Mycophenolic acid interferes the transcriptional regulation and protein trafficking of maturation surface markers in dendritic cells.

Transcription & protein traffic by mycophenolate

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HIGHLIGHTS

- Mycophenolic acid reduced mature dendritic cells (mDC) functionality
- CD86 surface expression was diminished due to transcriptional downregulation on mDC
- An interference in CD83 and ICAM-1 trafficking to membrane was observed on mDC
- Reduced surface expression was caused to decreased RNA on immature dendritic cells
- These results could be explained by decreased GTP levels due to IMPDH inhibition

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ABSTRACT:

Background: The ability of dendritic cells (DCs) to regulate adaptive immunity makes them interesting cells to be used as therapeutic targets modulating alloimmune responses. Mycophenolic acid (MPA) is an immunosuppressor commonly used in transplantation, and its effect on DCs has not been fully investigated.

Methods: Monocyte-derived DCs were obtained from healthy volunteers and cultured for 7 days. Cells were treated with MPA on day 2 and matured by lipopolysaccharide (LPS) stimulation. Functionality of mature DC (mDCs) was evaluated by allogeneic mixed lymphocytes reaction. Surface expression of maturation markers (CD40, CD83, CD86, and ICAM-1) was analysed in both immature DCs (iDCs) and mDCs by flow cytometry. To assess transcriptional regulation and protein subcellular location, RT-PCR and confocal microscopy were used, respectively.

Results: MPA decreased surface expression of all maturation markers in mDCs and significantly abrogated DCs-induced allogeneic T-cell proliferation after MPA pre-treatment. In iDCs, the reduced surface protein expression after MPA paralleled with mRNA downregulation of their genes. In mDCs, the mRNA levels of ICAM-1, CD40 and CD83 were enhanced in MPA-treated mDCs with an increase in the expression of CD83 and ICAM-1 near the Golgi compared to non-treated mDCs. In contrast, mRNA levels of CD86 were diminished after MPA treatment.

Conclusions: The reduced surface markers expression in mDCs exerted by MPA produced a decline in their capacity to activate immune responses. Moreover, the inhibition of guanosine-derived nucleotide biosynthesis by MPA treatment leads to DC maturation interference by two mechanisms depending on the marker, transcriptional downregulation or disrupted intracellular protein trafficking.

KEYWORDS: mycophenolic acid, human dendritic cell, protein trafficking, transcriptional regulation, guanosine nucleotides, immunosuppression

Abbreviations: CBA, Cytometric bead array; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cells; FACS, fluorescence-activated cell sorting; rhGM-CSF, recombinant human granulocyte–macrophage; GTP, guanosine-triphosphate; Gua, guanosine; IL-4, human interleukin 4; iDC, immature dendritic cell; IMPDH, inosine monophosphate dehydrogenase; LPS, lipopolysaccharide; LPS-mDC, dendritic cells matured with lipopolysaccharide; LPS+MPA-mDC, dendritic cells pre-treated with mycophenolic acid and matured with lipopolysaccharide; LPS+MPA+Gua-mDC, dendritic cells pre-treated with mycophenolic acid and guanosine and matured with lipopolysaccharide; mDC, mature dendritic cell; mo-DC, monocyte-derived dendritic cell; MLR, mixed lymphocyte reaction; MPA, mycophenolic acid; MPA-iDCs, immature dendritic cells pre-treated with mycophenolic acid; MPA+Gua-iDCs, immature dendritic cells pre-treated with mycophenolic acid and guanosine; PBMC, peripheral blood mononuclear cells; PHA-P, phytohaemagglutinin;

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1. INTRODUCTION

Dendritic cells (DCs) are the most professional potent antigen-presenting cells being considered as the bridge between the innate and adaptive response. In the absence of inflammatory or infectious stimuli, DCs are in steady-state status [1]. Steady-state DCs have an immature phenotype and are able to function like sentinels because of their ability to migrate through peripheral tissues to capture antigens by different mechanisms. Activation of DCs by different stimuli (endogenous ligands or pathogens, lipopolysaccharides (LPS), pro-inflammatory cytokines, alloantigens and hypoxia, among others) leads to DCs maturation [2–4]. When DCs switch to a maturation state, they acquire a great capacity for antigen processing and presentation to naïve T-cells, in order to initiate adaptive immune responses. The maturation process involves a phenotypical change characterized by increased expression of co-stimulatory molecules, adhesion molecules, attractant chemokines and pro-inflammatory cytokines [5,6].

DCs have the ability to modulate their functions and phenotypes depending on the maturation state. The induction of regulatory T-cells and the diminished activation of reactive T-cells caused by incomplete DCs maturation may provide a peripheral tolerance in transplantation or autoimmunity diseases. Many studies are emerging to support the idea of modulating DCs for the induction of tolerance in allogeneic transplants to finally prevent allograft rejection [7]. In this sense, the interference in DCs maturation and the altered expression of co-stimulatory molecules caused by immunosuppressive drugs could be a tool for alloresponse tolerance induction by DCs.

Although the understanding of the immunosuppressive drugs' action has been traditionally focused on lymphocytes, several studies reported that these drugs also interfere in DCs function [8]. Mycophenolate mofetil/sodium is an immunosuppressant prodrug widely used in solid

organ transplantation and mycophenolic acid (MPA) is its active metabolite. MPA inhibits in a non-competitive and reversible manner the enzymatic activity of inosine monophosphate dehydrogenase (IMPDH). IMPDH is a key enzyme of *de novo* purine nucleotide biosynthesis, more precisely of guanosine-derived nucleotide pathway, and it is well known that lymphocytes T and B are especially dependent on this pathway to proliferate [9,10]. Moreover, it has been described that MPA interferes in DCs function of presenting antigens in the direct and indirect allostimulation pathways by diminishing the expression of co-stimulatory molecules on the cell surface and reducing the pro-inflammatory cytokines for T-helper 1 cells [11–13]. However, the mechanisms underlying the observed effects still remain to be elucidated. It has been suggested that the lower levels of guanosine-triphosphate (GTP) derived from IMPDH inhibition by MPA could interfere in the mRNA synthesis and protein processing by interfering with protein glycosylation (due to the impaired incorporation of mannose and fucose sugars into proteins) [14]. In this sense, Dubsky *et al* [14] described that the pre-treatment of DCs with exogenous guanosine abrogated the low activation of T-lymphocytes reached after MPA treatment.

The aim of this project was to *in vitro* study the modulation mechanism derived from IMPDH inhibition on DCs maturation after MPA treatment, focusing on the expression and localization of different surface maturation markers (CD40, CD83, CD86 and ICAM-1).

