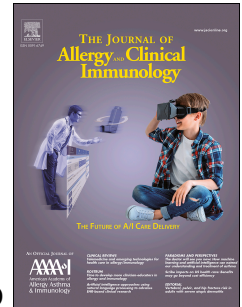


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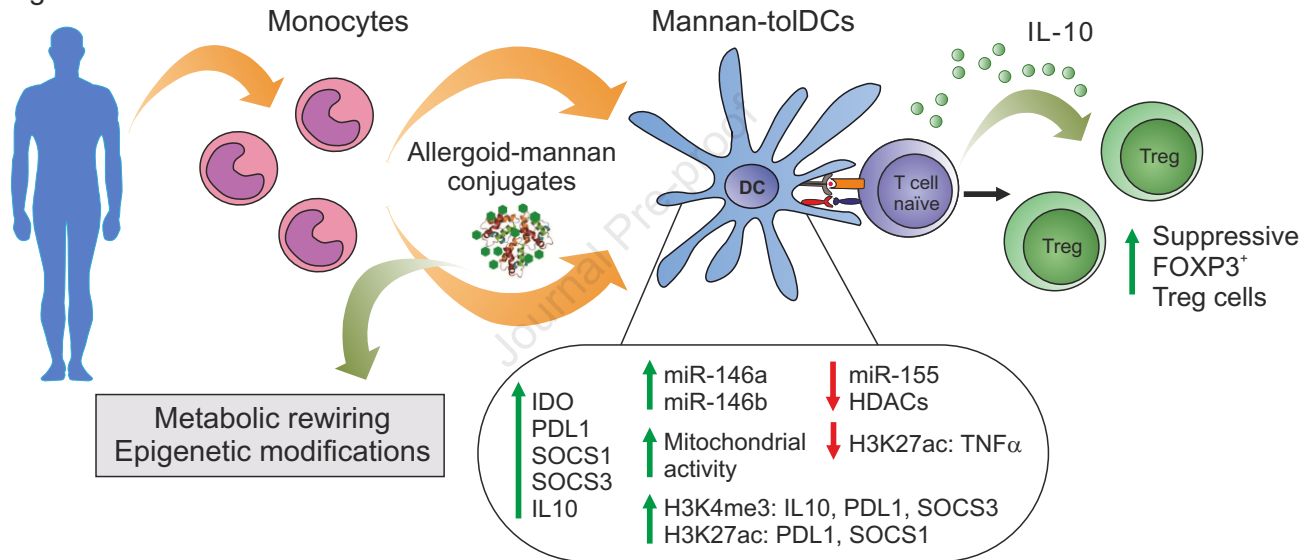
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Non-atopic or
Allergic donors



DCs: Dendritic cells; SOCS: Suppressor of cytokine signaling; HDACs: Histone deacetylases;
Mannan-tolDCs: Tolerogenic DCs generated by allergoid-mannan conjugates

Allergoid-mannan conjugates reprogram monocytes into tolerogenic DCs via epigenetic and metabolic rewiring

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ABSTRACT

Background: Allergoid-mannan conjugates are novel vaccines for allergen-specific immunotherapy (AIT) being currently assayed in phase II clinical trials. Allergoid-mannan conjugates target dendritic cells (DCs) and generate functional forkhead box P3 (FOXP3)⁺ regulatory T (Treg) cells, but their capacity to reprogram monocyte differentiation remains unknown.

Objective: We study whether allergoid-mannan conjugates could reprogram monocyte differentiation into tolerogenic DCs and the underlying molecular mechanisms.

Methods: Monocytes from non-atopic and allergic subjects were differentiated into DCs under conventional protocols in the absence or presence of allergoid-mannan conjugates. ELISAs, qPCR, cocultures, flow cytometry and suppression assays were performed. Metabolic and epigenetic techniques were also employed.

Results: Monocyte differentiation from non-atopic and allergic subjects into DCs in the presence of allergoid-mannan conjugates yields stable tolerogenic DCs. LPS-stimulated Mannan-tolDCs show a significantly lower cytokine production, lower TNF α /IL-10 ratio, higher expression of the tolerogenic molecules *PDL1*, *IDO*, *SOCS1*, *SOCS3* and *IL10*, and induce higher numbers of functional FOXP3⁺ Treg cells than conventional DCs counterparts. Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation. They also display epigenetic reprogramming involving specific histone marks within tolerogenic loci and lower expression levels of histone deacetylases genes (HDACs). Mannan-tolDCs significantly increase the expression of the anti-inflammatory miRNA-146a/b and decrease pro-inflammatory miRNA-155.

