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## **IL-10-producing macrophages preferentially clear early apoptotic cells**

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### **Summary**

To prevent the development of autoimmunity, professional phagocytes including subsets of dendritic cells (DCs) and macrophages (MØ) promptly and efficiently clear apoptotic cells. Here we identify that M-CSF-driven macrophages (MØ2) are potent phagocytes that have the unique capacity to preferentially bind and ingest early apoptotic cells. This macrophage subset has intrinsic anti-inflammatory properties, characterized by high IL-10 production in the absence of pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ . Importantly, whereas the IL-6 and TNF- $\alpha$  production by GM-CSF-driven macrophages (MØ1) is inhibited upon uptake of apoptotic cells, the anti-inflammatory status of MØ2 is retained during phagocytosis. MØ2 were shown to use CD14 to tether apoptotic cells, whereas recognition of phosphatidylserine (PS) contributed to uptake of early apoptotic cells. MØ2 showed more potent macropinocytosis compared to DCs and MØ1, and uptake of apoptotic cells was inhibited by a macropinocytosis inhibitor. Our studies suggest that, under steady-state conditions, IL-10-producing MØ2 are prominently involved in the clearance of early apoptotic cells.

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## Introduction

During normal homeostasis and tissue turnover large numbers of cells undergoing apoptosis are promptly removed and replaced. The removal of apoptotic material plays an important role in the suppression of inflammation and the regulation of immune responses<sup>1-3</sup>. Apoptotic cells are a rich source of autoantigens<sup>4</sup>, which are involved in the physiologic maintenance of self-tolerance. The uptake and processing of apoptotic cells has been proposed to be a silent process, meaning that release of pro-inflammatory cytokines by phagocytes is prevented<sup>5-8</sup>. Impaired clearance of apoptotic cells, resulting in an accumulation of late apoptotic and secondary necrotic cells, might provide a danger signal to antigen presenting cells (APCs), thus activating autoreactive T cells, and finally leading to the breakdown of peripheral tolerance<sup>3</sup>. Accumulating evidence has been provided that defective clearance of apoptotic cells can lead to exacerbation of inflammation and predisposes to the development of autoimmunity, such as in systemic lupus erythematosus (SLE)<sup>9</sup>.

The apoptotic cell clearance machinery includes professional phagocytes, i.e. immature dendritic cells (DCs) and macrophages (MØ), and non-professional phagocytes, including epithelial cells, fibroblasts and mesangial cells<sup>10</sup>. It has become clear that there are various subsets in both DCs and MØ<sup>11-13</sup>. Recent *in vitro* data show that MØ can be polarized into pro-inflammatory (MØ1) and anti-inflammatory cells (MØ2) by granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also termed CSF-1), respectively<sup>14,15</sup>. Classically, GM-CSF and M-CSF are thought to be the primary growth factors for the differentiation of macrophages<sup>16</sup>. Mice lacking M-CSF develop a general MØ deficiency<sup>17</sup>, whereas GM-CSF-knockout mice showed no major deficiency of MØ<sup>18,19</sup>. In humans, M-CSF, but not GM-CSF, is an ubiquitous cytokine circulating in the human body<sup>14,20</sup>. Thus, M-CSF could be the default cytokine to drive MØ differentiation under steady-state conditions.

The removal of apoptotic cells is an ongoing and constitutive process. Apoptotic cells provide "eat me signals" to phagocytes to promptly and efficiently engulf apoptotic cells during the very early stage of cell death, preventing them to release noxious intracellular contents<sup>21</sup>. Although the silent removal of early apoptotic cells is well appreciated, the immunological mechanisms involved are incompletely defined. There are several checkpoints conceivable which together determine the immunological response towards the clearance of apoptotic cells. First, the process and the stage of cell death and/or the specific death pathways triggered are closely related to the consequence of apoptotic cell clearance<sup>22</sup>. It has been established that late apoptotic or necrotic cells induce phagocyte activation whereas early apoptotic cells do not<sup>23,24</sup>. This is most likely due to differential expression of markers of cell death, such as heat shock proteins<sup>25</sup>, HMGB1<sup>26</sup> and

uric acid<sup>27</sup>. Second, opsonization of apoptotic cells by components of the innate immune system such as complement factors facilitates and modulates the clearance of apoptotic cells (reviewed in<sup>28</sup>). Third, the nature of phagocytes that take up apoptotic cells might provide a defined immunological response.

Due to the large heterogeneity of phagocytes, the contribution of different phagocyte subsets in the clearance of apoptotic cells in terms of cytokine signature and the polarization of immune regulation remains largely unknown. In this respect, we hypothesize that under steady-state conditions, the scavenging of apoptotic cells is largely confined to a specialized phagocyte subset with anti-inflammatory properties. To test this hypothesis, we compared three types of phagocytes, DC, MØ1 and MØ2, with respect to the phagocytosis of apoptotic cells at various stages of cell death. Our results strongly suggest that IL-10-producing MØ2 are prominently involved in the recognition and clearance of early apoptotic cells.

## Materials and Methods

### Generation of monocyte-derived DCs, MØ1 and MØ2.

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). DCs 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) supplemented with 5 ng/ml GM-CSF (Leucomax, Novartis Pharma BV, Arnhem, the Netherlands) and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) for at least 6 days, as previously described<sup>29</sup>. Two types of macrophages, namely MØ1 and MØ2, were generated in the same RPMI culture medium as DCs but supplemented with 5 ng/ml GM-CSF and 5 ng/ml M-CSF (R&D systems / ITK Diagnostics, Uithoorn, the Netherlands), respectively. In some experiments, MØ2 were generated in the presence of M-CSF and 10 µg/ml neutralizing anti-IL-10-receptor mAb<sup>30</sup>. In all experiments comparing these three types of phagocytes, they were generated in parallel from monocytes of the same donor. Separate unrelated donors were used for each independent experiment.

### Analysis of cell surface molecules 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 cytometry analysis to analyze the surface molecules of phagocytes: anti-CD1a (Leu-6), anti-CD14 (Leu-M3), anti-CD11b/Mac-1 (all from BD Biosciences, San Jose, CA), anti-DC-SIGN/CD209 (AZN-D1, a gift of Dr. Y. van Kooyk, VU Medical Center, Amsterdam, the Netherlands), and anti-mannose receptor (MR) /CD206 (D547.3, a gift of F. Koning, LUMC, Leiden, the Netherlands). Staining 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.

### **Induction of apoptosis and necrosis.**

Jurkat T cells were cultured in RPMI culture medium. Apoptosis of Jurkat T cells was induced by culture with 50  $\mu$ M etoposide (Vepesid, Bristol-Myers Squibb, New York, NY, USA) for 18 h in serum-free RPMI culture medium (RPMI 1640 containing 90 U/ml penicillin and 90  $\mu$ g/ml streptomycin). Alternatively, Jurkat cells were washed with PBS and exposed to ultra violet (UV)-C light (TUV lamp, predominantly 254 nm, Philips electronic Instruments Inc., Eindhoven, the Netherlands) at a dose of 50 J/m<sup>2</sup>. After UV irradiation, cells were cultured for 4 and 30 hours in serum-free RPMI medium to harvest the early and late apoptotic cells, respectively. Necrosis was induced by incubating Jurkat cells at 56°C in a water bath for 1 hour. Both apoptosis and necrosis were confirmed by double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI, VPS Diagnostics, Hoeven, the Netherlands) according to established methods<sup>31</sup>.