2. MATERIALS AND METHODS

2.1 Antibodies and reagents

Monocyte enrichment kit without CD16 depletion was obtained from StemCell Technologies (Grenoble, France). Recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) and fibronectin were purchased from R&D Systems (Minneapolis, MN, USA). Human serum from male AB plasma, LPS from *Escherichia coli serotype 011:B4*, phytohaemagglutinin-P lectin (PHA) from *Phaseolus Vulgaris*, human interleukin 4 (IL-4), MPA, non-phosphorylated guanosine were acquired from Sigma-Aldrich (St. Louis, MO, USA). X-VIVO medium was purchased from Lonza (Basilea, Switerland). Cytometric bead array (CBA) and carboxyfluorescein diacetate succinimidyl ester (CFSE) were from Molecular Probes (Madrid, Spain). The following mouse monoclonal anti-human antibodies were obtained from Becton Dickinson Pharmingen (San Diego, CA, USA): allophycocyanin (APC) labelled anti-CD3, ICAM-1 and CD86 antibodies (clone UCHT-1, HA58 and FUN-1, respectively), phycoerythrin (PE) labelled anti-MHC-II antibody (clone G46-6), PE-cyanin 5 (PE-Cy5) labelled anti-CD11c (clone B-ly6) and fluorescein isothiocyanate (FITC) labelled anti-CD83 and CD40 antibodies (clone HB15e and 5C3, respectively). The probe/primers for ICAM-1 (Hs00164932_m1), CD40 (Hs01002913_g1), CD83 (Hs00188486_m1), CD86 (Hs01567026_m1), and GAPDH (Hs99999905_m1) were purchased from Applied Biosystems (Foster City, CA, USA). Mouse anti-human CD83 (clone HB15a) was from Santa Cruz Biotechnology (Dallas, TX, USA) whereas ICAM-1 (clone MEM-111) and GM-130 (immunogen: 1-990 amino acids of GM-130) were from Abcam (Cambridge, UK). Rabbit anti-human CD40 (immunogen: peptide from the C-terminal CD40), CD86 (clone EP1158Y) and

GM-130 (clone EP892Y) were purchased also from Abcam. DRAQ-5 nuclei staining was obtained from Biostatus (Loughborough, UK).

2.2 Dendritic cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by density gradient centrifugation on Ficoll-Hypaque. Human cells were obtained from *Banc de Sang i Teixits* (Barcelona, Spain) in accordance with protocols approved by the Ethics Committee of the Hospital Bellvitge of Barcelona and the principles of the Declaration of Helsinki. An enriched monocyte population (70-75 %) was obtained by negative selection for CD14+ positive cells without CD16 depletion.

After PMBCs isolation, monocytes were cultured in a 6-well plate ($0.75 – 1 \times 10^6$ cells/mL) in 2 mL of X-VIVO medium containing 2% of inactivated human serum, 15 ng/mL rhGM-CSF and 10 ng/mL IL-4 at 37°C in a humidified atmosphere with 5% CO_2. After two days, more than 95 % of the monocytes derived to DCs (monocyte-derived dendritic cells, mo-DCs). On day 2, the medium was refreshed and the mo-DCs were treated with MPA at different concentrations (1–100 µM). In some experiments exogenous non-phosphorylated guanosine at 100 µM was also added on day 2. On day 6, immature mo-DCs (iDCs) were stimulated with LPS at 1 µg/mL to obtain mature DCs (mDCs), as previously described [4]. Finally, on day 7 these cells were harvested and used for the different experiments (Figure 1).

2.3 Cell phenotype analysis by flow cytometry

Flow cytometry (fluorescence-activated cell sorting, FACS) analysis was performed using a FACS Canto and Diva software. DCs phenotype was analyzed by FACS after 7-day culture as previously reported by our group [4].

Firstly, non-adherent cells were collected, whereas trypsinization procedure was used for adherent cells. Adherent and non-adherent mo-DCs were mixed and two washing steps using PBS were realized. Around 2 – 3 x 10^5 cells were stained with different fluorescent antibodies. Cells were stained with PE-MHC-II antibody, PE-Cy5-CD11c antibody, FITC-CD83 and CD40 antibodies and APC-ICAM-1 and CD86 antibodies for 25 min at room temperature protected from light. After characterizing DCs population as positive for MHC-II and CD11c, the mean fluorescence intensity (MFI) and the positive percentage of cells (%) expressing the different maturation markers was assessed. At least, 10.000 events were obtained in each tube.

2.4 Allogeneic mixed lymphocyte reaction

Allogeneic PBMCs from different buffy coat were stained with intracellular fluorescent dye CFSE and co-cultured with DCs collected at the end of the 7-day culture. Mixed lymphocyte reaction (MLR) culture was carried out in duplicate in round-bottom 96-well plates.

The DCs: PBMCs (1:10) were co-cultured (2 x 10^4 vs 2 x 10^5, respectively) in X-VIVO medium with 2 % of inactivated human serum. PBMCs with medium alone was used as negative controls whereas the addition of PHA-P (1 µg/mL) was used as positive controls. After 5 days, cells were collected and stained with APC-CD3 antibody for T-lymphocyte characterization for 25 min at room temperature.

2.5 Cell cytokine analysis by flow cytometry

After the 5 days of MLR assay, the co-culture medium was collected and frozen at - 80 ºC until analysis. IL-2, IL-6, IL-10 and IL-17A secretion was determined by FACS using the Human Th1/Th2/Th17 CBA kit. Cytokines were analyzed by FCAP Array™ software (BD Biosciences, Franklin Lakes, NJ, USA).

2.6 Transcriptional analysis by RT-PCR

Extraction of the total mRNA was performed using Trizol isolation reagent. Subsequently, at least 0.7 µg of RNA was retrotranscribed to cDNA with the High Capacity cDNA reverse transcription kit. Finally, quantitative real-time-PCRs were carried out using the ABI Prism 7900 HT Fast Real-Tine PCR System. The relative expression of ICAM-1, CD40, CD83 and CD86 were normalized using GAPDH gene as the best house-keeping gene in front of 18S gene. All the samples reached the specific gene threshold, that was estimated between 19-25 C_t depending on the gene. Data analysis was based on the $\Delta\Delta C_t$ method.

2.7 Subcellular localization analyses of dendritic maturation markers

DCs (2–3 x 10^5) were harvested on cover glass slips coated previously with 50 µg/mL of fibronectin in a 24-well plate. After 7-days culture, cells were fixed for 10 min with paraformaldehyde. Cells were permeabilized with 0.1 % Triton X-100 for 10 min and blocked with 1.5 % normal goat serum and 3 % bovine serum albumin in PBS for 60 min. Then, cells were incubated overnight at 4 ºC with mouse anti-human CD83 (1:150), ICAM-1 (1:75) and GM-130 (1:150) and rabbit anti-human CD40 (1:100), CD86 (1:100) and GM-130 (1:250)

antibodies. On the next day, secondary anti-mouse and anti-rabbit 488nm and 555nm were incubated at room temperature in the dark during 45 min. Nuclei were stained with DRAQ-5 (1:1000) in the dark during 30 min. Images were acquired with a Spectral Confocal Microscope (TCS-SL; Leica Microsystems, Wetzlar, Germany) using a Plan-Apochromat 63×/1.7 N.A. immersion oil objective. He/Ne laser beams (Lasos, Jena, Germany) at 633 nm for GM-130 and 456 nm for ICAM-1, CD40, CD83, CD86 and DRAQ-5 with a pinhole of 115 μm was used as excitation source. Images were captured using Leica Confocal software and merged using Adobe Photoshop CS (Adobe Systems, CA, USA).

2.8 Statistical analysis

Results are shown as the mean ± standard error of the mean (SEM) of the values obtained from at least three independent experiments ($n \geq 3$). Differences between conditions were analyzed by ANOVA test and using Bonferroni correction for the post-hoc analysis. To perform the statistical analysis Prism 6.0 (GraphPad, San Diego, CA, USA) was used.

3. RESULTS

3.1 MPA effect on DCs maturation phenotype switch: dose-response study

Characterization of DCs after MPA treatment was performed by analysing the expression of different maturation markers (CD40, CD83, CD86 and ICAM-1) on cell surface by flow cytometry. LPS stimulation on DCs (LPS-mDCs) enhanced statistically significant the surface expression of all markers, characterised by an increase on MFIs and on the % of cells expressing them (Table 1 and S1).

