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Allergoid-mannan conjugates imprint tolerogenic features in human macrophages

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CAPSULE SUMMARY: Allergoid-mannan conjugates promote the generation of human IL-10-producing macrophages with regulatory phenotypes, which might well contribute to the tolerogenic responses induced by this novel allergen-specific immunotherapy vaccine.

KEYWORDS: allergen-specific immunotherapy, allergoid-mannan conjugates, immune regulation, innate immunity, human macrophages.

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MATERIALS AND METHODS

Material, media and reagents

For cell cultures, we used RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL normocin (InvivoGen, San Diego, Calif), 50 µg/mL penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mmol/L sodium pyruvate (Life Technologies, Grand Island, NY). For experiments with THP1 cells, also glucose up to 4.5 g/L was added. LPS (0127:B8) (Sigma-Aldrich, St Louis, Mo) was used. Allergoid-mannan conjugates (Inmunotek) are performed by conjugation of grass pollen *Phleum pratense* allergens with mannan from *Saccharomyces cerevisiae* through the lysine residues from its mannoprotein in a single step. Briefly, glutaraldehyde (25 mmol/L) is added to a solution containing the mixture of allergen and mannan (1:0.5 wt/wt) in PBS for 6 hours at 4°C in continuous stirring. The reaction is stopped with glycine (1.25 mol/L), followed by tangential flow filtration with distilled water (membrane cutoff, 100 kDa). Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan (PM) were recovered in the concentrated retained fraction (>100 kDa).

THP1 macrophages generation and polarization

THP1 cells were grown in culture medium. Prior to the experiments, cells were differentiated to macrophages by incubation with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in culture medium for 24 hours. They were then washed with medium and grown in culture medium for 24 hours. Then, THP1 macrophages were polarized by incubation overnight with 50 ng/mL IFN γ (Preprotech, Rocky Hill, NJ) plus 100 ng/mL LPS (INF/LPS), 40 ng/mL IL4 (Preprotech), 50 ng/mL IL10 (Preprotech) or 50 µg/ml allergoid-mannan conjugates (PM). The control population was incubated in culture medium (Ctrl-) without polarizing cytokines. Subsequently, cells were collected, centrifuged and used for phenotype characterization by quantitative real-time RT-PCR analysis. In all the cases, viable cells were counted using Trypan blue with an optical microscope and analyzed by propidium iodide by flow cytometry.

Primary macrophages generation and polarization

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors by Ficoll density gradient centrifugation (800g, 20 minutes). Monocytes were obtained from total PBMC using anti-CD14 microbeads (Miltényi Biotec, Bergisch Gladbach, Germany). To generate human monocyte-derived macrophages, monocytes were cultured at 0.5×10^6 cells/mL in 48 well microplates for 6 days with cRPMI medium containing GM-CSF (100 ng/mL, PeproTeck, GM-MØ)

or M-CSF (10 ng/mL, PeproTeck, M-MØ). Addition of cytokine took place every 2 days. To generate Mannan-MØ, 50 µg/mL of PM was added at days 0 and 4 of the differentiation. In all the cases, viable cells were counted using Trypan blue with an optical microscope and analyzed by propidium iodide by flow cytometry. Then, macrophages were stimulated with medium (negative control) or LPS (10 ng/ml) for 24 hours, where indicated. Subsequently, cells were collected and centrifuged. Cell-free supernatants were used for cytokine quantification by ELISA and pellets were used for phenotype characterization by flow cytometry or quantitative real-time RT-PCR analysis.

To analyze the induction of allergen-specific Treg cells, unstimulated GM-MØ or Mannan-GM-MØ from allergic patients were cocultured with autologous peripheral blood lymphocytes (PBLs) at a DC/PBL ratio of 1:5 in the presence of native grass pollen allergens (50 µg/mL) to analyze FOXP3⁺ Treg cells. The 2 grass pollen allergic patients included in this study were previously clinically characterized.¹

Cytokine quantification

Concentrations of IL-10, TNF-α, and IL-6 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, CA). In all cases, manufacturer's instructions were followed with minor modifications.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

RNA was isolated from harvested cells by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For mRNA analysis, cDNA was generated with a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR was performed on cDNA by using FastStart Universal SYBR Green Master (Rox; Roche). The sequences of the used primer pairs are listed in **Table 1**. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems, Foster City, Calif). Data were normalized to EF1α and displayed as $2^{-\Delta CT}$ values multiplied by 10^4 , with Δ cycle threshold (ΔCT) defined as the difference between the CT value for the gene of interest and EF1α. To obtain the normalized profiles, we normalized the mean arbitrary units from each gene to the macrophage phenotype with the highest expression of that gene.