Next to apoptotic and necrotic cells, apoptotic blebs were used. Blebs were isolated from Jurkat cells treated with 50  $\mu$ M etoposide for 48 h, as described before<sup>32</sup>. Blebs from  $2.5 \times 10^5$  cells were used for the phagocytosis assay (see below) per  $1 \times 10^5$  phagocytes.

### **Phagocytosis assay.**

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>33</sup>. In brief, Jurkat cells were suspended in PBS at  $2 \times 10^7$  cells/ml and incubated for 30 min at 37°C with 5  $\mu$ M CFSE. Cells were washed and resuspended at  $2 \times 10^6$  cells/ml in serum-free RPMI culture medium, used for apoptosis induction, as described above. For the phagocytosis assay, labeled apoptotic cells ( $1 \times 10^5$ ) were co-cultured with DCs, MØ1 or MØ2 at 1:1 ratio for different time periods at 37°C or 4°C in 250  $\mu$ l RPMI culture medium in round-bottom glass tubes. DCs, MØ1 or MØ2 were stained with a PE-conjugated mAb against CD11b or APC-conjugated mAb against CD14 (BD Biosciences) and uptake was analyzed by two-color flow cytometry. The percentage of CD11b-positive cells that stained positive for CFSE was used as a measure for the percentage of DCs, MØ1 and MØ2 that ingested (37°C) and/or bound (4°C) apoptotic cells.

Phagocytosis of apoptotic cells by MØ1 and MØ2 was further investigated by confocal laser scanning microscopy with a BioRad MRC1024 ES krypton–argon ion laser scanning imaging system (Hercules, CA), using appropriate filter settings. Images were visualized using a 40x/0.75 numeric aperture (NA) oil objective, were acquired using Laser Sharp 2000 software (BIO-Rad), and were processed using ImageJ software version 1.33 (NIH Image, Bethesda, MD). For this purpose, CFSE-labeled apoptotic cells ( $1 \times 10^5$ ) and phagocytes were co-cultured at 1:1 ratio on the Lab-TEK™ chamber slides (NUNC/Sanbio, Uden, the Netherlands) for 2 h at 37°C, followed by washing to remove the non-ingested apoptotic cells, and staining with PE-conjugated mAb against CD11b. Cells were fixed with 4%

paraformaldehyde before analysis. More than 600 single cells of MØ1 or MØ2 were randomly scored. Phagocytosis was presented as phagocytic index (percentage of phagocytosing MØ × average number of apoptotic cells per MØ)<sup>34</sup>.

To measure a general phagocytic capacity of MØ1 and MØ2, sheep red blood cells (SRBC, erythrocytes) were obtained and opsonized with rabbit anti-sheep red blood cell IgG (E<sub>IgG</sub>). MØ1 and MØ2 were pre-cultured on Lab-TEK™ chamber slides at 37°C, followed by the addition of E<sub>IgG</sub> or non-opsonized erythrocytes at 1:50 ratio for 0.5 h. Unbound erythrocytes were washed away with PBS and uningested erythrocytes were lysed by lysis buffer. Cells were fixed and stained with May-Grünwald /Giemsa. More than 300 single cells of MØ1 or MØ2 were scored by the light microscopy and phagocytosis was presented as phagocytic index.

### **Cytokine detection.**

DCs, MØ1 and MØ2 were stimulated with 200 ng/ml lipopolysaccharide (LPS, *Salmonella Typhosa*, Sigma-Aldrich) for 24 h and supernatants were harvested. Cytokines were detected in the supernatants using ELISA. The measurements of IL-6 and TNF- $\alpha$  were performed as described<sup>35</sup>. The analysis of IL-10 was performed according to the manufacturer's instructions (Sanquin Research, Amsterdam, the Netherlands).

Co-culture was performed by incubating DCs, MØ1 and MØ2 with etoposide-induced apoptotic cells at a 1:1 ratio in RPMI culture medium. After 24 h, supernatants were harvested and tested for IL-6, IL-10 and TNF- $\alpha$  production.

### **Endocytosis and macropinocytosis assays.**

Lectin-mediated endocytosis was examined following co-incubation of phagocytes ( $1 \times 10^5$ ) with 100  $\mu$ g/ml Dextran<sup>FITC</sup> (Molecular Probes) for 30 or 60 min at 37°C in RPMI culture medium. Control experiments were done at 4°C. Blocking experiments were performed by pre-incubation of phagocytes for 20 min with 100  $\mu$ g/ml mannan, or 50 mM D-mannose (both from Sigma-Aldrich). Incubation with 50 mM L-mannose (Sigma-Aldrich) or culture medium only was used as a negative control. Macropinocytosis was measured as the cellular uptake of 100  $\mu$ g/ml lucifer yellow (LY) dipotassium salt (Molecular Probes) or 0.2  $\mu$ g/ml BSA<sup>FITC</sup> (Sigma-Aldrich). Cells were washed extensively with cold PBS containing 1% FCS and 0.02% NaN<sub>3</sub>. Before analysis with flow cytometry, the cell surface fluorescence was quenched with trypan blue (Sigma-Aldrich). To quantify the uptake, mean fluorescence intensity (MFI) values obtained at 4°C were subtracted from MFI values obtained at 37°C.

### **Inhibition of uptake of apoptotic cells**

To assess the role of phosphatidylserine (PS) in the uptake of early apoptotic cells by MØ2, early apoptotic cells were pre-incubated with recombinant annexin V (Sigma-Aldrich) (up to 50  $\mu$ g/ml) at 4°C for 20 min to mask PS, before incubation with MØ2. To assess the role of CD14 in the apoptotic cell

clearance, MØ1 and MØ2 were treated with an anti-CD14 blocking mAb (61D3, a kind gift of Dr. C. Gregory, University of Edinburgh, U.K.). MØ were pre-incubated with 61D3 (20 µg/ml) for 20 min at 4°C before addition of UV-induced early apoptotic cells or etoposide-induced apoptotic cells.

To investigate the role of macropinocytosis in the uptake of apoptotic cells by MØ1 and MØ2, 5-(N, N-Dimethyl)amiloride hydrochloride (DMA, Sigma-Aldrich) was used to inhibit macropinocytosis. MØ1 and MØ2 were pre-incubated with DMA (up to 1 mM) at 37°C for 20 min before early apoptotic cells or apoptotic blebs were added.