The addition of MPA treatment in stimulated DCs (MPA+LPS-mDCs) reduced differently the expression of all these proteins, evaluated by the MFIs, in a concentration-dependent manner (Table 1 and Table S1). CD86 showed the highest response to MPA treatment compared with the other markers, diminishing their MFIs at concentrations over 5 μM. Likewise, CD83 surface expression was reduced by MPA at concentrations ≥ 10 μM. Finally, CD40 and ICAM-1 surface expression was only significantly diminished after using high concentrations of MPA (50 and 100 μM) (Table 1). Moreover, the positive % of cells expressing CD83 and CD86 was reduced after the treatment with MPA at doses over 10 μM, whereas no significant interference was observed in the positive % of cells expressing CD40 and ICAM-1 (Table S1).

3.2 MPA effect on functional capacity of mature DCs

To address the functional effect of MPA treatment on mDC's ability to activate T-cell responses, MLR was performed using mDCs as stimulator cells. As expected, statistically significant higher lymphocyte proliferation response was observed when allogeneic PBMCs

were co-cultured with LPS-mDCs in comparison to non-stimulated iDCs ($p = 0.015$) (Figure 2A).

The lymphocyte proliferation decreased after MPA treatment on LPS-mDCs and it was concentration dependent. Low doses of MPA (5 µM) showed a trend to reduce T-lymphocyte proliferation in comparison to LPS-mDCs although this decrement was not statistically significant. Nevertheless, MPA inhibition effect on the alloimmune response achieved statistical significance at concentrations of MPA ≥ 10 µM (Figure 2A). Considering the results obtained in the MLR and in the phenotype characterization, doses of 10 and 50 µM of MPA were chosen for further experiments.

The cytokine secretion was assessed in the MLR supernatant (Figure 2B-E). A slight increase of IL-2, IL-6 and IL-10 cytokines was observed in LPS-mDCs as compared to non-stimulated iDCs. In contrast, the levels of IL-17A remained similar in both conditions. On the other hand, IL-2 was diminished after MPA treatment (50 µM) as compared to LPS-mDCs, whereas IL-17A levels increased, although both not reaching statistical significance. In addition, similar levels of IL-6 and IL-10 secretion were obtained between LPS-mDCs and MPA+LPS-mDCs.

3.3 Effect of guanosine on MPA-treated DCs

To assess the dependence of MPA effects on guanosine-derived nucleotide synthesis pathway, exogenous guanosine was added in both iDCs and mDCs.

iDCs treated with MPA (MPA-iDCs) showed a statistically significant reduction of their MFIs and positive % of cells for all the studied surface markers (Table 2 and Tables S2). The effect

of MPA was reverted when guanosine was added with MPA in iDCs (MPA+Gua-iDCs) (Table 2 and Tables S2). Conversely, the mere addition of exogenous guanosine to iDCs culture did not change the MFI or the positive % of cells expressing any surface marker (Table 2 and Table S2).

When guanosine addition was studied in mDCs, the effect of MPA treatment after guanosine (MPA+Gua+LPS-mDCs) was partially reverted for CD83, CD86 and ICAM-1 surface expression, although only CD86 reached statistically significant differences ($p<0.05$) (Table 1 and Table S1). In contrast, guanosine addition did not revert the effect of MPA treatment for CD40 expression (Table 1 and Table S1).

3.4 Regulation effect of MPA on mRNA expression

To evaluate the dependence of diminished markers' surface expression after MPA treatment on a transcriptional regulation, the mRNA expression of their corresponding genes was assessed.

iDCs showed a statistically significant decrease in gene expression of all the markers (CD40, CD83, CD86 and ICAM-1) after MPA treatment compared to non-treated iDCs, regardless of the concentration used (Figure 3A). In contrast, the addition of guanosine significantly reverted the effect of MPA treatment on iDCs (Figure 3A).

After LPS stimulation to iDCs, two different transcriptional regulations were observed. On the one hand, after 6 hours of LPS stimulation (LPS-mDCs) there was not any appreciable regulation on mRNA levels of CD86, showing similar values than iDCs (Figure 3B). Furthermore, after MPA treatment (10 and 50 µM), the transcriptional levels of CD86 were

statistically significant reduced compared to non-treated LPS-mDCs (Figure 3B). In contrast, CD40, CD83 and ICAM-1 displayed a different pattern in mRNA regulation, showing a significant enhancement in mRNA expression after 6 hours of LPS stimulation compared to non-stimulated iDCs (Figure 3B). Moreover, in all these genes, different concentrations of MPA significantly increased the mRNA levels compared to non-treated LPS-mDCs, observing higher enhancement with higher MPA concentrations (Figure 3B).

3.5 Effect of MPA on subcellular localization of maturation markers

As an mRNA regulation did not explain the decrease of CD40, CD83 and ICAM-1 on cell surface after MPA treatment in mDCs, the role of MPA on interfering protein trafficking was explored by analysing the intracellular localization of these markers. The cytosolic or cell surface localization of these proteins was evaluated using confocal microscopy. Moreover, GM-130 staining was used to identify Golgi apparatus inside the cell cytosol.

In iDCs, MPA treatment showed a general reduction in the fluorescence staining content regardless of the marker and its specific localization compared to non-treated iDCs (Figure 4 and 5, and Figure S1 and S2). After guanosine addition to MPA-treated iDCs, the fluorescence staining was enhanced compared to MPA-iDCs and appreciably it seemed to return to non-treated iDCs levels in all the proteins (Figure 4 and 5, and Figure S1 and S2).

The two different patterns observed in mRNA regulation after LPS stimulation were studied by analysing the interference exerted by MPA on intracellular protein trafficking to the cell membrane. On the one hand, CD86 was mainly localized in the cell cytosol in iDCs and LPS-mDCs. Fluorescence intensity staining was not appreciable affected after LPS stimulation in

comparison to iDCs (Figure S2). After MPA treatment, parallel to the transcriptional regulation, CD86 fluorescence staining diminished considerably compared to non-treated LPS-mDCs regardless of its localization. The addition of guanosine appeared to revert the MPA-derived interference on protein staining (Figure S2). In contrast to CD86 marker, the staining of CD83 on LPS-mDCs was higher in the membrane and near the Golgi compared to non-stimulated iDCs (Figure 4). After MPA treatment, its expression was substantially reduced in the membrane compared to LPS-mDCs while was similarly retained near the Golgi. The addition of guanosine reverted the different protein localization produced by MPA effect (Figure 4). Moreover, ICAM-1 was mainly localized in the cell surface membrane after LPS stimulation and its fluorescence staining was appreciable higher than non-stimulated iDCs (Figure 5). After MPA treatment, as CD83, the fluorescence staining was reduced in the cell membrane but, increased near the Golgi. The addition of guanosine appeared to revert the MPA-derived interference on protein localization near the Golgi (Figure 5). Concerning CD40, it was predominantly expressed on the cell surface membrane or in the cytosol near to extracellular membrane in iDCs and LPS-mDCs (Figure S1). In contrast to CD83 and ICAM, the CD40 fluorescence staining diminished especially in the cytosol after MPA treatment on LPS-mDCs. In contrast to all the other markers, the addition of guanosine did not assumably interfere on the effect produced by MPA treatment (Figure S1).

