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Xu, W.

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Human peritoneal macrophages show functional characteristics of M-CSF-driven anti-inflammatory type-2 macrophages

Wei Xu¹, Nicole Schlagwein¹, Anja Roos^{1,2}, Timo K. van den Berg³, Mohamed R. Daha¹, and Cees van Kooten¹

¹Department of Nephrology, ²Department of Clinical Chemistry, Leiden University Medical Center, Leiden, the Netherlands; ³Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam, the Netherlands

Summary

We have recently shown that *in vitro*-polarized M-CSF-driven anti-inflammatory macrophages (M ϕ 2) have the unique capacity to preferentially bind and ingest early apoptotic cells. However, these data are based on *in vitro* polarized cells and it is unclear whether M ϕ 2-like cells exist *in vivo*. Here we used CD163 as a cell surface marker to distinguish M ϕ 2 from the pro-inflammatory M ϕ 1. We show that human peritoneal M ϕ (pM ϕ) freshly isolated from patients on peritoneal dialysis have the phenotypical characteristics of M ϕ 2, including CD163 surface expression and lack of CD16. Like M ϕ 2, pM ϕ have the capacity for endocytosis and macropinocytosis, are able to preferentially bind and ingest early apoptotic cells, and produce large amounts of IL-10 upon stimulation with LPS. Moreover, upon LPS stimulation both pM ϕ and M ϕ 2 down-regulate CD86, resulting in a reduced capacity to stimulate proliferation of allogeneic T cells and an inhibition of Th1 cytokine release of these T cells. Our data provide the evidence for the first time that *in vitro*-polarized M ϕ 2 exist *in vivo*, and human pM ϕ resemble the anti-inflammatory M ϕ 2. We propose that pM ϕ have the potential to maintain an anti-inflammatory condition in the peritoneal cavity.

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Introduction

The removal of apoptotic cells by phagocytes plays an important role in the suppression of inflammation and the regulation of immune responses^{1,2}. Since professional phagocytes including dendritic cells (DCs) and macrophages (M ϕ) represent a very heterogeneous population of cells^{3,4}, the extent of clearance of apoptotic cells and its immunological consequence may depend on the nature of phagocyte subsets in a certain tissue compartment⁵. We and others have recently shown that human M ϕ can be polarized *in vitro* into pro-inflammatory (M ϕ 1) and anti-inflammatory cells (M ϕ 2) by granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also known as CSF-1), respectively⁶⁻⁸. Particularly, M ϕ 2 were shown to have the unique capacity to preferentially bind and ingest early apoptotic cells⁸. This raises the question whether the *in vivo* clearance of apoptotic cells is confined to a specialized subset of phagocytes with anti-inflammatory properties, such as M ϕ 2^{5,8}.

Since the results mentioned above are based on *in vitro* studies, a challenging task is to obtain insight in the physiological relevance of *in vitro*-polarized M ϕ 1 and M ϕ 2. Classically, most resident M ϕ are derived from circulating bone marrow-derived monocytes *in vivo*⁴. GM-CSF and M-CSF are major growth factors for M ϕ differentiation *in vivo*⁹. Peritoneal M ϕ (pM ϕ) represent resident tissue M ϕ of the peritoneal cavity, and constitute one of the few human resident M ϕ subpopulations that are easily accessible for isolation¹⁰. It has been shown earlier that in mice, M-CSF, distributed locally, plays a critical role in the differentiation of resident pM ϕ *in vivo*¹¹. In humans, the M-CSF level in the peritoneal fluid is 2.5-fold higher as compared to plasma, and it has been shown that the levels of M-CSF are correlated to pM ϕ numbers¹². Considering the importance of M-CSF as a major factor in the differentiation of resident M ϕ , we undertook the current study as a further step to identify the *in vivo* counterpart of M-CSF-driven M ϕ 2. We found that human resident pM ϕ , freshly isolated from patients on peritoneal dialysis (PD), share several characteristics of M ϕ 2. Moreover, like M ϕ 2 and in contrast to M ϕ 1, resident pM ϕ down-regulate CD86 upon stimulation with LPS and this is associated with a reduction of their allogeneic T cell stimulatory capacity. We hypothesize that pM ϕ have the potential to maintain an anti-inflammatory status in the peritoneal cavity.

