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

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

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Summary

We have recently shown that *in vitro*-polarized M-CSF-driven anti-inflammatory macrophages (Mφ2) 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 Mφ2-like cells exist *in vivo*. Here we used CD163 as a cell surface marker to distinguish Mφ2 from the pro-inflammatory Mφ1. We show that human peritoneal Mφ (pMφ) freshly isolated from patients on peritoneal dialysis have the phenotypical characteristics of Mφ2, including CD163 surface expression and lack of CD16. Like Mφ2, pMφ 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 pMφ and Mφ2 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 Mφ2 exist in vivo, and human pMφ resemble the anti-inflammatory Mφ2. We propose that pMφ 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 (Mφ) 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 compartment [5]. We and others have recently shown that human Mφ can be polarized *in vitro* into pro-inflammatory (Mφ1) and anti-inflammatory cells (Mφ2) by granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also known as CSF-1), respectively [6-8]. Particularly, Mφ2 were shown to have the unique capacity to preferentially bind and ingest early apoptotic cells [8]. 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 Mφ2 [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 Mφ1 and Mφ2. Classically, most resident Mφ are derived from circulating bone marrow-derived monocytes *in vivo* [4]. GM-CSF and M-CSF are major growth factors for Mφ differentiation *in vivo* [9]. Peritoneal Mφ (pMφ) represent resident tissue Mφ of the peritoneal cavity, and constitute one of the few human resident Mφ subpopulations that are easily accessible for isolation [10]. It has been shown earlier that in mice, M-CSF, distributed locally, plays a critical role in the differentiation of resident pMφ *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 pMφ numbers [12]. Considering the importance of M-CSF as a major factor in the differentiation of resident Mφ, we undertook the current study as a further step to identify the *in vivo* counterpart of M-CSF-driven Mφ2. We found that human resident pMφ, freshly isolated from patients on peritoneal dialysis (PD), share several characteristics of Mφ2. Moreover, like Mφ2 and in contrast to Mφ1, resident pMφ down-regulate CD86 upon stimulation with LPS and this is associated with a reduction of their allogeneic T cell stimulatory capacity. We hypothesize that pMφ have the potential to maintain an anti-inflammatory status in the peritoneal cavity.

Materials and Methods

Patients and pMφ

pMφ 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 pMφ was performed according the method described elsewhere [13]. 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 μg/ml streptomycin). After 2 h incubation at 37°C, non-adherent cells were removed by washing with PBS. Adherent cells (pMφ) were harvested by cell scraper (Costar).

Generation of Mφ1 and Mφ2.

Generation of Mφ1 and Mφ2 from human peripheral blood monocytes was performed using the methods described previously [8]. Briefly, Mφ1 and Mφ2 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 Mφ: 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 previously [8]. Briefly, fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE, Molecular Probes, Leiden, the Netherlands) labeled Jurkat cells were rendered into early (Annexin V^+ /PI^-, routinely ± 70%). and late apoptosis (Annexin V^+ /PI^+, routinely ± 95%). by irradiating them with ultra violet (UV)-C light (Philips TUV lamp, predominantly 254 nm) at a dose of 50 J/m^2 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 pMφ in 1:1 ratio at 37°C or 4°C for 0.5 h in 100 μl RPMI culture medium in round-bottom glass tubes. pMφ 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 pMφ was further investigated by confocal laser scanning microscopy with a LSM 510 (Carl Zeiss AG), as described

previously [8]. pMφ 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. Mφ1, Mφ2 and pMφ, 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 µCi (37 kBq) of [methyl-^{3}H]thymidine (NEN, Boston, MA). Results are presented as the mean cpm ± SD obtained from triplicate cultures.

Cytokine detection.

Mφ1, Mφ2 and pMφ (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 previously [8]. The supernatants harvested from MLR were measured for interferon-γ (IFN-γ), according the method described before [15].

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

pMφ share similar phenotype with Mφ2

The purity of pMφ 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 /Mφ (not shown). Flow cytometric analysis revealed a phenotype for human Mφ, 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).

