφ2-like cells is essential to ensure a silent clearance of apoptotic cells when overloaded apoptosis occurs.

We showed that exposure of pro-inflammatory Mφ1 to GM-CSF completely inhibited their production of IL-6 and TNF-α, and strongly enhanced their capacity to stimulate T cell proliferation, whereas treatment of anti-inflammatory Mφ2 with M-CSF induced their production of IL-6 and TNF-α, 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 [31]. Therefore, our data support the notion that during the due course of an inflammatory reaction, Mφ1 that sustain and stimulate inflammation may convert their function to participate in the healing phase of the reaction [32].

Emerging evidence show that tissue resident Mφ including alveolar [12,13], intestinal [14] and peritoneal Mφ [15] have anti-inflammatory properties. In chronic diseases such as tumors, Mφ have been suggested to have a dual role either in killing tumor or in promoting tumor survival [33]. Tumor associated Mφ acquire CD163 expression and have anti-inflammatory properties [34-36], which potentially promotes immune escape of tumor cells. Our data indicated that GM-CSF treatment on Mφ2 strongly increased their capacity to stimulate T cell proliferation.

In conclusion, *in vitro*-polarized GM-CSF-driven pro-inflammatory Mφ1 and M-CSF-driven pro-inflammatory Mφ2 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 Mφ upon exposure to interferon-gamma (IFN-γ) [37], or IL-6 and M-CSF [29,38], and a transdifferentiation of monocyte-derived DCs into osteolcasts upon culture in M-CSF combined with receptor activator of nuclear factor-κB ligand (RANKL) [39]. 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 Mφ and other myeloid cells in tissue homeostasis and disease pathogenesis.

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