Materials and Methods

Patients and pM ϕ

pM ϕ were isolated from peritoneal dialysate effluents from in total fifteen PD patients at the department of Nephrology of the Leiden University Medical Center (the Netherlands). The underlying primary diseases were renal vascular disease, diabetes, hypertension, cystic nephritis and systemic lupus

erythematosus (SLE). All patients had been free of infection for at least 4 weeks before the collection of dialysate effluents. Effluents were collected from 3 to 4 h dwells. The isolation of pM ϕ was performed according to the method described elsewhere¹³. Briefly, peritoneal cells were collected by centrifugation (RPM 1500, 10 min.) from chilled dialysate effluents. After washing two times with PBS, cells were seeded at 1×10^6 /ml in 6-well culture plates (Costar, Cambridge, MA) in RPMI culture medium (RPMI1640 containing 10% heat-inactivated FCS, 90 U/ml penicillin and 90 μ g/ml streptomycin). After 2 h incubation at 37°C, non-adherent cells were removed by washing with PBS. Adherent cells (pM ϕ) were harvested by cell scraper (Costar).

Generation of M ϕ 1 and M ϕ 2.

Generation of M ϕ 1 and M ϕ 2 from human peripheral blood monocytes was performed using the methods described previously⁸. Briefly, M ϕ 1 and M ϕ 2 were generated from CD14⁺ monocytes in RPMI culture medium in the presence of 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, for 6 days.

Analysis of cell surface molecules by flow cytometry.

The following mAbs were used for flow cytometry analysis to analyze the surface molecules on different M ϕ : anti-DC-SIGN/CD209 (AZN-D1, a gift of Dr. Y. van Kooyk, VU Medical Center, Amsterdam, the Netherlands), anti-CD86 (IT2.2; Pharmingen, San Diego, USA), anti-CD16 (3G8, a gift from Dr. J.G.J. van de Winkel, University of Utrecht, the Netherlands), and anti-CD163 (EDhu1,¹⁴). Staining was visualized by PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark), and compared to appropriate isotype controls. PE-conjugated anti-CD14 (Leu-M3), and PE-conjugated anti-CD11b/Mac-1 were purchased from BD Biosciences (San Jose, CA). Cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences). Dead cells, identified by propidium iodide (PI) uptake, were excluded from analysis.

Phagocytosis assay.

Phagocytosis of early or late apoptotic cells was assessed using a protocol described previously⁸. Briefly, fluorescently labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands) labeled Jurkat cells were rendered into early (Annexin V⁺/PI⁻, routinely \pm 70%) and late apoptosis (Annexin V⁺/PI⁺, routinely \pm 95%) by irradiating them with ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m² and then cultured for 3 and 28 hours in serum-free RPMI medium, respectively. Labeled early or late apoptotic cells (1×10^5) were co-cultured with pM ϕ in 1:1 ratio at 37°C or 4°C for 0.5 h in 100 μ l RPMI culture medium in round-bottom glass tubes. pM ϕ were stained with a PE-conjugated mAb against CD11b and uptake was analyzed by a two-color flow cytometry. Phagocytosis of early apoptotic cells by pM ϕ was further investigated by confocal laser scanning microscopy with a LSM 510 (Carl Zeiss AG), as described

previously⁸. pM ϕ were stained with mAb against CD11b or CD163 followed by Alexa red-conjugated goat-anti-mouse Abs (Molecular Probes).

Allogeneic mixed lymphocyte reaction.