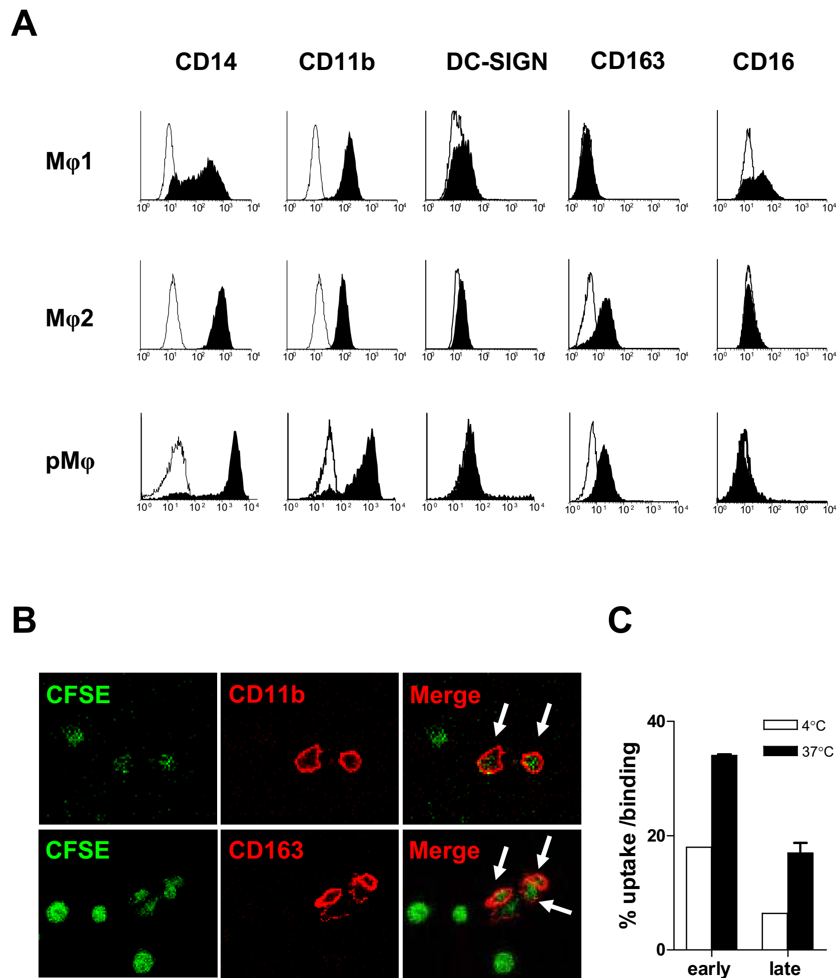

Figure 1. Characterization of Mφ1, Mφ2 and pMφ. Mφ1 and Mφ2 were generated in parallel from the same healthy donor cultured for 6 days. pMφ were freshly isolated from dialysate effluents from PD patients. Surface expression (closed histograms) was determined by flow cytometry. Open histograms represent matched isotype controls. (A) Surface expression of CD14, CD11b, DC-SIGN, CD163 and CD16 (FcγRIII) on Mφ1, Mφ2 and pMφ. 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 pMφ (see arrows). Red cells represent the CD11b- or CD163-positive pMφ and green ones are the CFSE-labeled apoptotic cells. (C) The uptake of early and late apoptoic cells was measured by flow cytometry. The percentage of uptake and binding (at 37°C) or binding (at 4°C) were calculated as 100% × (($CD11b^+CFSE^+$)/$CD11b^+$). Data are representative of 3 independent experiments. $p<0.01$, one sample *t* test.

Initially, to distinguish Mφ1 and Mφ2, 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 Mφ2, but not by Mφ1 (Fig. 1A). In contrast, CD16 (Fcγ receptor III) is expressed by Mφ1 or subsets of Mφ1, but not by Mφ2. pMφ are characterized as $CD163^{+}CD16^{-}$, sharing this phenotype with Mφ2 (Fig. 1B). CD163 has been shown to be associated with an anti-inflammatory Mφ phenotype, either alternative activated Mφ or those tissue Mφ isolated from patients with granulomatous disease [16]. Our finding that Mφ2 express CD163 confirms the association of CD163 with anti-inflammatory activities. The underling 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 Mφ2 phenotype of peritoneal macrophages can be attributed to a specific underlying pathology.

pMφ have high capacity for phagocytosis, endocytosis and macropinocytosis

An important functional characteristic of Mφ2 is that they have a higher capacity for antigen uptake, in particular that of early apoptotic cells, as compared to Mφ1 and DCs [8]. Therefore we assessed the capacity for uptake of early and late apoptotic cells by pMφ directly after isolation from dialysate effluents. Uptake of early apoptotic cells was confirmed by confocal microscopy, showing that $CD11b^{+}$ and $CD163^{+}$ pMφ had ingested CFSE-labeled early apoptotic cells (Fig. 1B). Quantification of uptake by flow cytometry shows that pMφ were capable to bind early or late apoptotic cells at 4°C and to engulf them at 37°C (Fig 2B). Importantly, like Mφ2 [8], pMφ 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, pMφ were active in lectin-mediated uptake of $Dextran^{FITC}$ and showed strong macropinocytosis-mediated uptake of luficer yellow (data not shown), which resembles Mφ2. Collectively, these data suggest that pMφ share their strong phagocytic properties with Mφ2.