### **Statistical analysis.**

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

## **Results**

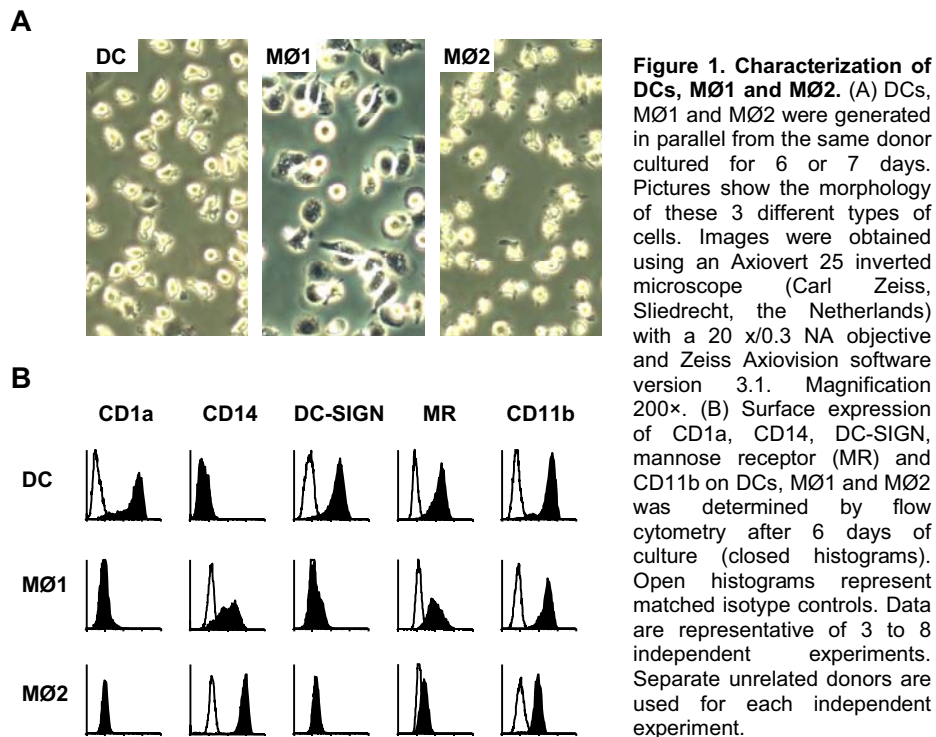
### **Characterization of DCs, MØ1 and MØ2.**

Three types of phagocytes were generated from peripheral blood monocytes by different cytokines, i.e. IL-4 and GM-CSF for DCs, GM-CSF for MØ1, and M-CSF for MØ2, respectively. MØ2 were less-adherent cells, with irregular shapes compared with MØ1 (Fig. 1A). In contrast to DCs, MØ1 and MØ2 shared the typical macrophage phenotype, being positive for CD14, but negative for CD1a and DC-SIGN (Fig. 1B). Notably, MØ2 expressed higher levels of CD14, but much lower level of mannose receptors (MR) than MØ1. All three cell types showed expression of CD11b.

### **Uptake of apoptotic cells by DCs, MØ1 and MØ2.**

To investigate the phagocytosis of these three phagocyte subsets, apoptosis was induced in Jurkat cells using etoposide for 18 hours at 37°C in serum-free RPMI culture medium, as described before<sup>32</sup>. According to annexin V and PI staining, early apoptotic cells (Annexin<sup>+</sup>PI<sup>-</sup> populations) were routinely  $27.52 \pm 6.08$  % and late apoptotic cells (PI<sup>+</sup> populations) were  $48.61 \pm 10.76$  % (n=8). Apoptosis was confirmed by nuclear fragmentation using Hoechst staining (data not shown). Exposure of apoptotic cells to DCs, MØ1 and MØ2 resulted in a time- and temperature-dependent binding and ingestion, as shown by the appearance of CFSE- and CD11b-double positive populations (Fig. 2A). Notably, from the three types of cells, MØ2 demonstrated the highest capacity for binding of apoptotic cells at 4°C, and for uptake of apoptotic cells at 37°C (Fig. 2A). MØ2 have a 1.5 to 2-fold

higher capacity for both binding and uptake of apoptotic cells compared to DCs and MØ1 (Fig. 2B; ANOVA,  $P < 0.001$ ). Similar results were obtained when different

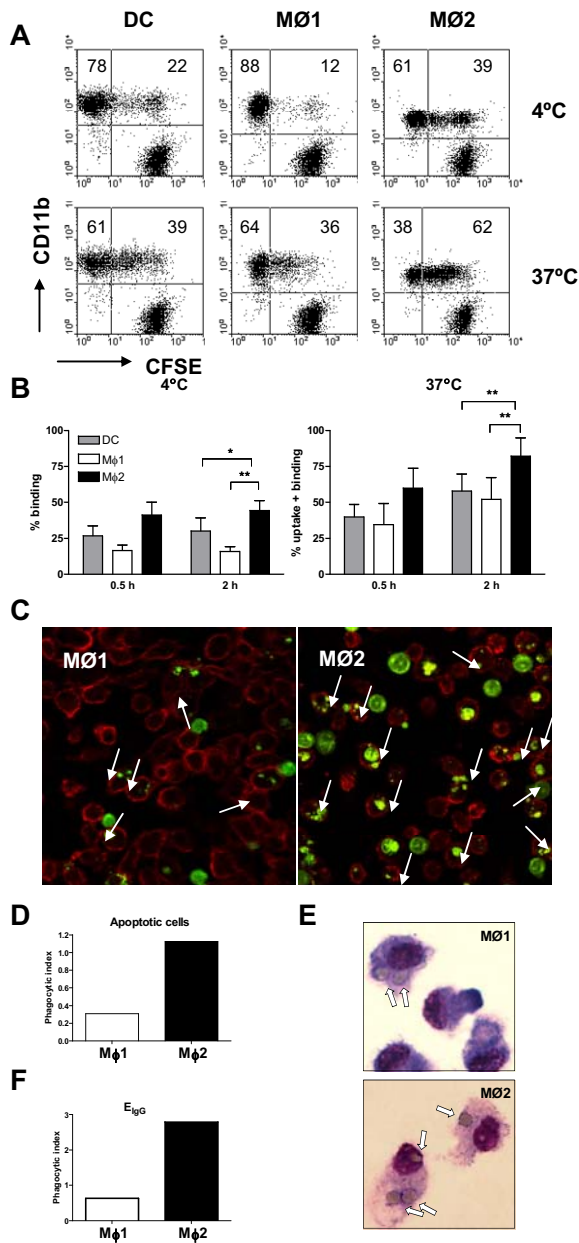


ratios of phagocytes:apoptotic cells (4:1, 16:1, and 64:1) were applied during co-culture (data not shown). The uptake was significantly increased in time at 37°C (ANOVA,  $p < 0.05$  for all three type of phagocytes), but the binding at 4°C was stable (Fig. 2B).