Responder T cells used for allogeneic mixed lymphocytes reaction (MLR) assays were isolated by sheep erythrocyte rosetting of mononuclear cells obtained from healthy donors. Stimulator cells, i.e. M ϕ 1, M ϕ 2 and pM ϕ , were first cultured with or without 200 ng/ml lipopolysaccharide (LPS, Salmonella Typhosa, Sigma-Aldrich) for 24 h. Prior to MLR, stimulator cells were irradiated (50 Gy) and added in graded doses to 1.5×10^5 allogeneic T cells in 96-well round-bottom tissue culture plates in RPMI culture medium. Cell proliferation was quantified by incubating the cells during the last 8 h of the 6-day cultures with 1 μ Ci (37 kBq) of [methyl-³H]thymidine (NEN, Boston, MA). Results are presented as the mean cpm \pm SD obtained from triplicate cultures.

Cytokine detection.

M ϕ 1, M ϕ 2 and pM ϕ (2×10^5) were stimulated with or without 200 ng/ml LPS for 24 h in 48-well-plate and supernatants were harvested. Cytokines were quantified in the supernatants using ELISA. The measurements of IL-6 and IL-10 were performed as described previously⁸. The supernatants harvested from MLR were measured for interferon- γ (IFN- γ), according the method described before¹⁵.

Statistical analysis.

Statistical analysis was performed by two-way ANOVA 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 and discussion

pM ϕ share similar phenotype with M ϕ 2

The purity of pM ϕ isolated from the dialysate effluents of PD patients was determined by analysis of morphology and flow cytometry for surface markers. These cells showed typical morphology for monocytes /M ϕ (not shown). Flow cytometric analysis revealed a phenotype for human M ϕ , i.e. positive for CD14 and CD11b, but negative for DC-SIGN, a marker for DCs (Fig. 1A). The major contaminating cells are CD3⁺ T lymphocytes which represented approximately 40% of total peritoneal cells before removing non-adherent cells and remained 5-15% thereafter (data not shown).