Conclusion: Allergoid-mannan conjugates reprogram monocyte differentiation into stable tolerogenic DCs via epigenetic and metabolic reprogramming. Our findings shed light into novel mechanisms by which allergoid-mannan conjugates might contribute to allergen tolerance induction during AIT.

CAPSULE SUMMARY

We show that next generation AIT vaccines based on allergoids conjugated to mannan drive monocyte differentiation from non-atopic and allergic subjects into stable tolerogenic DCs that prime functional Treg cells via epigenetic and metabolic reprogramming.

CLINICAL IMPLICATIONS

We uncover innate immune mechanisms contributing to allergen tolerance induction that might help to identify novel AIT biomarkers and to design alternative DC-based strategies for allergy and other inflammatory diseases.

KEY WORDS

Allergen-specific immunotherapy, allergoid-mannan conjugates, monocytes, tolerogenic dendritic cells, regulatory T cells, metabolism, epigenetics

ABBREVIATIONS

AIT: Allergen-specific immunotherapy

APC: Allophycocyanin

ChIP: Chromatin immunoprecipitation

DCs: Dendritic cells

FOXP3: Forkhead box 3

HDAC: Histone deacetylase

H3K27ac: Histone H3 acetylation on lysine 27

H3K27me3: Histone H3 trimethylation on lysine 27

H3K4me3: Histone H3 trimethylation on lysine 4

hmoDCs: Human monocyte-derived DCs

- 89 IDO: Indoleamine 2,3-dioxygenase
- 90 LDHA: Lactate dehydrogenase A
- 91 LNA: Locked nucleic acid
- 92 Mannan-tolDCs: Tolerogenic DCs generated by allergoid-mannan conjugates
- 93 miRNA: MicroRNA
- 94 MTA: 5'-methylthioadenosine
- 95 NCoR1: Nuclear receptor co-repressor 1
- 96 OXPHOS: Oxidative phosphorylation
- 97 PBMC: Peripheral blood mononuclear cells
- 98 PD-L1: Programmed death ligand 1
- 99 PE: Phycoerythrin
- 100 PerCP: Peridinin chlorophyll protein complex
- 101 ROS: Reactive oxygen species
- 102 SOCS: Suppressor of cytokine signaling
- 103 TCA: Tricarboxylic acid
- 104 Treg: Regulatory T
- 105

INTRODUCTION

Allergen-specific immunotherapy (AIT) is currently the only disease-modifying treatment with potential curative capacity for allergy.^{1, 2} The better understanding of the immune mechanisms involved in allergen tolerance induction during AIT is of paramount importance not only to design safer and more effective AIT vaccines but also to identify potential novel biomarkers for the management of allergic patients undergoing the treatment.^{3, 4} After a very rapid desensitization of effector cells, the immunological changes associated with successful AIT include the generation and maintenance of functional allergen-specific regulatory T (Treg) cells, which is promoted by the restoration of tolerogenic dendritic cells (DCs).^{5, 6} This is also linked to the induction of B regulatory cells secreting allergen-specific blocking IgG antibodies that neutralize allergens preventing degranulation of mast cells, basophils and eosinophils and also inhibit IgE-facilitated allergen presentation by antigen presenting cells, thus impairing the activation of allergen-specific memory Th2 responses.^{3, 7} Although the data about the role of monocytes and other innate cells in the context of AIT is still limited, it has been recently shown that after the first year of AIT increased numbers of intermediate monocytes in parallel with reduced numbers of nonclassical monocytes are observed.⁸