4. DISCUSSION

The present study was focused on the effects of MPA on the activation of DCs' maturation. Our results reveal that MPA treatment leads to a weaker capacity of DCs to present antigens to T-cell and to activate them as a consequence of reduced levels of co-stimulatory molecules (CD40 and CD86) and adhesion proteins (ICAM-1) on the cell surface. This decrease could be explained by the mRNA downregulation of these genes and/or by the interference on their protein trafficking to the cell membrane observed in our results. To our knowledge, this is the first study that analyse the transcriptional regulation of maturation genes (CD40, CD83, CD86 and ICAM-1) and that explore the subcellular localization of these proteins.

Phenotype analysis of DCs after MPA treatment was performed both in iDCs and mDCs. After LPS stimulation, the surface expression of all maturation markers was enhanced compared to iDCs. In mDCs, MPA treatment was able to reduce the surface expression of all these markers in a concentration-dependent manner. These results are in accordance with the Colic *et al* findings after treatment of MPA at 10 μM concentration on LPS-stimulated DCs [12]. Likewise, diminished expression of maturation markers after increasing MPA concentrations between 2.5 and 100 μM was also observed after TNF-α stimulation on DCs [13,14]. Most studies were focused only on examining the MPA effect on mDCs and little is known about the role of this drug on circulating steady-state iDCs [12]. In this regard, our results revealed that MPA treatment also decreased the MFI of all maturation markers on iDCs in comparison with non-treated iDCs. The results observed after 10 and 50 μM of MPA treatment develop clinical relevance because these concentrations are commonly managed in transplant patients as pre-dose and high-peak concentration in plasma, respectively [12,15].

This phenotypical switch after MPA treatment led to a dysfunctional modulation in the MLR assay. The inhibition of DCs maturation by MPA after LPS stimulus clearly resulted in a lower proliferation of T-lymphocytes in the MLR compared to non-treated mDCs. Moreover, this effect of MPA depended on the concentration used. The concentration-dependent reduction observed in the MLR assay could be explained by the parallel diminished expression of the co-stimulatory molecules on the cell surface, that was also related to the MPA concentrations used. This result was previously described in the literature with different concentrations of MPA and with different stimulation agents (LPS, TNF-α, poly I:C and zymosan), evidencing the interference on mDCs capacity in the direct and indirect alloresponses [11–13,16]. Moreover, a reduction in pro-inflammatory cytokines implicated in T-helper 1 activation (IL-2) [17–20] accompanied by an increment of cytokines implicated in regulatory T-cells (IL-17A) was observed under MPA treatment. Previous reports have shown similar results, however, they were mainly focused on a decrease in other T-helper 1 activation cytokines like IFN-γ, TNF-α and IL-12 [11,12,20,21]. Other results of cytokine secretion in the MLR assay are confusing. It appears that MPA caused changes of IL-2 and IL-17A, but do not influence the levels of IL-6 and IL-10 after MLR. To our knowledge, concerning IL-6, Iwaszkiewicz-Grzes *et al* found similar results describing no interferences on IL-6 secretion after MPA treatment on mDCs [19]. On the other hand, concerning IL-10, other authors reported that MPA treatment diminished IL-10 secretion [12,19] while others observed an enhanced secretion [13,20,21]. Moreover, Faugaret *et al* described that the diminished T-cell proliferation by DCs in the MLR after MPA treatment was dependent on IL-2 secretion and not due to IL-10 [22]. The secretion of this cytokine pattern reaffirms the effect of MPA on mDCs leading to functional changes in the MLR as other studies described [11–13].

The transcriptional levels of all the markers after treatment with 10 or 50 µM of MPA on iDCs were significantly reduced, explaining those results observed in FACS analysis and the lower fluorescence intensity staining of these proteins observed in the confocal microscopy images. The diminished protein staining and mRNA expression observed in iDCs after MPA treatment was countered when LPS stimulate iDCs, although surface maturation marker expression remained lower than non-treated LPS-mDCs. Two different regulations produced by MPA treatment on mDCs were observed depending on the marker.

First, as happened with all the markers in MPA-treated iDCs, the diminished cell surface expression of CD86 observed after MPA treatment on mDCs could be related to the decreased mRNA levels obtained in this gene. Accordingly, the cytoplasmic staining observed on LPS-mDCs was reduced without accumulation in the Golgi after MPA treatment, suggesting no interference in the intracellular protein trafficking.

Second, in contrast with CD86, MPA treatment increased the gene expression of CD40, CD83 and ICAM-1 compared to LPS-stimulated DCs. This transcriptional upregulation was not translated in an increment on protein cell surface expression, suggesting that other regulation beyond transcription might take place. For better understanding the decrease on cell surface expression of CD40, CD83 and ICAM-1 after MPA treatment on mDCs, their intracellular localization was explored to elucidate an interference in protein trafficking to membrane produced by disturbed protein glycosylation. A decrease of CD83 and ICAM-1 in cell membrane expression accompanied with cytosolic accumulation was observed in MPA-treated mDCs compared with non-treated mDCs. These results suggest disrupted intracellular protein trafficking that may be produced by the interference on protein glycosylation. Similarly, Cao *et al* [23] observed that the interference on protein glycosylation produced by tunicamycin

diminished CD83 expression on the cell surface with an increment in the Golgi compartment. Moreover, He *et al* [24] showed that a deficiency on *N*-glycosylation also produced a lower expression of functional ICAM-1 in the cell membrane. Therefore, we hypothesized that in these markers, after LPS stimulation, DCs could be using GTP nucleotides to promote transcriptional upregulation, but probably there is not enough GTP to fully complete mannose and fucose incorporation for protein glycosylation. Some authors revealed that interferences in proper protein glycosylation produces an aberrant or misfolded glycoprotein that promotes the ubiquitination of these proteins and their proteasomal degradation [25–28]. This could explain why not accumulation of CD40 was observed in the Golgi or cytosol after MPA treatment on mDCs.

Nowadays, the molecular mechanism implicated in the reduction of maturation markers in DCs surface after MPA treatment has not been fully elucidated. Some authors supported that the reduction of intracellular GTP levels due to the inhibition of IMPDH induces these downstream effects [14]. Our data reinforced the studies which suggested that the interference on DCs maturation is produced by the low levels of GTP nucleotides that restricts the mRNA production and the incorporation of mannose and fucose sugars for protein glycosylation [12,29–31]. In contrast, other authors suggested that MPA could interfere in the MAPK/p-38 signalling pathway, an important pathway implicated in the maturation of iDCs after TNF-α or LPS stimuli [22,32]. We suggest that although there might be some interference in this signalling pathway, in our model seems not to be as relevant as the reduction in the levels of GTP observed in other studies [13,16,20,22]. Moreover, the enhanced T-cell proliferation described by Dubsky *et al* [14] when exogenous guanosine was pre-added in DCs and the increased expression of surface markers observed in our study compared to non-treated with guanosine, reinforce the hypothesis that MPA interferes on DCs maturation by the reduction of GTP levels

derived from IMPDH inhibition. In fact, our results showed that guanosine reverted better the mRNA downregulation than the interference in protein trafficking produced by MPA treatment.

A limitation of this study is that all surface markers were not chronologically individualized, and they were analysed at the same time points (6 and 24 hours). Additionally, the lower adherence after MPA at 50 µM did not allow determining MPA effect at high concentrations by confocal microscopy.