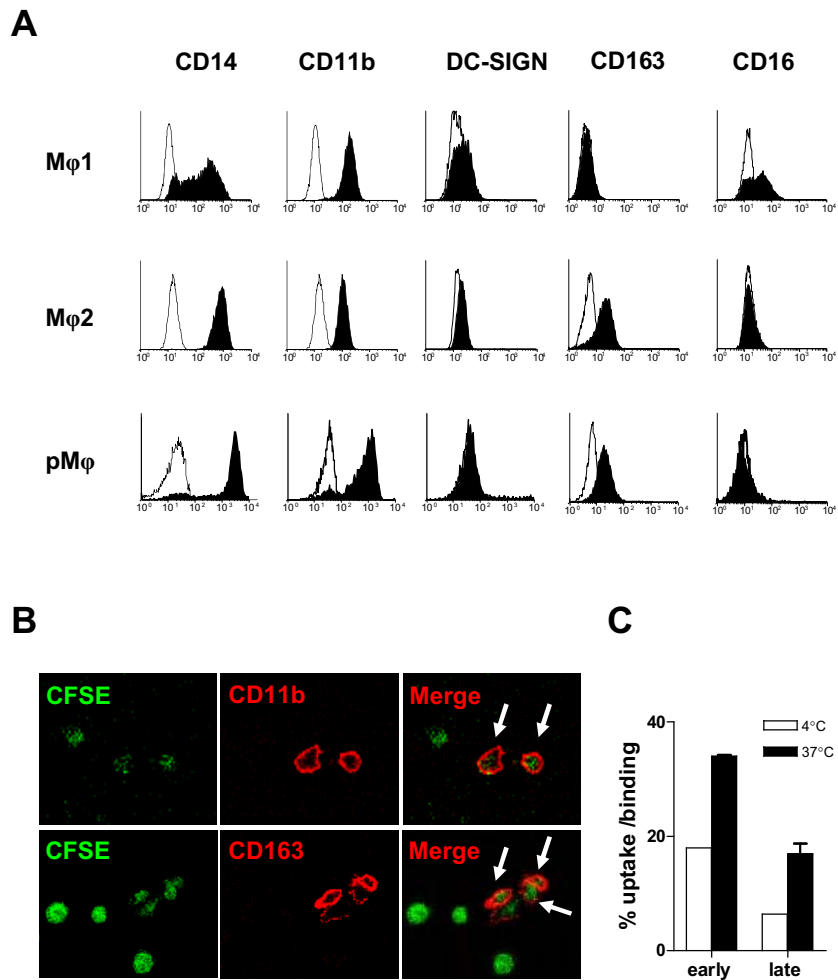


Figure 1. Characterization of M ϕ 1, M ϕ 2 and pM ϕ . M ϕ 1 and M ϕ 2 were generated in parallel from the same healthy donor cultured for 6 days. pM ϕ were freshly isolated from dialysate effluents from PD patients. Surface expression (closed histograms) was determined by flow cytometry. Open histograms represent matched isotype controls. (A) Surface expression of CD14, CD11b, DC-SIGN, CD163 and CD16 (Fc γ RIII) on M ϕ 1, M ϕ 2 and pM ϕ . Data are representative of at least 3 independent experiments using separate unrelated donors. (B) Confocal microscopy images show the uptake of early apoptotic cells by pM ϕ (see arrows). Red cells represent the CD11b- or CD163-positive pM ϕ and green ones are the CFSE-labeled apoptotic cells. (C) The uptake of early and late apoptotic cells was measured by flow cytometry. The percentage of uptake and binding (at 37°C) or binding (at 4°C) were calculated as $100\% \times ((\text{CD11b}^+\text{CFSE}^+)/\text{CD11b}^+)$. Data are representative of 3 independent experiments. $p < 0.01$, one sample t test.

Initially, to distinguish M ϕ 1 and M ϕ 2, we analyzed an array of surface makers, and found that CD163, a cell surface glycoprotein belonging to the group B cysteine-

rich scavenger receptor family, is exclusively expressed by M ϕ 2, but not by M ϕ 1 (Fig. 1A). In contrast, CD16 (Fc γ receptor III) is expressed by M ϕ 1 or subsets of M ϕ 1, but not by M ϕ 2. pM ϕ are characterized as CD163⁺CD16⁻, sharing this phenotype with M ϕ 2 (Fig. 1B). CD163 has been shown to be associated with an anti-inflammatory M ϕ phenotype, either alternative activated M ϕ or those tissue M ϕ isolated from patients with granulomatous disease¹⁶. Our finding that M ϕ 2 express CD163 confirms the association of CD163 with anti-inflammatory activities. The underlying pathology of the patients used for analysis was diverse, including renal vascular disease, diabetes, hypertension, cystic nephritis and systemic lupus erythematosus. Therefore we feel that it is unlikely that the M ϕ 2 phenotype of peritoneal macrophages can be attributed to a specific underlying pathology.

pM ϕ have high capacity for phagocytosis, endocytosis and macropinocytosis

An important functional characteristic of M ϕ 2 is that they have a higher capacity for antigen uptake, in particular that of early apoptotic cells, as compared to M ϕ 1 and DCs⁸. Therefore we assessed the capacity for uptake of early and late apoptotic cells by pM ϕ directly after isolation from dialysate effluents. Uptake of early apoptotic cells was confirmed by confocal microscopy, showing that CD11b⁺ and CD163⁺ pM ϕ had ingested CFSE-labeled early apoptotic cells (Fig. 1B). Quantification of uptake by flow cytometry shows that pM ϕ were capable to bind early or late apoptotic cells at 4°C and to engulf them at 37°C (Fig 2B). Importantly, like M ϕ 2⁸, pM ϕ exhibited a superior capacity for the binding and uptake of early apoptotic cells, compared with late apoptotic cells (mean fold difference: 1.87±0.17, p<0.01, ANOVA) (Fig 1C). Furthermore, pM ϕ were active in lectin-mediated uptake of Dextran^{FITC} and showed strong macropinocytosis-mediated uptake of lucifer yellow (data not shown), which resembles M ϕ 2. Collectively, these data suggest that pM ϕ share their strong phagocytic properties with M ϕ 2.