Although AIT is a successful treatment for many allergic patients, it still remains underused due to several drawbacks in terms of safety, efficacy, long duration and patients' adherence. To overcome these issues, new vaccines using novel adjuvants and/or the use of alternative routes of administration are being developed.⁹⁻¹¹ Glutaraldehyde-polymerized allergoids conjugated to non-oxidized mannan (allergoid-mannan conjugates) by an alternative single-step production approach represent next-generation AIT vaccines targeting DCs.^{12, 13} Allergoid-mannan conjugates are captured by DCs by mechanisms depending on mannose receptor and DC-SIGN-mediated internalization. Allergoid-mannan conjugates induce blocking antibodies and promote

the generation of human and mice functional forkhead box 3 (FOXP3)⁺ Treg cells through programmed death ligand 1 (PD-L1) both *in vitro* and *in vivo*.^{14, 15} Its molecular mechanism of action on human DCs involve mTOR, glycolysis and reactive oxygen species (ROS) production, and the presence of alum interferes those signaling pathways impairing the imprinted tolerogenic properties.¹⁶ Subcutaneous administration of *D. farinae* allergoid-mannan conjugates in dogs with atopic dermatitis demonstrated a rapid clinical improvement within the first 3 months of treatment.¹⁷ In humans, four phase II clinical trials, for grass pollen, birch pollen and mites allergy (EudraCT Numbers: 2014-005471-88, 2015-000820-27, 2018-002522-23, 2020-004126-32) have been performed or are currently ongoing.

Despite these mechanistic and clinical advances, whether allergoid-mannan conjugates are able to reprogram human monocyte differentiation into tolerogenic DCs and the underlying molecular mechanisms remain unknown. Monocytes are one of the first immune cells to be recruited to vaccine administration sites where they might differentiate into immunogenic or tolerogenic DCs depending on the encountering signal.¹⁸⁻²² Different studies have shown that mechanisms involving epigenetic and metabolic reprogramming drive the differentiation of human monocyte-derived DCs (hmoDCs) into immunogenic or tolerogenic DCs.^{20, 23, 24}

We sought to investigate whether allergoid-mannan conjugates could influence the differentiation of human monocytes into tolerogenic DCs. The immunogenicity vs tolerogenicity in human DCs is finely regulated by metabolic and epigenetic reprogramming. Therefore, we also wanted to study whether these mechanisms might be underlying the phenotypic and functional features imprinted by allergoid-mannan conjugates during monocyte differentiation. Herein, we uncover novel mechanisms by which allergoid-mannan conjugates could restore tolerance to allergens during AIT.

METHODS

Material, media and reagents

Glutaraldehyde-polymerized grass pollen (*Phleum pratense*) allergoids conjugated to nonoxidized mannan (Allergoid-mannan conjugates) and mannan-free glutaraldehyde-polymerized grass pollen allergoids (Allergoids) were provided by Immunotek SL, Spain. Peripheral blood mononuclear cells (PBMC) for monocyte purification were obtained by Ficoll density gradient centrifugation (800 g, 20 minutes) from buffy coats obtained from “Centro de Transfusiones de la Comunidad Autónoma de Madrid (CAM)” of healthy non-atopic donors (negative specific-IgE against grass pollen and other common aeroallergens and total serum IgE levels below 30 ng/ml as determined by ELISA). Grass pollen allergic patients, which demographic and clinical features are collected in table E2, had recurrent clinical symptoms during the grass pollen season and displayed specific-IgE against grass pollen allergens. The 6 patients included in this study were previously clinically characterized.¹⁴ The study was approved by the corresponding Regional Ethics Committee of CAM (EudraCT: 2014-005471-88). For the *in vitro* experiments performed in this work new fresh blood samples were withdrawn upon proper informed consent.

Statistical analysis

In all experiments, data represent the mean $\pm$ SEM of the indicated parameters. Statistical differences were determined with the paired or unpaired Student *t* test when data follow normal distribution and Wilcoxon matched-pairs test when they not using Prism software 6.0 (GraphPad Software). Significance is indicated in each figure.

All procedures used in this study are fully described in the Methods section in this article's Online Repository.

RESULTS

Monocyte-derived dendritic cells differentiated in the presence of allergoid-mannan conjugates display tolerogenic features