To confirm and quantify the uptake of apoptotic cells by MØ1 and MØ2, confocal microscopy was used. This analysis clearly showed the presence of CFSE-labeled apoptotic material inside the cells (Fig. 2C). Quantification of phagocytic index confirmed our FACS data that MØ2 were more potent in the uptake of apoptotic cells than MØ1 (Fig. 2D). The higher phagocytic index in MØ2 was due to a significantly higher percentage of MØ2 contributing to the uptake as compared to MØ1 (Chi-square test,  $p < 0.0001$ ), whereas the number of apoptotic cells taken up per macrophage between MØ1 and MØ2 were similar (2.33 vs 2.55, respectively). We realize that the difference of uptake between the quantification measured by FACS and by confocal microscopy is large, which is probably due to several factors. First of all, data presented are from separate sets of experiments with cells derived



from different donors. We have not performed a side-by-side comparison of both techniques. Second, for FACS analysis, we were using the cells in solution, which might affect their phagocytic behavior. However, in all cases MØ1 and MØ2 were generated in parallel from the same donor. Therefore we think we can draw firm conclusions from the comparisons between MØ1 and MØ2.



**Figure 2. Uptake of apoptotic cells by DCs, MØ1 and MØ2.** (A) Jurkat T cells were labeled with CFSE and induced into apoptosis by treating cells with etoposide for 18 hours. Apoptotic cells ( $0.1 \times 10^6$  cells) were co-incubated with DCs, MØ1 or MØ2 at 1:1 ratio, for 0.5 h and 2 h at 37°C or 4°C. Prior to flow cytometry analysis, cells were stained with PE-conjugated mAb against CD11b. CFSE- and CD11b-double positive populations represent the phagocytes that have bound and/or ingested apoptotic cells. Dot-plots represent the phagocytosis of apoptotic cells at 2 h. (B) The percentage of uptake and binding (at 37°C) or binding (at 4°C) were calculated as  $100\% \times ((CD11b^+CFSE^+)/CD11b^+)$ . Data indicate the mean  $\pm$  SD from 4 independent experiments performed in duplicate. Statistics were performed with two-way ANOVA. \*,  $p < 0.01$ , \*\*,  $p < 0.001$ . (C) Confocal microscopy images show the uptake of apoptotic cells by MØ1 and MØ2 (see arrows). Red cells represent the CD11b-PE positive MØ and green ones are the CFSE-labeled apoptotic cells. (D) Based on the confocal images, more than 600 cells of MØ1 and MØ2 were scored. Data are presented as phagocytic index (percentage of phagocytosing MØ  $\times$  average number of apoptotic cells per MØ). A Chi-square test was performed to evaluate the difference in the capacity of apoptotic cell uptake between MØ1 and MØ2 ( $p < 0.0001$ ). (E) Sheep erythrocytes were opsonized with rabbit anti-sheep red blood cell IgG (E<sub>IgG</sub>) and co-cultured with MØ1 and MØ2 on Lab-TEK™ chamber slides at 37°C for 0.5 h, followed by May-

To determine whether the observed highly phagocytic capacity of MØ2 represent a generalized increase on phagocytosis, uptake of E<sub>IgG</sub> was investigated. We found that the phagocytic index of MØ2 is 6.84 fold higher than that of MØ1 (mean of 3 independent experiments) (Fig. 2E, F). This is especially explained by the fact the percentage of MØ2 contributing to the uptake was significantly higher than that of MØ1 (Chi-square test,  $p < 0.0001$  for all three independent experiments), whereas the number of erythrocytes taken up per macrophage between MØ1 ( $1.83 \pm 0.17$ ) and MØ2 ( $2.23 \pm 0.72$ ) showed no difference (paired *t* test,  $p = 0.40$ ). The non-opsonized erythrocytes were not ingested by MØ1, whereas the uptake by MØ2 was of low efficiency (phagocytic index  $< 0.1$ , data not shown). Together, these data clearly show that MØ2 have a more generalized higher capacity of phagocytosis than MØ1.

### **MØ2 retain their anti-inflammatory status after uptake of apoptotic cells.**

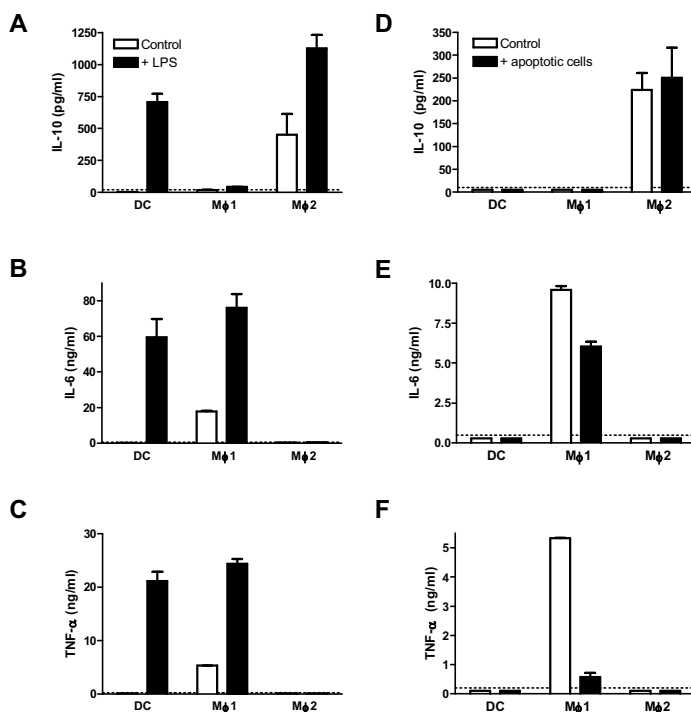
It has been proposed that clearance of apoptotic material is a non-inflammatory process<sup>1-3</sup>. Therefore we investigated the functional consequences of uptake of apoptotic cells by MØ1 or MØ2 by measuring the cytokine release in supernatants. The capacity of cytokine production by the three phagocyte subsets was first investigated using LPS stimulation (Fig. 3A-C). LPS-stimulated MØ2 produced large amounts of IL-10, but failed to secrete IL-6 and TNF- $\alpha$ . In contrast, MØ1 produced high amounts of IL-6 and TNF- $\alpha$  after LPS stimulation, whereas IL-10 was hardly detectable. DCs derived from the same donor were able to produce IL-10, IL-6 and TNF- $\alpha$  following LPS stimulation.

MØ2 showed intrinsic IL-10 production (Fig. 3A, D), which was retained after uptake of apoptotic cells (Fig. 3D), whereas production of IL-6 or TNF- $\alpha$  was not induced (Fig. 3E-F). Importantly, uptake of apoptotic cells by MØ1 resulted in a down-regulation of IL-6 and TNF- $\alpha$  production, but no induction of IL-10 (Fig. 3D-F). Upon exposure to apoptotic cells, DCs did not secrete IL-10, IL-6 or TNF- $\alpha$ .