pM ϕ produce high amount of IL-10, as do M ϕ 2

The anti-inflammatory M ϕ 2 are characterized by high IL-10 production, whereas the pro-inflammatory cytokines such as IL-6, TNF- α are hardly produced^{7,8}. We investigated whether freshly isolated pM ϕ also predominantly produce anti-inflammatory cytokines. M ϕ 2, but not M ϕ 1, produced high amounts of IL-10 upon LPS stimulation (ANOVA, p<0.01) (Fig. 2A left panel). pM ϕ produced high amounts of IL-10, and LPS stimulated pM ϕ to produce significantly more IL-10 (mean fold increase: 5.19 ± 4.67, p<0.05, one sample *t* test) (Fig. 2A right panel).

Unlike the M ϕ 2 which produce little or no IL-6 (Fig. 2B left panel)⁸, pM ϕ produced considerable amounts of IL-6 after LPS stimulation (ANOVA, p<0.01) (Fig. 3D).

However, LPS was unable to induce a further significant increase in IL-6 by these pM ϕ (mean fold increase: 1.47 ± 0.88 , $p > 0.05$) (Fig. 2B right panel). IL-6 production by pM ϕ has been documented before¹⁷ and this was dramatically elevated in pM ϕ from peritonitis patients¹⁸. These data suggest that pM ϕ may perhaps not be as polarized as *in vitro*-generated M ϕ 2, but, nevertheless, they do have the high IL-10 producing capacity of typical of M ϕ 2, suggesting a potential anti-inflammatory activity.