pMφ produce high amount of IL-10, as do Mφ2

The anti-inflammatory Mφ2 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 pMφ also predominantly produce anti-inflammatory cytokines. Mφ2, but not Mφ1, produced high amounts of IL-10 upon LPS stimulation (ANOVA, $p<0.01$) (Fig. 2A left panel). pMφ produced high amounts of IL-10, and LPS stimulated pMφ 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 Mφ2 which produce little or no IL-6 (Fig. 2B left pannel) [8], pMφ 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 pMφ (mean fold increase: 1.47 ± 0.88, p>0.05) (Fig. 2B right panel). IL-6 production by pMφ has been documented before [17] and this was dramatically elevated in pMφ from peritonitis patients [18]. These data suggest that pMφ may perhaps not be as polarized as *in vitro*-generated Mφ2, but, nevertheless, they do have the high IL-10 producing capacity of typical of Mφ2, suggesting a potential anti-inflammatory activity.

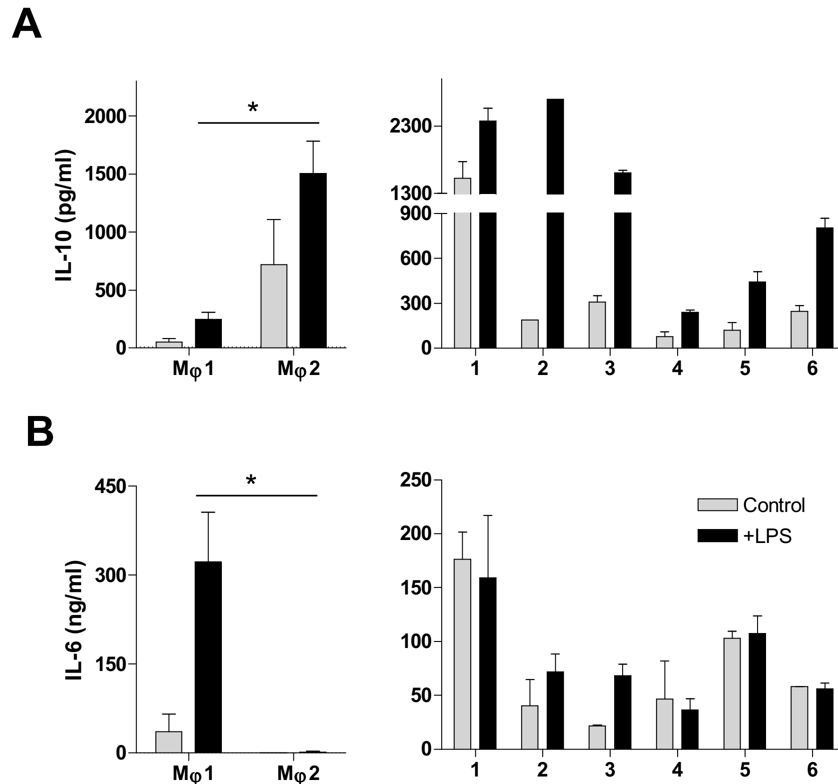

Figure 2. Cytokine production by pMφ after LPS stimulation. Isolated pMφ (2×10^5) were immediately cultured with or without LPS (200 ng/ml) for 24 h in RPMI culture medium. In parallel, Mφ1 and Mφ2 were stimulated by LPS in the same way. Supernatants were harvested and measured by ELISA for IL-10 (A) and IL-6 (B) of Mφ1 and Mφ2 and pMφ. Data shown (Mφ1 and Mφ2) are mean ± SD from 4 independent experiments. *, p<0.01, ANOVA. For pMφ, data shown are from 6 separate unrelated donors. Data represent the mean ± SD from duplicate cultures.

Mφ2 and pMφ show hampered T cell stimulatory capacity

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

LPS stimulation increased CD80 and CD86 expression on Mφ1 but failed to do so on Mφ2 (Fig. 3A). Instead, a significant down-regulation of CD86 was observed on both Mφ2 and pMφ upon LPS stimulation (Fig. 3B, $p<0.01$, one sample *t* test). In line with this, in an allogeneic MLR, activation of Mφ1 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 Mφ2 and pMφ resulted in a hampered T cell proliferation (mean of fold increase for Mφ2: 0.90 ±0.42, $p<0.01$, and for pMφ: 0.88 ±0.13, $p<0.01$) (Fig. 3C). Measurement of IFN-γ production by activated T cells showed induction of IFN-γ by Mφ1 and pMφ, but not by Mφ2 (Fig. 3D). However, similar to T cell proliferative responses, LPS activation of Mφ1 increased IFN-γ production (mean fold increase: 2.83, $p<0.01$), whereas LPS activation of pMφ decreased the IFN-γ response (mean fold increase: 0.42, $p=0.012$) (Fig. 3D). Taken together, Mφ2 and pMφ 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 pMφ have been studied earlier, showing that pMφ are competent antigen presenting cells [19,20]. Our data confirm that pMφ can stimulate allogeneic T cell proliferation. However, our finding that LPS stimulation hampered the T cell stimulatory capacity and inhibited IFN-γ production by pMφ provides new insight in this type of resident Mφ. It has been well accepted that both *in vitro* and *in vivo* Mφ are heterogeneous in immune activities, i.e. activating versus suppressing [10]. Our data suggests that pMφ might be suppressor Mφ 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 Mφ were also found in other tissue Mφ, such as murine alveolar Mφ [21,22] and human intestinal Mφ [23].