Flow cytometry

Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 minutes at room temperature in the darkness with human anti-CD14-PerCP (clone M5E2, dilution 1:50). For analysis of FOXP3 expression in human T cells primed with MØs, cells were first subjected to

surface staining with anti-human CD4-PerCP (clone OKT4, dilution 1:100), CD127-PE (clone A019D5, dilution 1:200), and CD25-APC (clone BC96, dilution 1:200) antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488 (clone 259D, dilution 1:30), according to manufacturer's recommendations. The corresponding isotype control was included in each staining. Flow cytometry analysis was performed in a FACSCalibur cytometer (Beckton Dickinson) in the Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

Statistical analysis

In all experiments, data represent the mean $\pm$ SEM of the indicated parameters. Statistical analysis was calculated using one-way ANOVA, whose P value summary was statistically significantly ($P < 0.05$) in all the cases, or Wilcoxon test/ Mann-Whitney test when comparing just two different conditions using Prism software 6.0 (GraphPad Software). Significance is indicated in each figure.

BIBLIOGRAPHY

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Table 1. Primer sequences

Name	Forward	Reverse
CCR7	CATGGACCTGGGGAAACCAA	AAAGTTCCGCACGTCCTTCT
CD80	GATAACCTGCTCCCATCCTGG	CTTGGGGCAAAGCAGTAGGT
CD206	ACACAAACTGGGGGAAAGGTT	TCAAGGAAGGGTCGGATCG
IL10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT
CD163	AAATTACCTGCTCAGCCCACA	ACGTGTCACCATGCTTCACT
CCL2	TCTGTGCCTGCTGCTCATAG	GGGCATTGATTGCATCTGGC
IDO	AGAAGTGGGCTTTGCTCTGC	TGGCAAGACCTTACGGACATCTC
PDL1	AAGATGAGGATATTTGCTGTCTTTATATTC	GTCCTTGGGAACCGTGACAGT
SOCS1	CCCTGGTTGTTGTAGCAGCTT	CAACCCCTGGTTTGTGCAA
SOCS3	CCTCAGCATCTCTGTCGGAAGA	GCATCGTACTGGTCCAGGAACT

<i>IL6</i>	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
<i>EF1α</i>	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT