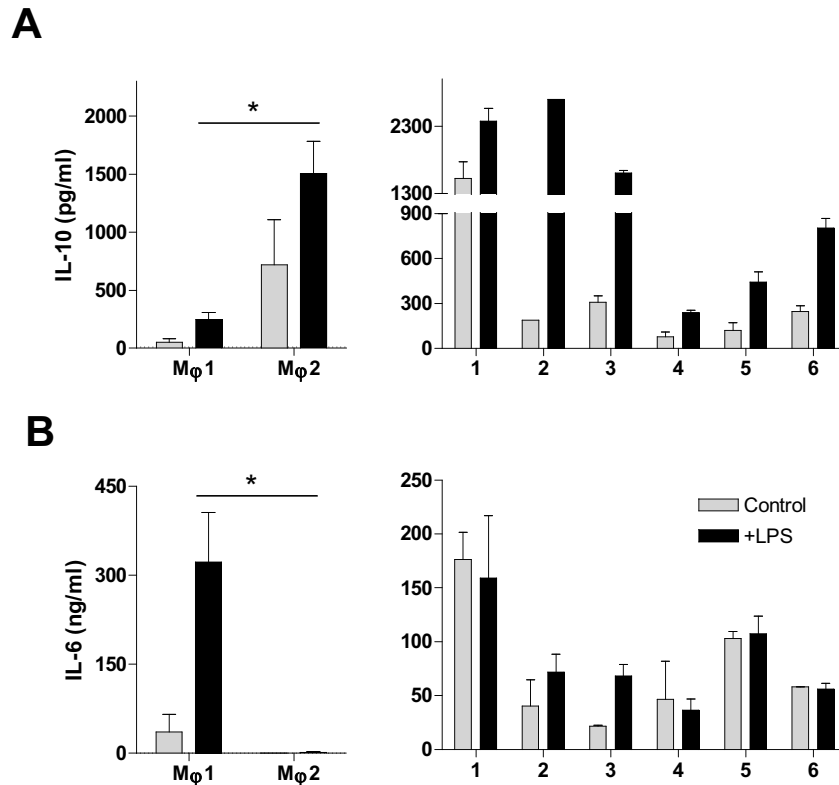


Figure 2. Cytokine production by pM ϕ after LPS stimulation. Isolated pM ϕ (2×10^5) were immediately cultured with or without LPS (200 ng/ml) for 24 h in RPMI culture medium. In parallel, M ϕ 1 and M ϕ 2 were stimulated by LPS in the same way. Supernatants were harvested and measured by ELISA for IL-10 (A) and IL-6 (B) of M ϕ 1 and M ϕ 2 and pM ϕ . Data shown (M ϕ 1 and M ϕ 2) are mean \pm SD from 4 independent experiments. *, $p < 0.01$, ANOVA. For pM ϕ , data shown are from 6 separate unrelated donors. Data represent the mean \pm SD from duplicate cultures.

M ϕ 2 and pM ϕ show hampered T cell stimulatory capacity

We next investigated the expression of costimulatory molecules among these M ϕ . Basal levels of CD80 and CD86 were comparable for all three M ϕ populations.

LPS stimulation increased CD80 and CD86 expression on M ϕ 1 but failed to do so on M ϕ 2 (Fig. 3A). Instead, a significant down-regulation of CD86 was observed on both M ϕ 2 and pM ϕ upon LPS stimulation (Fig. 3B, $p < 0.01$, one sample t test). In line with this, in an allogeneic MLR, activation of M ϕ 1 with LPS resulted in a strongly increased T cell stimulatory capacity (mean of fold increase: 1.32 ± 0.35 , $p < 0.01$, one sample t test) (Fig. 3C). In contrast, LPS activation of both M ϕ 2 and pM ϕ resulted in a hampered T cell proliferation (mean of fold increase for M ϕ 2: 0.90 ± 0.42 , $p < 0.01$, and for pM ϕ : 0.88 ± 0.13 , $p < 0.01$) (Fig. 3C). Measurement of IFN- γ production by activated T cells showed induction of IFN- γ by M ϕ 1 and pM ϕ , but not by M ϕ 2 (Fig. 3D). However, similar to T cell proliferative responses, LPS activation of M ϕ 1 increased IFN- γ production (mean fold increase: 2.83 , $p < 0.01$), whereas LPS activation of pM ϕ decreased the IFN- γ response (mean fold increase: 0.42 , $p = 0.012$) (Fig. 3D). Taken together, M ϕ 2 and pM ϕ showed a similar pattern to activate allogeneic T cells, in particular with respect to the reduced T cell stimulatory capacity upon LPS exposure.

The antigen presentation activities of pM ϕ have been studied earlier, showing that pM ϕ are competent antigen presenting cells^{19,20}. Our data confirm that pM ϕ can stimulate allogeneic T cell proliferation. However, our finding that LPS stimulation hampered the T cell stimulatory capacity and inhibited IFN- γ production by pM ϕ provides new insight in this type of resident M ϕ . It has been well accepted that both *in vitro* and *in vivo* M ϕ are heterogeneous in immune activities, i.e. activating versus suppressing¹⁰. Our data suggests that pM ϕ might be suppressor M ϕ with a role in the maintenance of peripheral tolerance upon scavenging self antigen derived from for instance apoptotic cells. Indeed, the anti-inflammatory and immune suppressive capacities of resident M ϕ were also found in other tissue M ϕ , such as murine alveolar M ϕ ^{21,22} and human intestinal M ϕ ²³.

Concluding remarks

In conclusion, we show that human pM ϕ display a strong resemblance to *in vitro*-generated anti-inflammatory M ϕ 2, thereby representing an *in vivo* counterpart of M ϕ 2. These CD163⁺ pM ϕ have similar capacities as M ϕ 2 for endocytosis, macropinocytosis, and a superior uptake of early apoptotic cells compared to the uptake of late apoptotic cells. We suggest that human resident pM ϕ are a subset of the primary phagocytes involved in the silent clearance of apoptotic cells. Furthermore, stimulated pM ϕ show a hampered capacity in allogeneic T cell proliferation and induce inhibition of Th1 cytokines. Thus we propose that pM ϕ have the potential to maintain an anti-inflammatory condition in the peritoneal cavity.