To summarize, these results showed for the first time that phenotypical changes produced by MPA interference observed in previous studies [11–13] could be explained by a transcriptional downregulation or by an intracellular protein trafficking blockage, depending on the maturation marker. Both mechanisms could be explained by the lower GTP levels observed by Dubsky *et al* [14]. Finally, the potential tolerogenic effects induced by DCs after MPA treatment makes these treated DCs a suitable tool to be explored in cellular therapy on transplantation or autoimmune diseases, where most patients receive MPA therapy.

5. FIGURE CAPTIONS

Figure 1. **Experimental design.** Monocytes were cultured in X-VIVO medium supplemented with 10 ng/mL of IL-4, 15 ng/mL of GM-CSF and 2 % of human serum for 7 days. Non-stimulated immature DCs (iDCs) were used as negative control whereas DCs stimulated with LPS 1 mg/mL at day 6 (LPS-mDCs) become positive controls for treatment groups. Different concentrations of mycophenolic acid (MPA) were added on day 2 of culture with/without the addition of exogenous non-phosphorylated guanosine (Gua) at 100 µM.

Figure 2. **Dendritic cell functional capacity after mixed lymphocyte reaction**. A) T T-cell proliferation after 5 days of mixed lymphocyte reaction (MLR) between pre-treated dendritic cells with mycophenolic acid (MPA) at different concentrations and allogeneic peripheral blood mononuclear cells. The percentage of CD3+ positive cells (T-cells) that proliferates after MLR is represented. Non-stimulated immature DCs (iDCs) were used as negative control whereas DCs stimulated with LPS become positive controls for treatment groups (LPS). B-E) Cytokines released in the co-culture supernatant after 5 days of MLR. The concentration (pg/mL) of cytokines (IL-2, IL-6, IL-10, IL-17A) after MLR is represented. Each column illustrates the median [interquartile range]. *# $p < 0.05$ vs iDC; * $p < 0.05$ vs LPS.*

Figure 3: **Gene expression of maturation markers (CD40, CD83, CD86 and ICAM-1) in stimulated and non-stimulated dendritic cells.** A) Fold induction in the mRNA expression of maturation markers on dendritic cells treated with 10 and 50 µM of mycophenolic acid (MPA) and with/without exogenous guanosine (Gua) with respect to non-treated immature dendritic cells group (iDCs) after 7 days of culture. Data represent the mean ± standard error.

B) Fold induction after 6 hours of LPS stimulation (LPS) in the mRNA expression of maturation markers with respect to iDC group after 7 days of culture.

Figure 4: **Subcellular localization of CD83 in stimulated and non-stimulated dendritic cells.** Confocal microscopy of immature (iDCs) and stimulated (LPS) dendritic cells treated with 10 µM of mycophenolic acid (MPA) and with/without exogenous guanosine (Gua) after 7 days of culture. In green is illustrated the expression of CD83, whereas in red is showed GM-130 marker.

Figure 5: **Subcellular localization of ICAM-1 in stimulated and non-stimulated dendritic cells.** Confocal microscopy of immature (iDCs) and stimulated (LPS) dendritic cells treated with 10 µM of mycophenolic acid (MPA) and with/without exogenous guanosine (Gua) after 7 days of culture. In green is illustrated the expression of ICAM-1, whereas in red is showed GM-130 marker.

6. TABLES

Table 1:

	CD40	CD86	CD83	ICAM-1
iDC	1.00 (880.1 ± 97.3)	1.00 (1437.4 ± 182.7)	1.00 (152.7 ±17.8)	1.00 (1833.4 ± 117.0)
LPS	2.11 ± 0.08# (1742.5 ± 215.2)	1.21 ± 0.07# (1755.5 ± 256.8)	2.59 ± 0.18# (413.8 ± 47.2)	3.98 ± 0.09# (6917.8 ± 377.1)
MPA 1 + LPS	2.29 ± 0.03 (2031.0 ± 201.4)	1.21 ± 0.05 (1953.9 ± 451.9)	2.50 ± 0.09 (473.9 ± 69.1)	4.43 ± 0.04 (7150.5 ± 640.9)
MPA 5 + LPS	2.12 ± 0.21 (1897.5 ± 292.5)	0.93 ± 0.07* (1491.9 ± 385.0)	2.16 ± 0.19 (398.4 ± 41.4)	4.03 ± 0.39 (6283.1 ± 545.2)
MPA 10 + LPS	1.86 ± 0.09 (1565.1 ± 173.1)	0.70 ± 0.04* (999.3 ± 139.1)	1.93 ± 0.17* (304.1 ± 32.6)	3.67 ± 0.16 (6356.6 ± 379.5)
MPA 50 + LPS	1.74 ± 0.07* (1455.2 ± 160.5)	0.64 ± 0.04* (890.7 ± 107.8)	1.99 ± 0.19* (314.9 ± 33.8)	3.24 ± 0.14* (5587.7 ± 323.0)
MPA 100 + LPS	1.56 ± 0.13* (1442.8 ± 197.0)	0.62 ± 0.10* (952.2 ± 225.8)	1.86 ± 0.25* (337.0 ± 27.2)	3.23 ± 0.22* (5031.7 ± 290.1)
MPA 10 + LPS + Gua	1.42 ± 0.12† (1125.5 ± 228.8)	1.00 ± 0.08† (1339.3 ± 212.2)	2.47 ± 0.42 (328.0 ± 63.7)	3.52 ± 0.13 (6475.8 ± 373.2)
MPA 50 + LPS + Gua	1.49 ± 0.10‡ (1213.0 ± 235.6)	1.06 ± 0.06‡ (1418.3 ± 206.2)	2.58 ± 0.34 (335.2 ± 48.6)	3.35 ± 0.07 (6176.5 ± 325.5)

Table 1. Surface expression of maturation markers after LPS and different concentrations of mycophenolic acid (µM) (MPA) with or without the addition of exogenous guanosine (Gua). Data represent the fold induction mean with respect to non-stimulated iDCs ± standard error. In brackets is represented the mean fluorescence intensity (MFI) ± standard error. *# $p < 0.05$ vs iDC; * $p < 0.05$ vs LPS; † $p < 0.05$ vs MPA 10; ‡ $p < 0.05$ vs MPA 50.*

Table 2:

	CD40	CD86	CD83	ICAM-1
iDC	1.00 (880.1 ± 97.3)	1.00 (1437.4 ± 182.7)	1.00 (152.7 ±17.8)	1.00 (1833.4 ± 117.0)
Gua	0.91 ± 0.10 (790.7 ± 152.7)	1.12 ± 0.11 (1367.8 ± 246.0)	0.91 ± 0.08 (135.2 ± 11.6)	1.04 ± 0.12 (1759.5 ± 189.8)
MPA 10	0.63 ± 0.04***** (595.2 ± 63.3)	0.59 ± 0.03***** (774.8 ± 88.0)	0.63 ± 0.05***** (79.7 ± 9.4)	0.50 ± 0.06***** (952.8 ± 106.5)
MPA 50	0.57 ± 0.04***** (545.6 ± 58.1)	0.51 ± 0.04***** (665.0 ± 76.6)	0.61 ± 0.05***** (76.2 ± 5.3)	0.36 ± 0.03***** (678.8 ± 65.8)
MPA 10 + Gua	0.84 ± 0.02† (814.6 ± 102.5)	0.94 ± 0.03† (1248.0 ± 147.0)	1.00 ± 0.04† (127.25 ± 13.5)	0.96 ± 0.04† (1804.0 ± 53.4)
MPA 50 + Gua	0.71 ± 0.03‡ (694.2 ± 94.8)	0.92 ± 0.06‡ (1238.8 ± 183.4)	0.87 ± 0.05‡ (111.0 ± 12.6)	0.77 ± 0.03‡ (1457.2 ± 52.4)