### **MØ2 preferentially take up early apoptotic cells.**

To assess whether there are differences among these three types of phagocytes in the uptake of apoptotic cells in different stages of cell death, Jurkat cells were exposed to UV light. Four hours after UV irradiation, around 50-70% early apoptotic cells (annexin V<sup>+</sup> / PI<sup>-</sup>) were obtained (Fig. 4A). Thirty hours after UV irradiation, around 70-95% of cells were late apoptotic (PI<sup>+</sup>). Necrotic cells generated by heat shock were all PI-positive. Viable cells were used as a negative control. All three types of phagocytes were unable to take up viable cells (Fig. 4B). Importantly, MØ2 were the only phagocytes that were able to distinguish early apoptotic cells from late apoptotic and necrotic cells. MØ2 have a preferential uptake of early apoptotic cells, compared to late apoptotic or necrotic cells (Fig. 4B). In contrast, DCs and

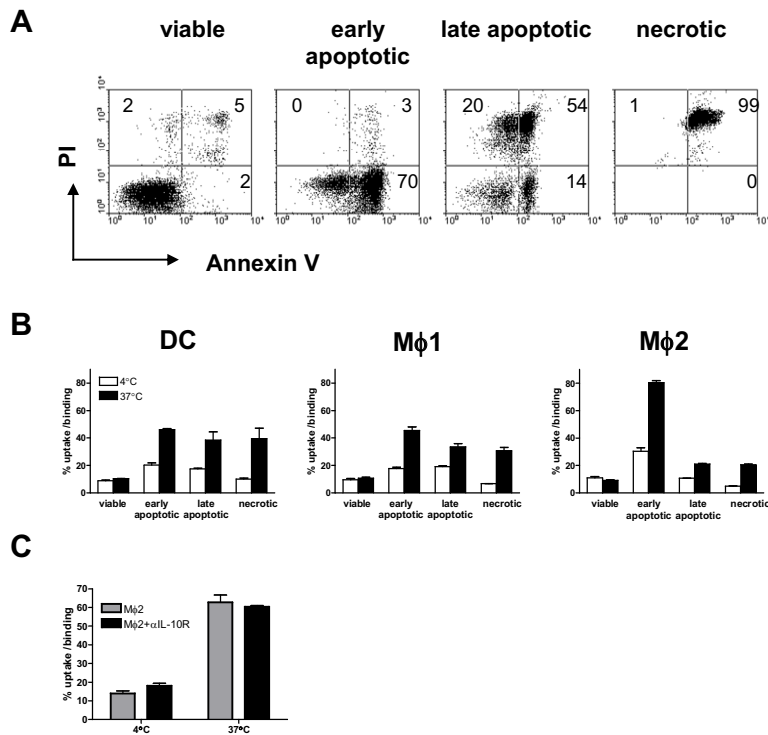
MØ1 show an equal efficiency in the uptake of early apoptotic, late apoptotic or necrotic cells. Furthermore, the efficiency of taking up early apoptotic cells by MØ2 is at least 2-fold higher than that by DCs and MØ1 (mean  $\pm$  SD of phagocytosis of MØ2: MØ1 from 5 independent experiments is:  $2.65 \pm 0.62$ ,  $p < 0.0001$ , two-way ANOVA).



**Figure 3. Cytokine production by DCs, MØ1 and MØ2 after LPS stimulation or uptake of apoptotic cells.** (A-C) Day-6 DCs, MØ1 and MØ2 were extensively washed, and subsequently stimulated with or without LPS for 24h. (D-F) DCs, MØ1 or MØ2 ( $1 \times 10^5$  cells) were co-cultured with etoposide-induced apoptotic cells at a 1:1 ratio for 24 h in RPMI culture medium. Supernatants were harvested and measured by ELISA for IL-10, IL-6, and TNF- $\alpha$ . Data are presented as mean  $\pm$  SD from duplicate cultures and are representative of at least 3 independent experiments where cells are generated from separate unrelated donors. Dashed lines represent the detection limits of ELISA.

It has been shown that IL-10-activated MØ have increased phagocytic capacity for the uptake of apoptotic cells<sup>36</sup>. To investigate whether IL-10-producing MØ2 become highly phagocytic to early apoptotic cells as a consequence of the IL-10 environment created by themselves, we generated MØ2 in the presence of neutralizing anti-IL-10-receptor mAb. Although the anti-IL-10-receptor mAb inhibited the endogenous IL-10 production of MØ2 (data not shown), it did not

inhibit phagocytosis of early apoptotic cells as compared to MØ2 generated in the absence of anti-IL-10-receptor mAb (Fig. 4C). We conclude that endogenous IL-10 produced by MØ2 is not involved in driving the development of high capacity for phagocytosis of early apoptotic cells.



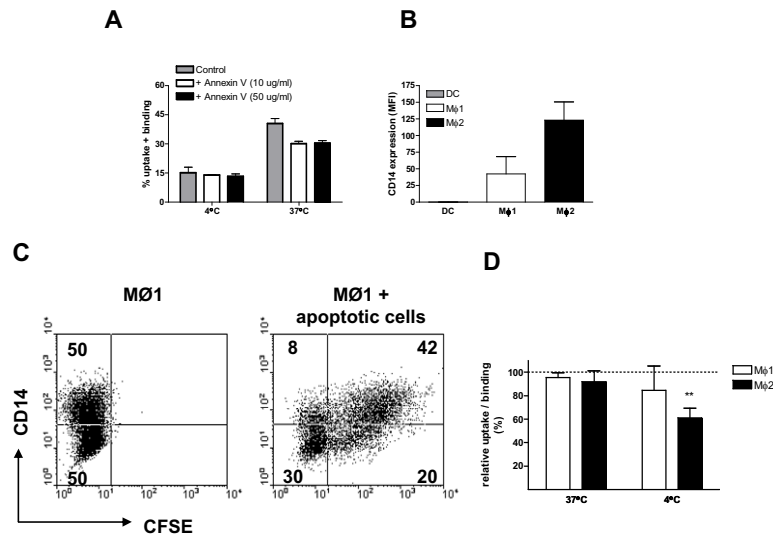
**Figure 4. Uptake of viable, early apoptotic, late apoptotic, and necrotic cells by DCs, MØ1 and MØ2.** (A) Early and late apoptotic cells were induced by UV-C light at a dose of 50 J/m<sup>2</sup>, then cultured for another 4 and 30 h, respectively. Necrosis was induced by incubating Jurkat cells at 56°C for 1 hour. The untreated viable cells were used a control. Apoptosis was confirmed by double staining with FITC-labeled annexin V and propidium iodide (PI). (B) These CFSE-labeled cells were co-cultured with DCs, MØ1 or MØ2 (1×10<sup>5</sup> cells) at 1:1 ratio, in the same way as described at Fig. 2. The percentages of uptake and binding (at 37°C) or binding (at 4°C) were quantified similarly as described at Fig. 2. Data represent at least 3 independent experiments performed in duplicate. (C) Uptake of early apoptotic cells by MØ2 generated in the presence or absence of 10 µg/ml neutralizing anti-IL-10-receptor mAb.