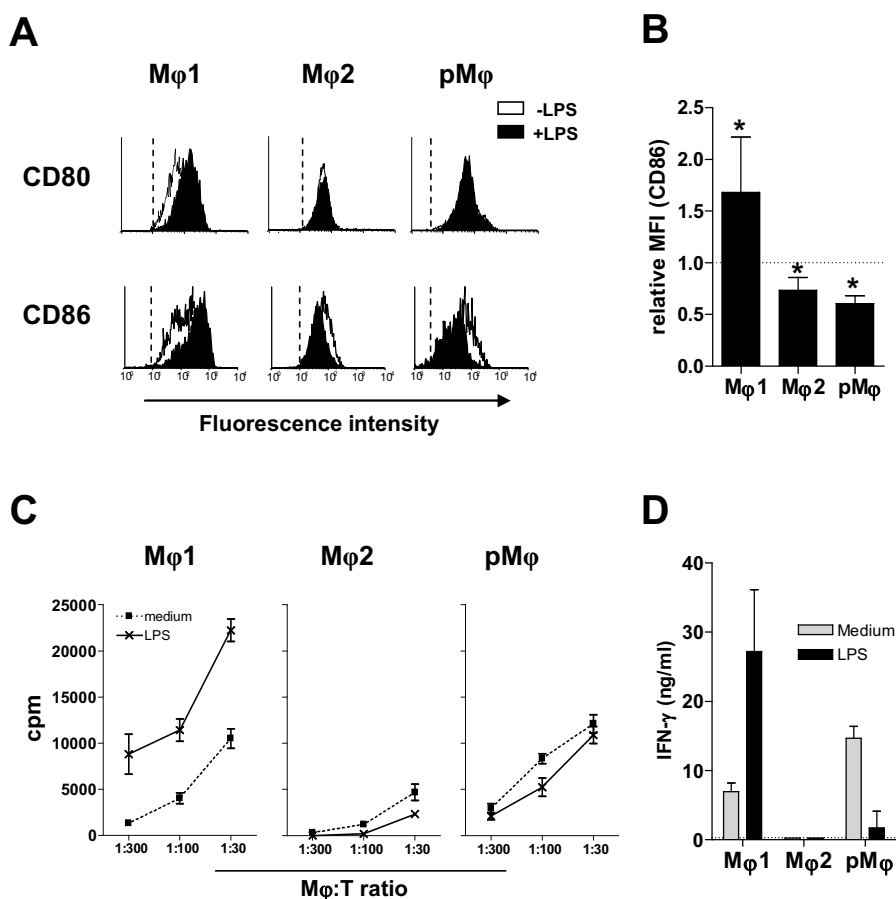


Figure 3. T cell stimulatory capacity of M ϕ 1, M ϕ 2 and pM ϕ . (A) CD80 and CD86 expression was determined by flow cytometry on cells with (closed histograms) and without LPS stimulation (open histograms) for 24 h. Dashed line represents the matched isotype control. (B) Relative expression of CD86 on M ϕ 1, M ϕ 2 and pM ϕ after LPS stimulation compared to the ones without stimulation. Data are the mean \pm SD of 3 independent experiments using 3 unrelated donors. *, $p < 0.01$, one sample t test. (C) Irradiated M ϕ 1, M ϕ 2 and pM ϕ 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. (D) Supernatants of MLR (M ϕ :T ratio at 1:10) were harvested and measured by ELISA for IFN- γ . Dashed line indicates the detection limit for ELISA. Data are mean of triplicate cultures and representative of at least 4 independent experiments.

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