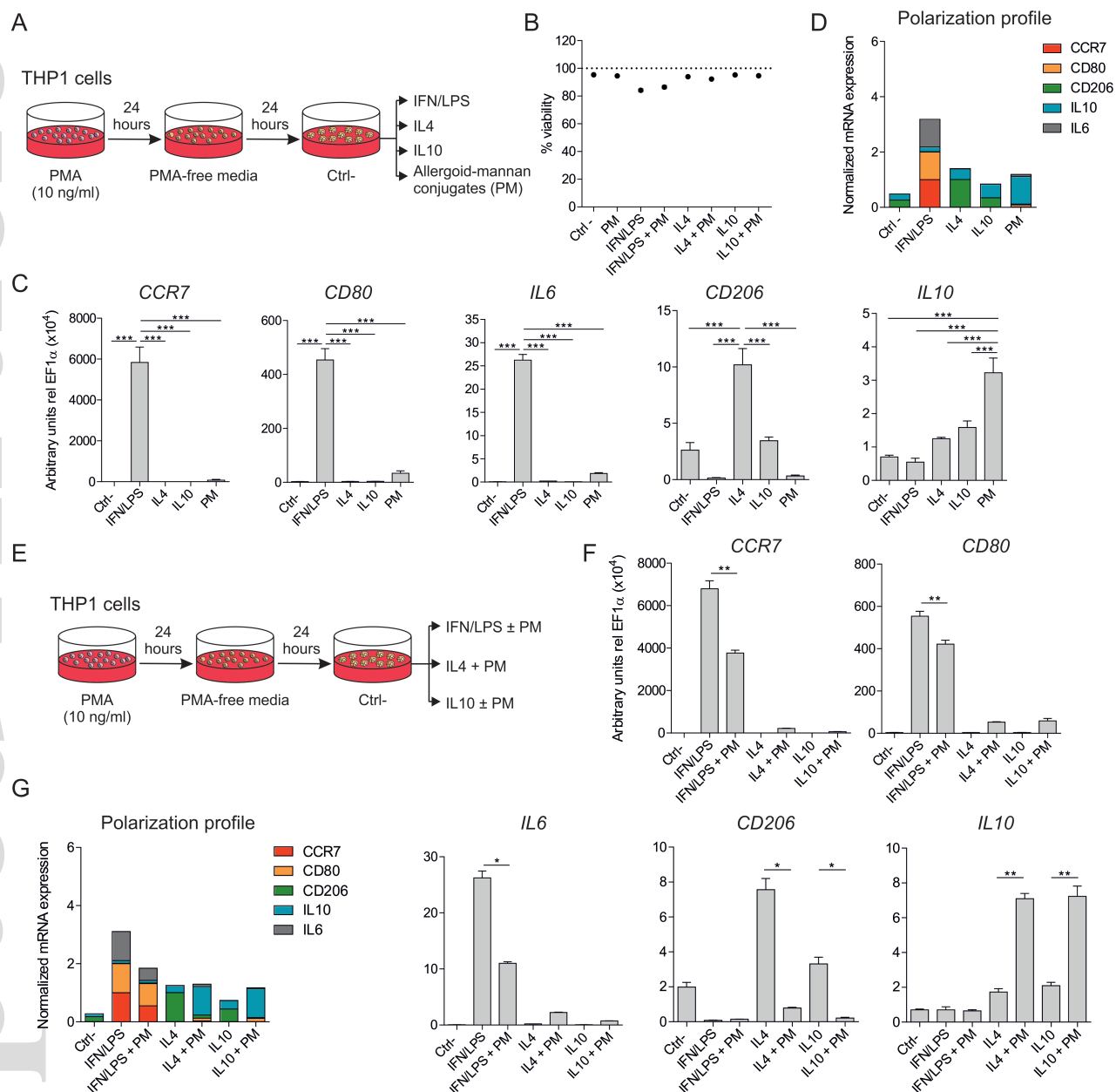


Figure 1. Benito-Villalvilla et al

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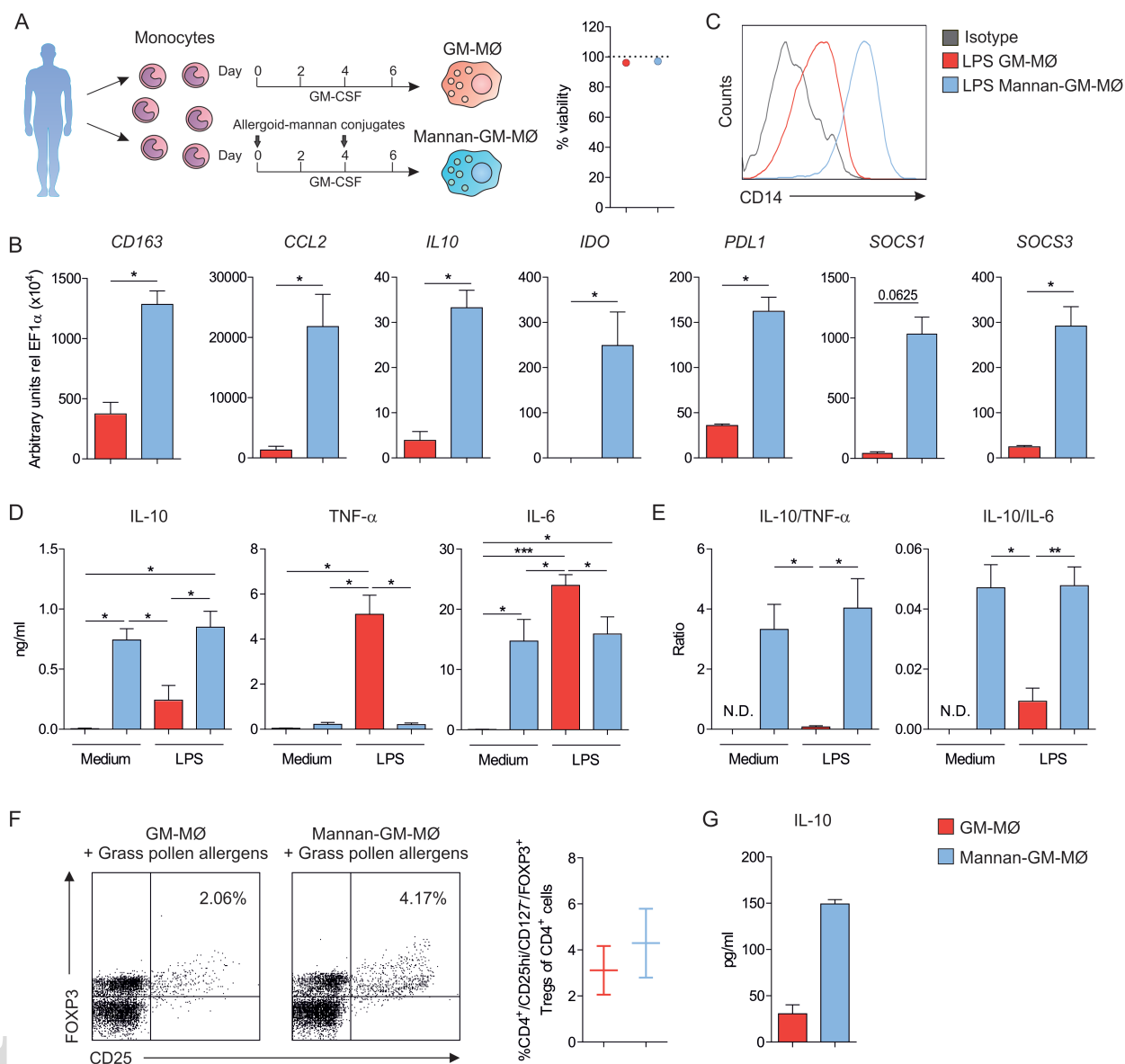
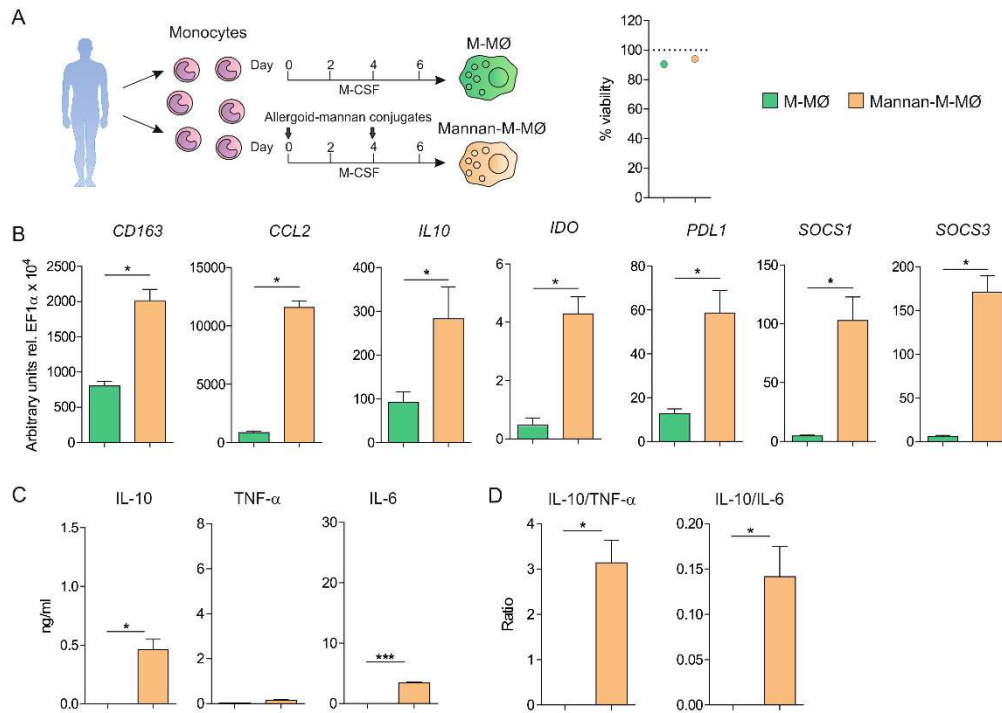


Figure 2. Benito-Villalvilla et al

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Supplementary Figure 1. Benito-Villalvilla et al

Figure S1. A, Mannan-M-MØs generation protocol from human monocytes and viability of the generated cells. **B**, mRNA expression levels of genes in freshly isolated M-MØ or Mannan-M-MØ. **C and D**, Cytokine (C) or cytokine ratios (D) produced after 24 hours in the presence of medium of conventional M-MØ or Mannan-M-MØ. Results are mean ± s.e.m. of 2 (A), 4-6 (B, C and D) independent experiments. Wilcoxon test. * $P < 0.05$, and *** $P < 0.01$.