Table 2. Surface expression of maturation markers after different concentrations of mycophenolic acid (μM) (MPA) with or without the addition of exogenous guanosine (Gua) in non-stimulated immature DCs (iDC). Data represent the fold induction mean with respect to non-stimulated iDCs ± standard error. In brackets is represented the mean fluorescence intensity (MFI) ± standard error. ** $p < 0.05$ vs iDC; † $p < 0.05$ vs MPA 10; ‡ $p < 0.05$ vs MPA 50.*

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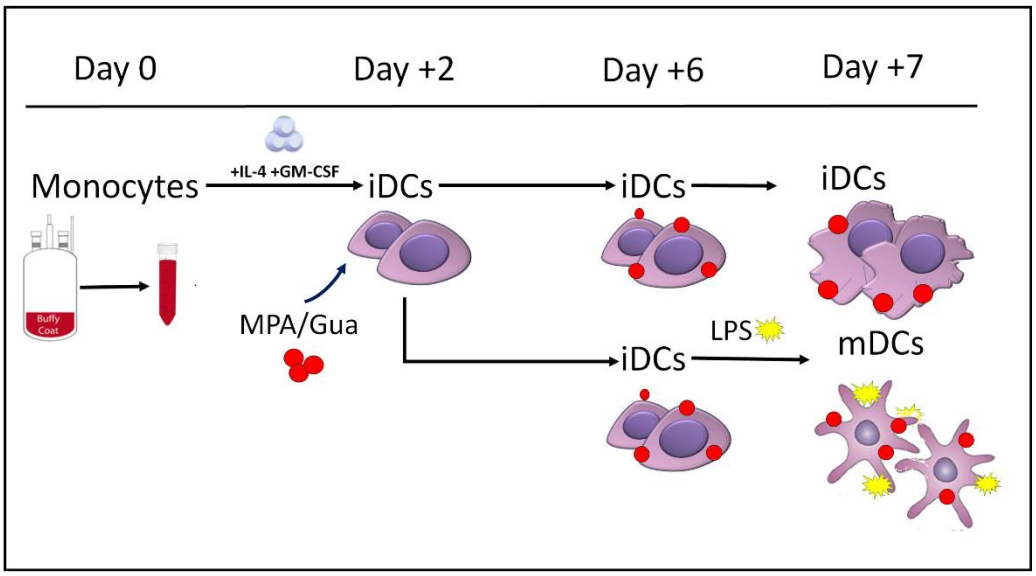
662 **Figure 1**



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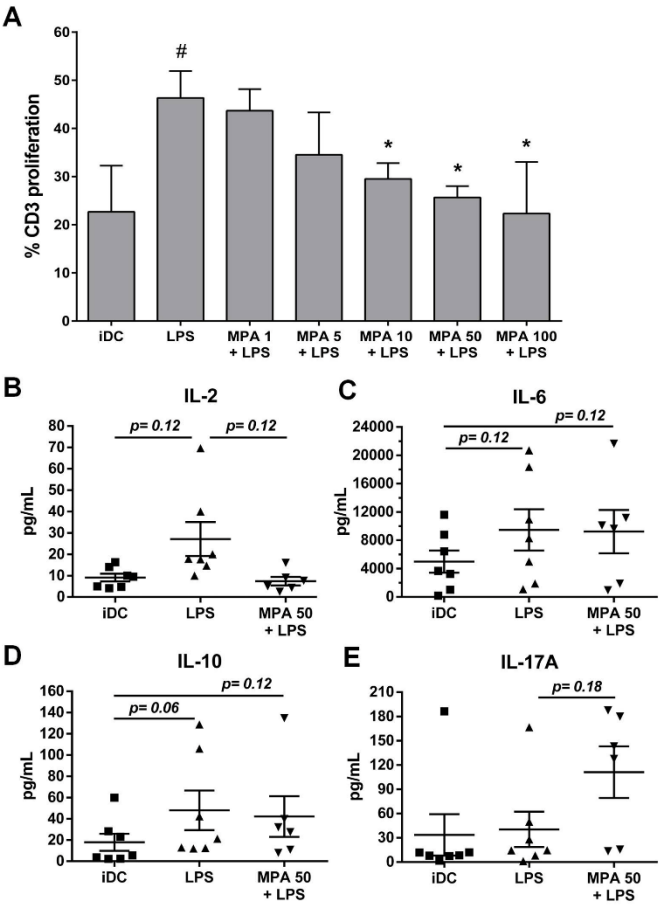

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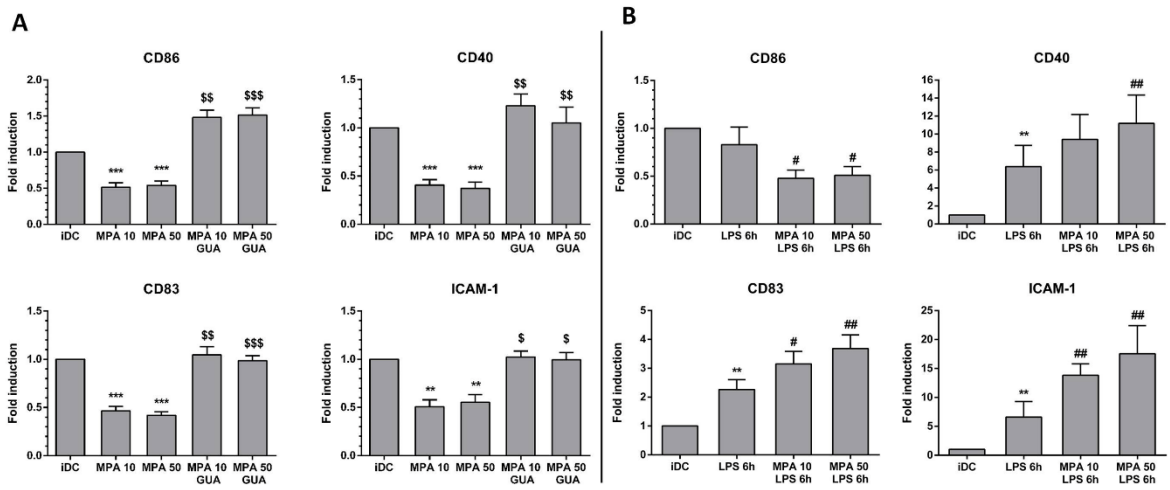