To analyze whether allergoids conjugated to mannan could reprogram the differentiation of human monocyte-derived DCs (hmoDCs), purified blood monocytes from non-atopic donors were differentiated into DCs under conventional protocols in the absence or presence of allergoid-mannan conjugates without affecting cell viability (**Fig 1, A and E1**). Neither conventional hmoDCs nor DCs generated in the presence of allergoid-mannan conjugates (Mannan-toIDCs) produced cytokines in the presence of medium (unstimulated condition referred as “Ctrl -” from here onwards) (**Fig 1, B**). After 18 hours of LPS stimulation, Mannan-toIDCs produced significantly lower levels of the pro-inflammatory cytokines TNF α and IL-6 than hmoDCs (**Fig 1, B**). The production of IL-6 was still significant as compared with control cultures, as also occurred, in a greater extent, for the anti-inflammatory IL-10 (Fig. 1B). Therefore, the TNF α /IL-10 and IL-6/IL-10 ratios were significantly lower in Mannan-toIDCs than hmoDCs, suggesting that Mannan-toIDCs display anti-inflammatory features (**Fig 1, C**). Freshly differentiated Mannan-toIDCs expressed significantly higher mRNA levels of the classical tolerogenic molecules *IDO*, *PDL1*, *SOCS1*, *SOCS3* and *IL10* than hmoDCs without differences for ICOSL (**Fig 1, D**). Remarkably, hmoDCs generated in the presence of allergoids without mannan conjugation (Allergoids) displayed a cytokine signature similar to that observed for conventional hmoDCs upon LPS stimulation. In contrast, LPS-stimulated hmoDCs generated in the presence of mannan alone induce a similar cytokine profile than Mannan-toIDCs (**Fig E2, A**). Allergoids-DCs did not increase the mRNA expression levels of *IDO*, *PDL1*, *SOCS1*, *SOCS3* or *IL10*. Mannan alone also induce the mRNA expression levels of the assayed tolerogenic markers (**Fig E2, B**), confirming that mannan is indispensable to imprint the anti-inflammatory and tolerogenic features observed in Mannan-toIDCs. Supporting these data, LPS-

stimulated Mannan-tolDCs but not Allergoids-DCs showed decreased expression of CD-86 and increased expression of PD-L1 compared to conventional hmoDCs (data not shown).

To assess the capacity of Mannan-tolDCs and hmoDCs to polarize CD4⁺ T cell responses, we performed allogeneic cocultures. Unstimulated Mannan-tolDCs generated T cells producing higher levels of IL-10 than T cells primed by unstimulated conventional DCs, without differences in IFN- γ and IL-5 production (**Fig 1, E**). Interestingly, LPS-stimulated Mannan-tolDCs generated T cells producing significantly higher levels of IL-10 and still lower levels of IFN- γ and IL-5 than T cells generated by LPS-stimulated hmoDCs (**Fig 1, E**). IL-17A production was not detected in any assayed condition (**Fig 1, E**). The IFN- γ /IL-10 and IL-5/IL-10 ratios were significantly lower when T cells were primed by LPS-stimulated Mannan-tolDCs than hmoDCs (**Fig 1, F**). Remarkably, Mannan-tolDCs also induced significantly higher numbers of CD4⁺CD25^{high}CD127⁺FOXP3⁺ Treg cells than conventional hmoDCs (**Fig 1, G**). Representative dot plots of Treg generation are displayed. For functional experiments, we sorted the generated Treg cells (CD4⁺CD25^{high}CD127⁺) and non-Treg cells (**Fig E3, A**). Purified Treg cells generated by Mannan-tolDCs but not CD4⁺CD25⁺CD127⁺ non-Treg cells inhibited the proliferation of autologous PBMCs in a dose-dependent manner (**Fig 1, H and E3, B**), demonstrating that the induced Treg cells display functional suppressive capacity. Collectively, all these data demonstrate that allergoids conjugated to mannan are able to reprogram monocyte differentiation and generate tolerogenic DCs able to prime functional Treg cells.

Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation

Immunogenicity vs tolerogenicity in human DCs is finely regulated by metabolic reprogramming.^{25, 26} Therefore, we studied changes in the metabolic state of the generated Mannan-tolDCs in comparison to conventional hmoDCs. Unstimulated