### Role of PS and CD14 in the uptake of apoptotic cells by MØ1 and MØ2.

A major feature of early apoptosis is the redistribution of PS from the inner layer of the cell membrane to the outside. Therefore we next investigated whether PS is involved in the phagocytosis by MØ2. Pre-incubation of early apoptotic cells with non-labeled annexin V prevented binding of FITC-labeled annexin V, confirming an effective masking of PS (data not shown), as described before<sup>32,37</sup>. The uptake of

early apoptotic cells by MØ2 was partially inhibited after PS masking (inhibition was between 6.5% to 23.2% in three independent experiments), and this effect could not be improved by applying a higher concentration of annexin V (Fig. 5A).

CD14 has been shown to be involved in the recognition of apoptotic cells by MØ<sup>38</sup>. As a possible explanation for their efficient uptake of apoptotic cells, we noticed that MØ2 express a significantly higher level of CD14 compared to MØ1 (Fig. 1B and Fig. 5B). Surface expression of CD14 on MØ1 is heterogeneous (Fig. 1B). Based on the FACS dot-plots, MØ1 were equally divided into two populations: CD14<sup>high</sup> and CD14<sup>low</sup> (Fig. 5C). Phagocytosis experiments showed that CD14<sup>high</sup> cells were twice as efficient in binding and uptake of apoptotic cells compared to CD14<sup>low</sup> cells (Fig. 5C). Thus we further investigated the role of CD14 in the engulfment of early apoptotic cells by using a blocking antibody against CD14, mAb 61D3<sup>38</sup>. The uptake of early apoptotic cells by both MØ1 and MØ2 was not significantly inhibited by this mAb. We did observe that the binding of early apoptotic cells by MØ2 at 4°C was inhibited ( $p < 0.001$ ) (Fig. 5D). Similar data were obtained when apoptosis was induced with either UV or etoposide. Together, these data indicate that CD14 is involved in tethering but not engulfing apoptotic cells.



**Figure 5. Role of PS and CD14 in the uptake of early apoptotic cells by MØ1 and MØ2.** (A) CFSE-labeled early apoptotic cells were pre-incubated with 10 µg/ml or 50 µg/ml unlabeled annexin V or medium as control at 4°C for 20 min, before the co-incubation with MØ2. The percentage of uptake and/or binding was calculated as described in Figure 2. Data shown are representative of 3 independent experiments performed in duplicate. (B) CD14 expression on DCs, MØ1, and MØ2 generated in parallel from the same donor. Bars show the mean fluorescence intensity (MFI). Data shown are the mean ± SD from 4 independent experiments. (C) After co-incubation of apoptotic cells with MØ1, cells were stained with a APC-conjugated anti-CD14 mAb, instead of anti-CD11b. CD14 expression was divided equally into two populations: CD14<sup>high</sup> and CD14<sup>low</sup>. Dot-plots of the FACS showed the distinct uptake of apoptotic cells by CD14<sup>high</sup> and CD14<sup>low</sup> cells. (D) MØ1 and MØ2 were pre-incubated with or without a blocking anti-CD14 antibody

(mAb 61D3) at 4°C for 20 min., before the co-incubation with early apoptotic cells for 2 h. Relative uptake / binding was calculated as  $100\% \times (\% \text{ in the presence of anti-CD14}) / (\% \text{ in the absence of anti-CD14})$ . Data are shown as mean  $\pm$  SD from 4 (MØ1) to 6 (MØ2) independent experiments performed in duplicate. Similar results were obtained when etoposide-induced apoptotic cells were applied. \*\*,  $p < 0.001$ , one sample *t* test. Separate unrelated donors are used for each independent experiment. Dashed line indicates 100% of relative uptake.

### **Uptake of early apoptotic cells by MØ2 is mediated by macropinocytosis.**

Phagocytes take up antigens via receptor-mediated endocytosis mostly by C-type lectins, such as mannose receptors (MR), or via macropinocytosis (fluid phase endocytosis)<sup>39,40</sup>. Therefore, we investigated lectin-mediated endocytosis of soluble antigens using Dextran<sup>FITC</sup>. Despite the fact that MØ2 showed a significantly lower expression of MR (Fig. 1B and Fig. 6A), the uptake of Dextran<sup>FITC</sup> by MØ1 and MØ2 was comparable, whereas uptake by DCs was more efficient (Fig. 6B). The uptake of Dextran<sup>FITC</sup> by DCs and MØ1 was completely inhibited by mannan or D-mannose, whereas L-mannose, used as a negative control, did not inhibit the uptake (Fig. 6C). In contrast, pre-treatment of MØ2 by mannan or D-mannose inhibited the uptake of Dextran<sup>FITC</sup> only by 50% (Fig. 6C), suggesting the involvement of lectin-independent mechanisms such as macropinocytosis. Indeed, for the uptake of both BSA<sup>FITC</sup> and LY, MØ2 were shown to be 2- to 3-fold more efficient compared to MØ1 and DCs (ANOVA,  $p < 0.001$ ) (Fig. 6D, 6E).

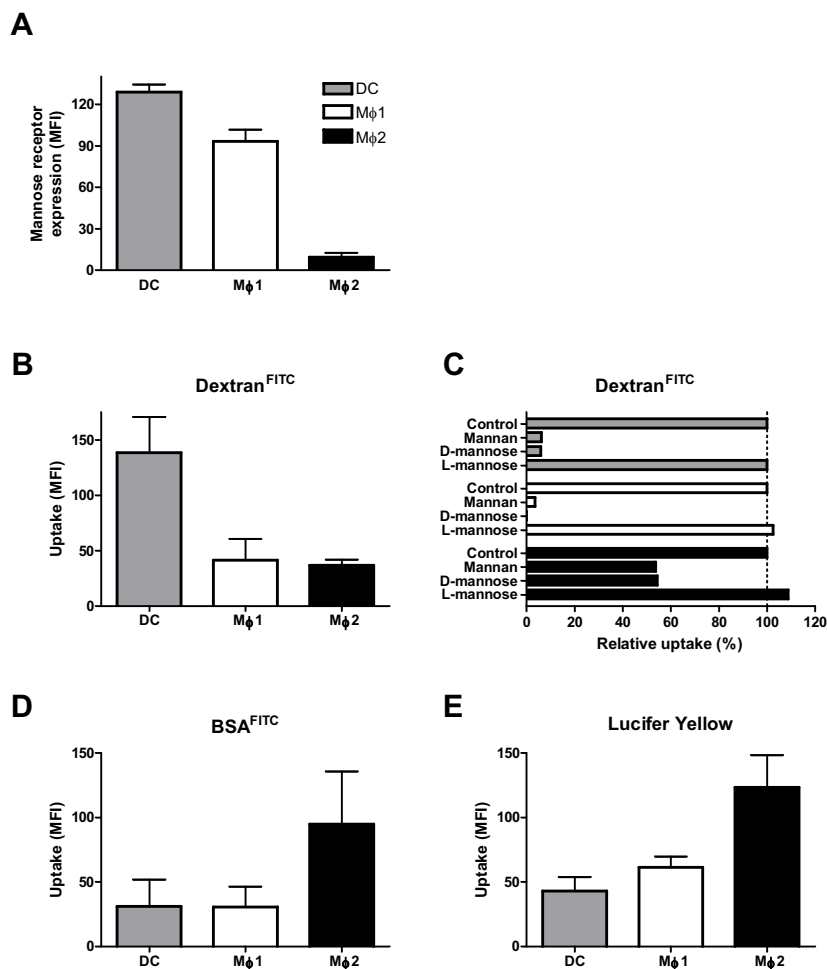
To investigate a possible contribution of macropinocytosis in the uptake of early apoptotic cells by MØ2, DMA, a Na<sup>+</sup>/H<sup>+</sup> antiporter blocker, was used to inhibit the fluid phase uptake by macropinocytosis<sup>39</sup>. DMA treatment of MØ2 not only inhibited the uptake of both BSA<sup>FITC</sup> and LY (Fig. 7A), but also inhibited the uptake of early apoptotic cells in a dose-dependent fashion (Fig. 7B), without interfering with the viability of the cells. Similarly, DMA also inhibited the uptake of early apoptotic cells by CD14<sup>high</sup> MØ1 (Fig. 7C).