Concluding remarks

In conclusion, we show that human pMφ display a strong resemblance to *in vitro*-generated anti-inflammatory Mφ2, thereby representing an in vivo counterpart of Mφ2. These $CD163^+$ pMφ have similar capacities as Mφ2 for endocytosis, macropinocytosis, and a superior uptake of early apoptotic cells compared to the uptake of late apoptotic cells. We suggest that human resident pMφ are a subset of the primary phagocytes involved in the silent clearance of apoptotic cells. Furthermore, stimulated pMφ show a hampered capacity in allogeneic T cell proliferation and induce inhibition of Th1 cytokines. Thus we propose that pMφ have the potential to maintain an anti-inflammatory condition in the peritoneal cavity.

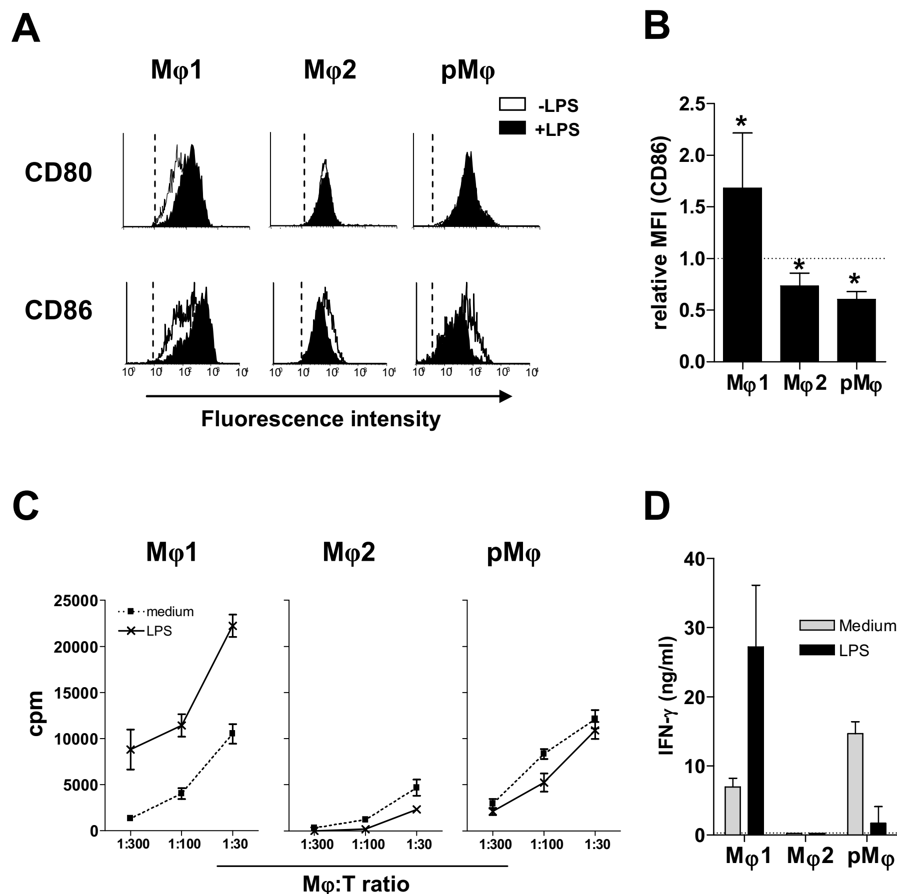

Figure 3. T cell stimulatory capacity of Mφ1, Mφ2 and pMφ. (A) CD80 and CD86 expression was determined by flow cytometry on cells with (closed histograms) and without LPS stimulation (open histograms) for 24 h. Dashed line represents the matched isotype control. (B) Relative expression of CD86 on Mφ1, Mφ2 and pMφ after LPS stimulation compared to the ones without stimulation. Data are the mean ± SD of 3 independent experiments using 3 unrelated donors. *, $p<0.01$, one sample *t* test. (C) Irradiated Mφ1, Mφ2 and pMφ 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 (Mφ: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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