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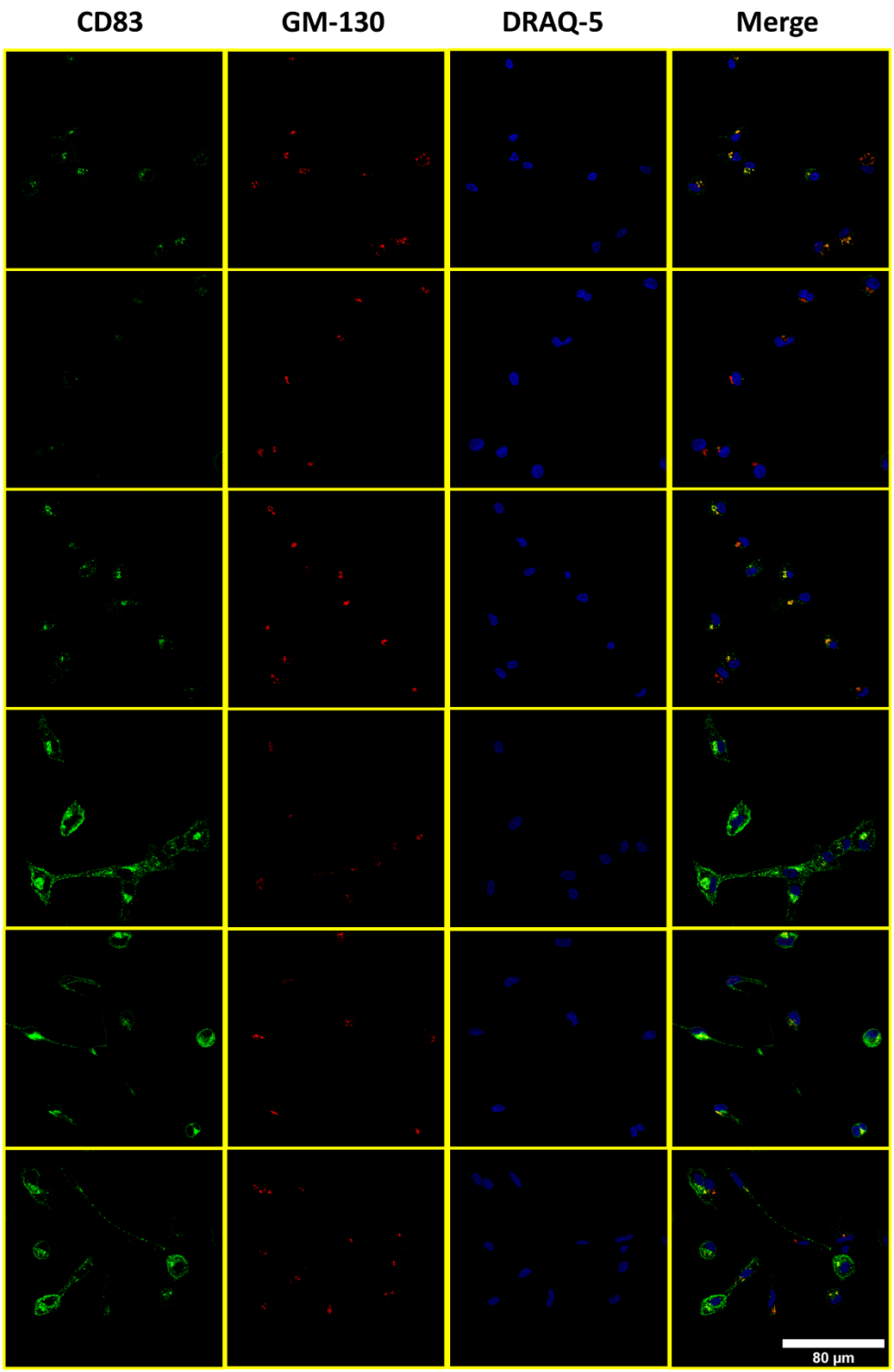

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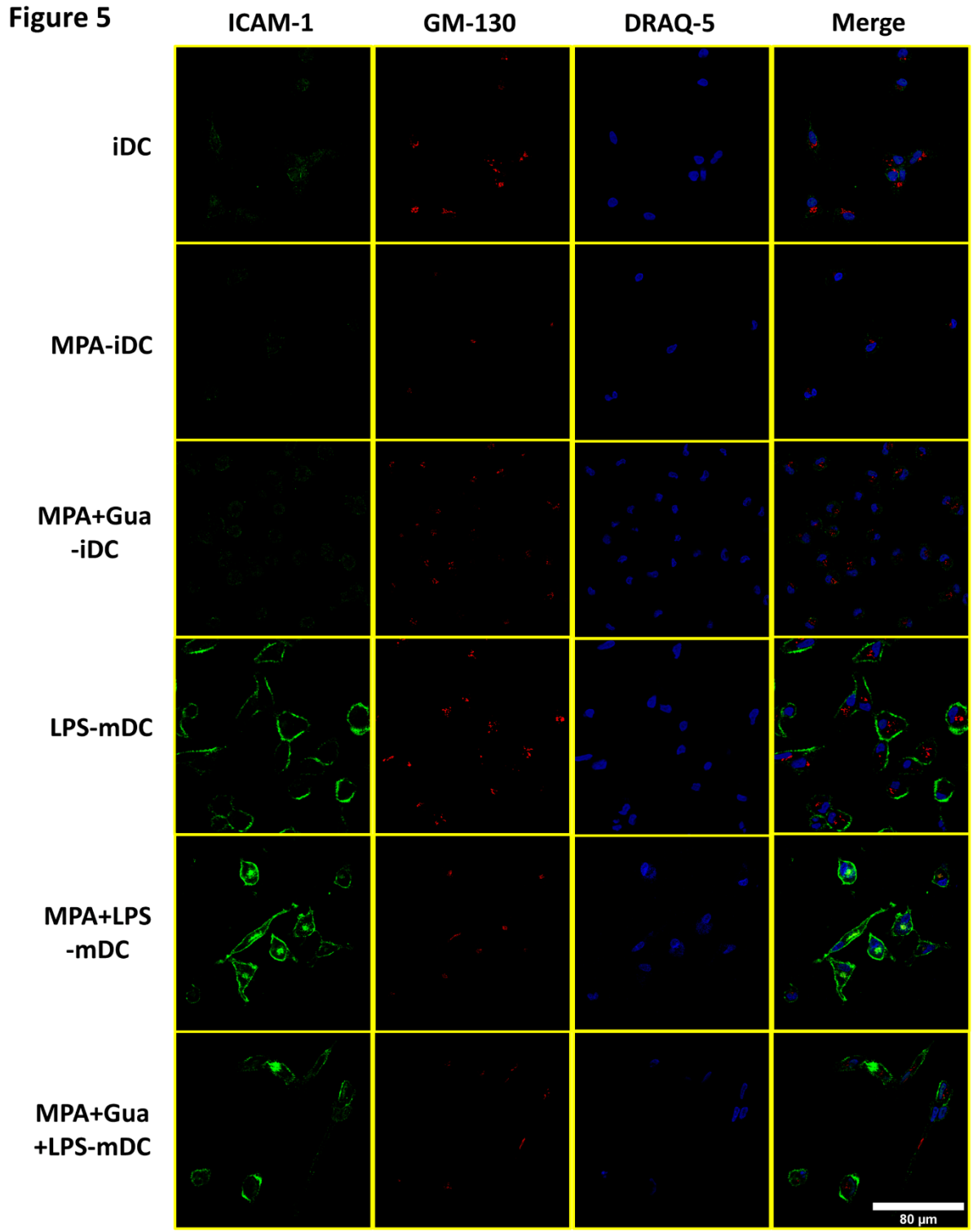