We next investigated the uptake of microparticles derived from apoptotic cells (apoptotic blebs). MØ2 were able to take up apoptotic blebs twice as efficiently as MØ1 (Fig. 7D). DMA inhibited the uptake of apoptotic blebs by MØ2 dose-dependently (Fig. 7E), and in concentrations that are lower than needed for the inhibition of uptake of early apoptotic cells.

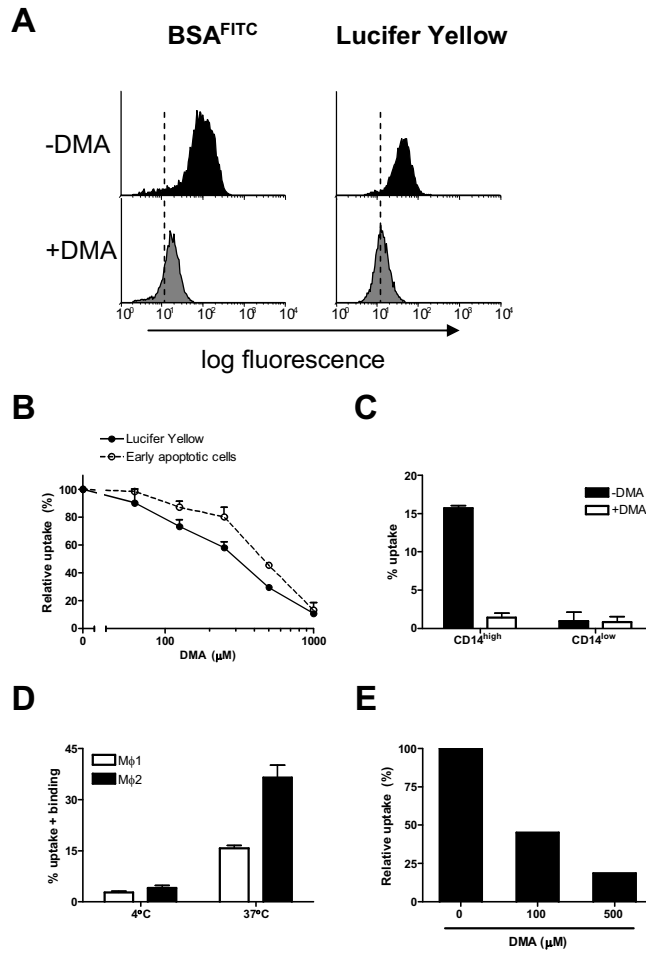
### **Discussion**

Over the last few years, it has become clear that both DCs and MØ show a large heterogeneity<sup>11,13</sup>. We hypothesized that distinct phagocyte subsets contribute differentially to the clearance of apoptotic cells. Here we identify that the IL-10-producing MØ2 are potent phagocytes that have the unique capacity to preferentially bind and ingest early apoptotic cells. This macrophage subset has

intrinsic anti-inflammatory properties, characterized by high IL-10 production in the absence of pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ . IL-10 is an important anti-inflammatory cytokine, which is able to limit and ultimately terminate inflammatory responses and plays a role in differentiation of regulatory T cells <sup>41</sup>.



**Figure 6. Lectin-mediated endocytosis and macropinocytosis by DCs, MØ1 and MØ2.** (A) Mannose receptor (MR)/CD206 expression on DCs, MØ1, and MØ2. Bars show the mean fluorescence intensity (MFI). Data shown are the mean ( $\pm$  SD) from 2 to 3 independent experiments. (B) The uptake of Dextran<sup>FITC</sup> (100  $\mu$ g/ml) by DCs, MØ1, and MØ2 was measured at 1 h. Data shown are the mean  $\pm$  SD from 3 independent experiments. (C) The uptake of Dextran<sup>FITC</sup> was measured by different phagocytes pre-incubated with 100  $\mu$ g/ml mannan, or 50 mM D-mannose or L-mannose. The uptake by untreated phagocytes (control) is considered as 100%, and data show the relative uptake against the control. Data represent 3 independent experiments. (D) The uptake of BSA<sup>FITC</sup> (0.2  $\mu$ g/ml) by DC, MØ1, and MØ2 at 1 h. Data shown are the mean  $\pm$  SD from 5 independent experiments. (E) The uptake of lucifer yellow (LY) (100  $\mu$ g/ml) by DCs, MØ1, and MØ2 at 1 h. Data shown are the mean  $\pm$  SD from 3 independent experiments (\*\*,  $p < 0.001$ , ANOVA).



**Figure 7. Uptake of early apoptotic cells and apoptotic blebs by MØ2 is prevented by an inhibitor of macropinocytosis.** MØ2 were pre-incubated with or without DMA at 37°C for 20 min. before the co-culture with BSA<sup>FITC</sup> (0.2 µg/ml), LY (100 µg/ml), or early apoptotic cells. (A) Data show the uptake of BSA<sup>FITC</sup> and LY at 1 h by MØ2 in the presence (gray histogram) or absence (black histogram) of 500 µM DMA. The dashed lines represent the background mean fluorescence of MØ2. (B) Data show the dose-dependent effect of DMA on uptake (37°C-4°C) of apoptotic cells and LY by MØ2 and represent the relative uptake of DMA-treated MØ2

against the controls (untreated cells). Data represent 4 independent experiments. (C) MØ1 were treated with or without 500 µM DMA at 37°C for 20 min before the co-incubation with early apoptotic cells for 30 min. Data show the quantification of uptake (37°C-4°C) by CD14<sup>high</sup> and CD14<sup>low</sup> cells. Data represent 3 independent experiments. (D) Apoptotic blebs were isolated from CFSE-labeled Jurkat cells and were used for the phagocytosis assay with MØ1 and MØ2. Data represent 4 independent experiments. (E) MØ2 were pre-treated with or without DMA (up to 500 µM). Relative uptake is shown. Similar results were obtained from 2 independent experiments. Separate unrelated donors are used for each independent experiment.