Mannan-tolDCs displayed a significantly lower production of lactate (1.7 ± 0.38 vs 2.7 ± 0.28 mM) and Warburg effect than hmoDCs (**Fig 2, A**), and the same tendency was observed after LPS-stimulation (2.6 ± 0.18 mM in Mannan-tolDCs vs 3.3 ± 0.34 mM in hmoDCs). Mannan-tolDCs displayed significantly higher metabolic rate than hmoDCs under unstimulated conditions, indicating a higher consumption of glucose from the culture medium (**Fig 2, B**). LPS-stimulated hmoDCs significantly increase their metabolic rate without no significant differences compared to LPS-stimulated Mannan-tolDCs (**Fig 2, B**). Both unstimulated and LPS-stimulated Mannan-tolDCs showed significantly higher mitochondrial mass and mitochondrial membrane potential than unstimulated or LPS-stimulated hmoDCs (**Fig 2, C**), indicating that the glucose metabolic fate in Mannan-tolDCs is shifted towards mitochondrial oxidative phosphorylation (OXPHOS). Supporting these data, both unstimulated and LPS-stimulated Mannan-tolDCs significantly increased ROS production compared to hmoDCs (**Fig 2, D**). NADH accumulation in LPS-stimulated Mannan-tolDCs was significantly reduced compared to hmoDCs, with the same tendency in unstimulated Mannan-tolDCs (**Fig 2, D**), suggesting a higher mitochondrial respiratory chain activity. Intracellular ATP production was higher in unstimulated Mannan-tolDCs than hmoDCs, without differences after LPS stimulation (**Fig 2, D**). Mannan-tolDCs expressed higher mRNA levels of key metabolic enzymes involved in glycolysis, TCA and OXPHOS after 4 hours of LPS-stimulation, except for lactate dehydrogenase A (LDHA), than hmoDCs (**Fig 2, E**). Collectively, these data indicate that Mannan-tolDCs shifted glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation.

Mannan-tolDCs epigenetic reprogramming is associated with tolerogenicity

Epigenetics plays an important role in the generation of sustained immunogenic or tolerogenic DCs during monocyte differentiation.²³ To assess the capacity of allergoid-mannan conjugates to imprint epigenetic reprogramming in the generated Mannan-

tolDCs, we initially studied chromatin status near genes of specific cytokines and tolerogenic molecules. We performed chromatin immunoprecipitation (ChIP) analysis of the active chromatin histone marks, H3K4me3 and H3K27ac, and the inhibited chromatin histone mark, H3K27me3. The histone modification H3K4me3 (open chromatin and transcription activation) was significantly more associated to *IL10*, *PDL1* and *SOCS3* genes in Mannan-tolDCs than hmoDCs (**Fig 3**). Similarly, active H3K27ac was more associated to *PDL1* and *SOCS1* and less associated to *TNF α* in Mannan-tolDCs than hmoDCs. In contrast, H3K27me3 (close chromatin and transcription inhibition) was less associated to *IDO* gene in Mannan-tolDCs than hmoDCs. These data indicate that generated Mannan-tolDCs display epigenetic reprogramming involving specific histone marks associated with the activation of a tolerogenic transcriptional program.

We also performed inhibition experiments using the histone methyltransferase inhibitor 5'-methylthioadenosine (MTA) or the histone demethylase inhibitor pargyline. LPS-stimulated Mannan-tolDCs produced significantly less IL-10 in the presence of MTA without significant changes observed in the presence of pargyline (**Fig 4, A**), supporting a role for histone methylation in IL-10 production by Mannan-tolDCs. We did not observe significant changes in *TNF α* production (**Fig 4, A**), suggesting other alternative mechanisms for this cytokine. In addition, the mRNA levels of the histone deacetylases (HDAC1 to 11) were lower in freshly generated Mannan-tolDCs than hmoDCs, suggesting that allergoid-mannan conjugates imprint a global epigenetic reprogramming that ensure histone acetylation and the subsequent open chromatin status within the specifically regulated tolerogenic genes in Mannan-tolDCs.

We also wanted to study other mechanisms involved in Mannan-tolDCs tolerogenicity. Previous studies have identified miRNA-146 as an anti-inflammatory and miRNA-155 as a pro-inflammatory miRNA in human DCs.^{27, 28} miRNA-146a and miRNA-146b expression levels were significantly higher in Mannan-tolDCs than hmoDCs, both under unstimulated and LPS-stimulated conditions (**Fig 4, C**). In

contrast, miRNA-155 levels were significantly lower in unstimulated Mannan-toIDCs than hmoDCs. LPS stimulation increased the expression of miRNA-155 with significantly higher levels observed in hmoDCs than Mannan-toIDCs. Mannan-toIDCs were transfected either with locked nucleic acids control (LNA cont) or miRNA-146 inhibitor (LNA-146) or with control (cont) or miRNA-155-5p for miRNA-155 overexpression, and then stimulated with LPS or medium for 24 hours without affecting cell viability (**Fig E4, A**). The effectiveness of the transfections were analyzed by quantifying the miRNA expression levels under the different conditions (**Fig E4, B**). Inhibition of miRNA-146 expression or miRNA-155 overexpression in Mannan-toIDCs increased TNF α production after LPS stimulation, with higher levels observed upon miRNA-155 overexpression (**Fig E4, C**). Changes in IL-10 production by LPS-stimulated Mannan-toIDCs were not observed in the assayed conditions (**Fig E4, C**), indicating that miRNA-146 and miRNA-155 contribute to regulate TNF α but not IL-10 production in LPS-stimulated Mannan-toIDCs.