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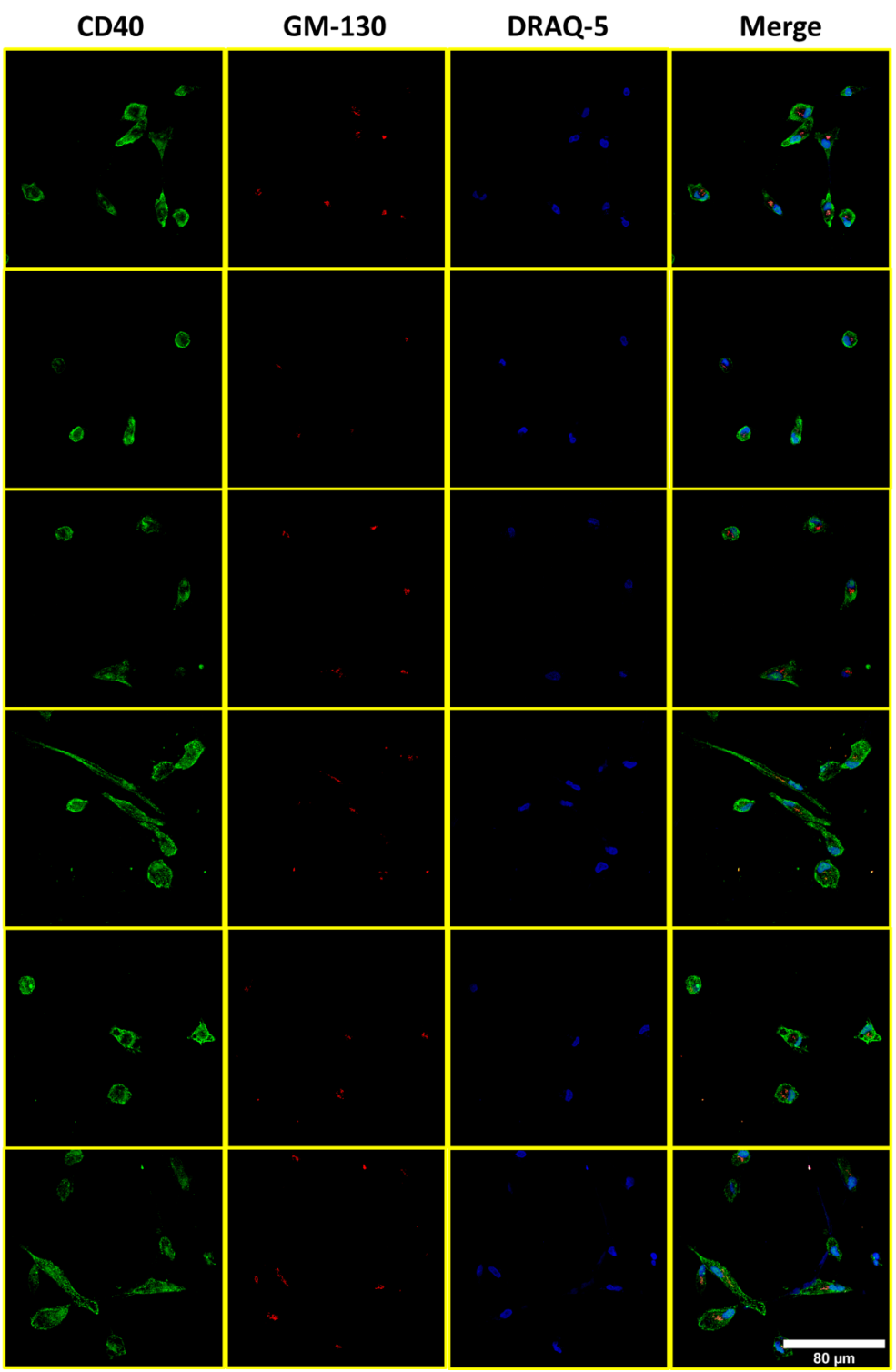

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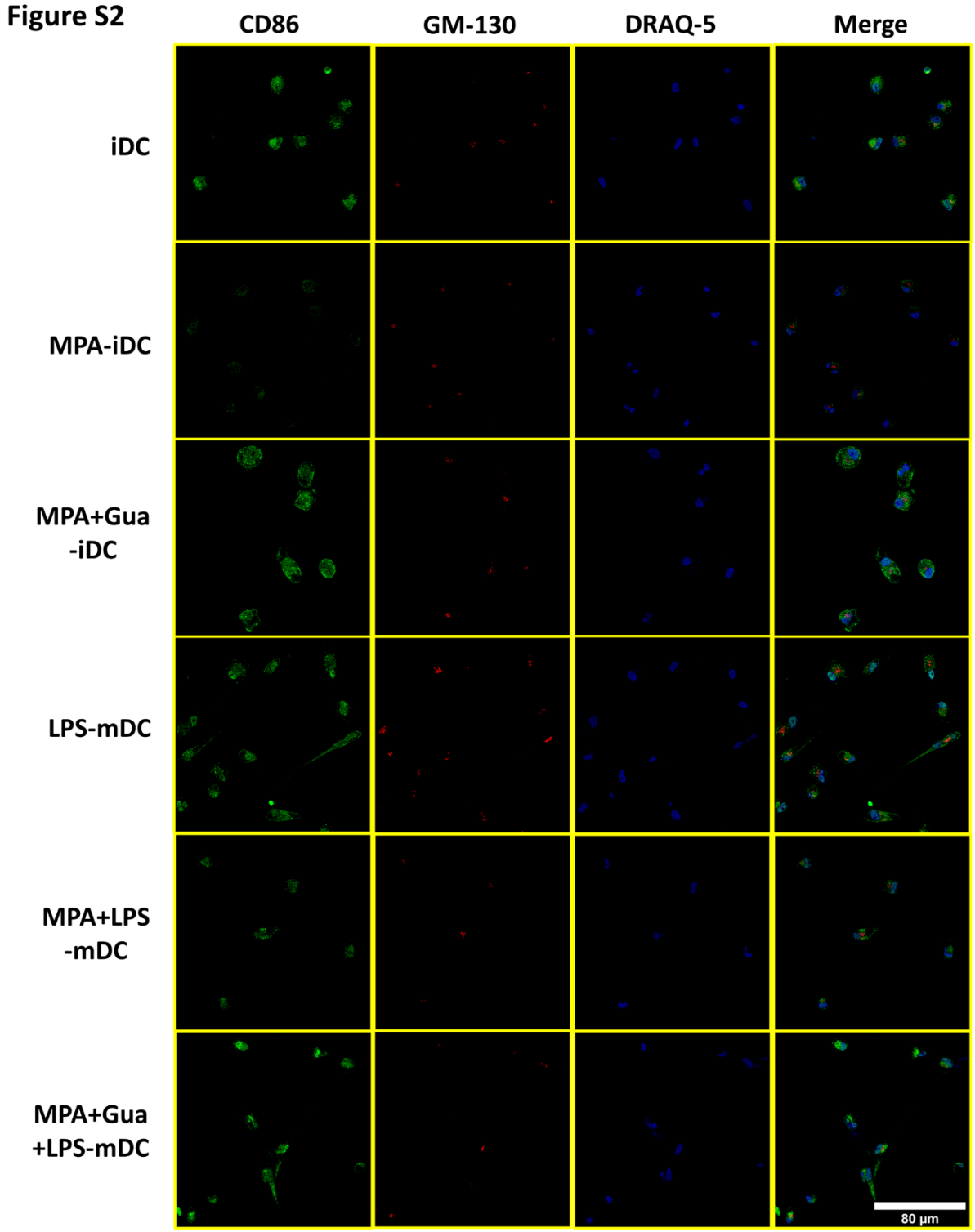

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