The generation of apoptotic cells is a continuous process. Although it is generally accepted that clearance of apoptotic cells is a non-inflammatory process, this most likely happens only when apoptotic cells are removed during early stages of apoptosis progression. Late apoptotic or necrotic cells activate APCs, promoting them to present self-antigen to T cells, potentially resulting in autoimmunity<sup>23,24</sup>. Thus under normal conditions, apoptotic cells should be cleared before they



progress into late stages. Our findings that MØ2 have the capacity to preferentially take up early apoptotic cells indicate that these IL-10-producing MØ subsets might be unique safeguards to promptly clear apoptotic cells in a silent way. To note, increased capacity of phagocytosis of apoptotic cells by MØ2 is not unique to apoptotic cells, but also to IgG-opsonized erythrocytes, suggesting MØ2 have a more generalized higher capacity of phagocytosis than MØ1. It remains to be established what the *in vivo* contribution is to apoptotic cell clearance by different myeloid subsets and which mechanisms underlie the specific clearance of early apoptotic cells.

It has been shown that IL-10-activated MØ obtain enhanced capacity for the uptake of apoptotic cells<sup>36</sup>. However, phagocytosis experiments using MØ2 generated in the presence of neutralizing anti-IL-10-receptor mAb suggested that endogenous IL-10 produced by MØ2 is not involved in driving the development of high capacity for phagocytosis of early apoptotic cells. This inconsistency could be due to the fact that Ogden *et al.*<sup>36</sup> generated MØ in the presence of IL-10 during differentiation process, whereas MØ produced low level of IL-10 during MCS-driven differentiation. It is surprising that neutralizing anti-IL-10-receptor mAb inhibited the IL-10 production during differentiation of MØ2. It remains unclear whether endogenous IL-10 is required to signal to IL-10R on MØ2, thus stimulating MØ2 to produce IL-10.

Both GM-CSF and M-CSF are key growth factors, not only for MØ generation *in vitro*, but also for MØ differentiation *in vivo*. *Op/op* mice lacking M-CSF develop a profound macrophage deficiency, and this could be partially corrected by the implantation of diffusion chambers containing M-CSF-producing cells<sup>17</sup>, confirming that M-CSF is crucial in MØ differentiation. GM-CSF knockout mice did not show major deficiency of MØ, although the MØ are smaller than normal<sup>18,19</sup>. Under steady-state conditions, M-CSF is the only primary MØ growth factor detectable in peripheral blood<sup>20</sup>. In contrast, GM-CSF is generally viewed as a pro-inflammatory cytokine involved in inflammation and is hardly detectable in circulation<sup>42</sup>. Thus under steady-state conditions, M-CSF could be the default cytokine driving MØ differentiation.

Cell-cell contact with apoptotic cells is sufficient to induce profound inhibition of IL-12 production by activated macrophages, thus preventing them to mount an immune response<sup>8</sup>. We show here that, upon uptake of apoptotic cells, MØ2 do not produce pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$ , while their IL-10 production was not inhibited. Importantly, and in line with previous results, uptake of apoptotic cells by MØ1 resulted in a down-regulation of IL-6 and TNF- $\alpha$  production. It has been shown that monocyte-derived MØ differentiated with M-CSF acquire the ability to selectively induce T cell apoptosis in an activation-specific fashion<sup>43</sup>, leading to the speculation that peripheral tolerance can be helped by selectively deleting autoreactive T cells that respond to self-antigen.

Therefore, at least two mechanisms might be operational to prevent that uptake of apoptotic cells leads to immune activation.

Recently, CD14 has been reported to play an important role in the recognition of apoptotic cells<sup>38</sup> and was further suggested as a tethering molecule for MØ to recognize apoptotic cells<sup>44</sup>. CD14<sup>-/-</sup> mice showed a persistence of apoptotic cells, supporting that CD14 plays a broad tethering role in the apoptotic cell clearance *in vivo*<sup>45</sup>. In our study, CD14 expression is remarkably different between MØ1 (CD14<sup>+</sup>) and MØ2 (CD14<sup>+++</sup>). Notably, MØ2 have a strong interaction with apoptotic cells already at 4°C. Blocking studies using anti-CD14 antibody showed that CD14 is mainly involved in the surface binding, but hardly in the uptake of apoptotic cells, supporting the notion that CD14 is a tethering molecule rather than an engulfing molecule<sup>38</sup>.

Next to the tethering process, other mechanisms are involved in the engulfment of apoptotic cells. Uptake of antigens (Ag) takes place via at least two different pathways: receptor-mediated endocytosis and macropinocytosis<sup>39,40</sup>. Although macropinocytosis is thought to be a specific characteristic of DCs<sup>39</sup>, it can be induced in other cells, including MØ stimulated with M-CSF<sup>46,47</sup>. Here we show in a side-by-side comparison that MØ2, generated in the presence of M-CSF but tested in the absence of M-CSF, exhibit more potent macropinocytosis compared with monocytes-derived DCs. The suggestion that macropinocytosis contributes to the uptake of apoptotic cells has already been put forward<sup>48</sup>. Engagement of MØ surface receptors by apoptotic cells, either directly (via PS) or indirectly (via C1q or mannose binding lectin (MBL) binding to apoptotic cells) actively promotes macropinocytosis<sup>34,49</sup>. Our studies using the inhibitor DMA suggest that the efficient uptake of early apoptotic cells or apoptotic blebs by MØ2 is mediated by Na<sup>+</sup>/H<sup>-</sup> antiporter-dependent macropinocytosis. Rac, Rho and Cdc42 have been described as the major regulators of actin-driven macropinocytosis<sup>50</sup>. Whether these regulators are involved in initiating macropinocytosis, particularly in the uptake of early apoptotic cells by MØ2, remains to be established. Our findings are in agreement with the proposal that the uptake of apoptotic cells is a two-step process<sup>3</sup>: first, apoptotic cells are engaged with phagocytes by receptor-dependent tethering processes such as CD14 for MØ2; Second, formation of fluid-filled macropinosomes allows MØ2 to promptly and efficiently engulf early apoptotic cells or microparticles-derived from apoptotic cells.

In conclusion, different subsets of phagocytes, such as DCs, MØ1 and MØ2 recognize and process early and late apoptotic cells in a differential manner. The cytokine environment and the nature of phagocytes at a certain location contribute to the decision whether apoptotic cells are cleared in a silent or inflammatory fashion. In this respect, we demonstrate that IL-10-producing MØ2 have the unique capability to preferentially bind and ingest early apoptotic cells. Our studies suggest that under steady-state conditions, M-CSF-driven MØ2 might be the default

phagocytes to preferentially clear early apoptotic cells in a silent way, thereby contributing to the induction and maintenance of peripheral tolerance after encountering self-antigen.

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