Allergoid-mannan conjugates also reprogram the differentiation of monocytes from allergic patients into tolerogenic DCs via epigenetic and metabolic rewiring

To verify that allergoids conjugated to mannan could also reprogram the differentiation of monocytes from allergic patients into tolerogenic DCs, we purified monocytes from allergic patients and generated DCs under different conditions as described in **Fig 1, A**. LPS-stimulated Mannan-toIDCs from allergic patients also produced significantly lower levels of TNF α and IL-6 than hmoDCs with lower TNF α /IL-10 and IL-6/IL-10 ratios (**Fig 5, A and B**). Mannan-toIDCs from allergic patients showed significantly higher mRNA expression levels of *IDO*, *PDL1*, *SOCS1*, *SOCS3* and *IL10* than hmoDCs (**Fig 5, C**). Unstimulated Mannan-toIDCs from allergic patients generated T cells producing higher IL-10 and lower IFN- γ than those generated by hmoDCs (**Fig 5, D**). Remarkably, LPS-stimulated Mannan-toIDCs from allergic patients generated T cells producing higher levels of IL-10 and lower levels of IFN- γ and IL-5 than T cells generated by hmoDCs

(**Fig 5, D**). The IFN- γ /IL-10 and IL-5/IL-10 ratios were also significantly lower in LPS-stimulated Mannan-tolDCs cocultures (**Fig 5, E**). Purified Treg cells (CD4⁺CD25⁺CD127⁻) generated by Mannan-tolDCs from allergic patients but not CD4⁺CD25⁻CD127⁻ non-Treg cells inhibited the proliferation of autologous PBMCs in a dose-dependent manner (**Fig 5, F and E3, C**). Interestingly, both Mannan-tolDCs unstimulated and loaded with native grass pollen allergens increase in the generation of FOXP3⁺ Treg cells compared to conventional hmoDCs, indicating that Mannan-tolDCs from allergic patients are able to induce and expand allergen-specific Treg cells (**Fig E5**).

Metabolically, LPS-stimulated Mannan-tolDCs from allergic patients also displayed lower production of lactate (Warburg effect) and higher mitochondrial mass and mitochondrial membrane potential than allergic hmoDCs (**Fig 6, A and B**). Mannan-tolDCs from allergic patients showed increased mRNA expression levels of glycolytic, TCA cycle and OXPHOS genes than hmoDCs (**Fig 6, C**). The mRNA expression levels for HDAC1 to 11 were also lower in Mannan-tolDCs than hmoDCs from allergic patients (**Fig 6, D**). Finally, the miRNAs expression pattern in Mannan-tolDCs from allergic patients was the same to that described in non-atopic donors (**Fig 6, E**). Collectively, these data demonstrated that allergoids conjugated to mannan are also able to differentiate monocytes from allergic patients into Mannan-tolDCs showing tolerogenic properties via metabolic and epigenetic reprogramming.

DISCUSSION

In this study, we show for the first time that next generation AIT vaccines based on allergoids conjugated to mannan reprogram monocyte differentiation and generate tolerogenic DCs via epigenetic and metabolic rewiring both in non-atopic donors and allergic patients. Mechanistically, generated Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation, a metabolic hallmark of tolerogenic DCs.²⁴ Allergoid-mannan conjugates imprint epigenetic reprogramming in the generated Mannan-tolDCs involving specific histone marks, thus ensuring sustained open chromatin status within the specifically regulated tolerogenic genes during monocyte differentiation, leading to tolerogenic DCs with stable phenotypes even upon pro-inflammatory stimulation. In addition, Mannan-tolDCs significantly increase the expression of the anti-inflammatory miRNA-146a/b whereas decrease pro-inflammatory miRNA-155, which also contribute to the imprinted tolerogenic features. Overall, we uncover unprecedented molecular mechanisms by which allergoid-mannan conjugates might well also restore tolerance to allergens during AIT.

We previously showed that allergoid-mannan conjugates target DCs via mannose receptor and DC-SIGN, enhancing allergen uptake, increasing IL-10 production, PD-L1 expression and promoting the generation of functional allergen-specific of FOXP3⁺ Treg cells both *in vitro* and *in vivo*, which was impaired by the conventional adjuvant alum.^{12, 14, 16} However, whether allergoid-mannan conjugates could also regulate human monocyte differentiation into DCs and the underlying molecular mechanisms remained fully unknown. Vaccine administration sites are usual places of monocyte recruitment,^{21, 22} and there is compelling experimental evidence showing that monocyte differentiation into inflammatory or tolerogenic DCs is highly conditioned by the signals encountered in the local tissue microenvironment.^{18-20, 29} For example, endogenous and synthetic molecules such as IL-10, TGF- β , glucocorticoids, rapamycin, minocycline,

ethyl pyruvate or Vitamin D3, among others, promote the *in vitro* generation of human monocyte-derived tolerogenic DCs.^{19, 20, 30-32} Some of them such as dexamethasone, minocycline, ethyl pyruvate or vitamin D3 also induce tolerogenic DCs in mice *in vivo*.^{20, 33} Here, we demonstrate that allergoid-mannan conjugates can also drive monocytes differentiation into tolerogenic DCs both in non-atopic and allergic subjects. The generated Mannan-tolDCs significantly increase the expression of classical tolerogenic molecules and polarize functional FOXP3⁺ Treg cells. Remarkably, Mannan-tolDCs from non-atopic subjects and allergic donors display a stable tolerogenic phenotype under inflammatory conditions as demonstrated by their lower production of pro-inflammatory cytokines and significantly lower TNF α /IL-10 ratio compared to conventional DCs upon LPS-stimulation. Supporting these data, LPS-stimulated Mannan-tolDCs also induced higher numbers of IL-10-producing Treg cells with concomitant lower generation of effector Th1 and Th2 cells than conventional DCs. These results are aligned with the observed higher expression of *SOCS1* and *SOCS3* genes in Mannan-tolDCs, which are among the most relevant cytokine signaling suppressor molecules.^{34, 35} Similarly, PD-L1 and IDO have been previously linked to the capacity of tolerogenic DCs to induce functional FOXP3⁺ Treg cells.^{14, 36-39} There might be some evidences suggesting potential mechanisms by which these processes might also occur *in vivo*: i) allergoid-mannan conjugates induce high levels of IL-10 but also IL-6 by already differentiated DCs,¹⁴ which could act as an initial pro-inflammatory signal enhancing monocyte recruitment; ii) allergoid-mannan conjugates are hypoallergenic, but their residual allergenicity¹⁴ might well induce the signals and cytokine milieu for monocyte recruitment and differentiation; iii) allergoid-mannan conjugates induce spleen Treg cells, which is impaired by oxidation of mannan or by the presence of alum at the injection site;^{14, 16} iv) allergoid-mannan conjugates are efficiently captured by human monocyte-derived DCs,¹⁴ which could then migrate to draining lymph nodes and transfer the antigens to other cells migrating from the injection site by different mechanisms, thus facilitating additional contacts with the

vaccine; v) allergoid-mannan conjugates, which are structurally very stable, are also larger in size than allergoids without mannan, thus showing a lower diffusion rate.¹³ While all these structural features may support a persistent presence of the allergoid-mannan conjugates at the injection site, infiltrating myeloid cells will be able to uptake these conjugates even if a low concentration does exist, as this uptake is favoured by a receptor-mediated mechanism.¹⁴

To gain further insights into the molecular mechanisms underlying the stable tolerogenic phenotype induced in Mannan-tolDCs, we sought to investigate potential metabolic and epigenetic reprogramming imprinted by allergoid-mannan conjugates during monocyte differentiation. Different studies have shown that the regulation of the functional properties of human DCs is highly connected with metabolic reprogramming.^{25, 26} Our data revealed that Mannan-tolDCs from non-atopic and allergic patients shift glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS both under steady state conditions and upon LPS stimulation, indicating a metabolic rewiring that has been previously associated to tolerogenic human DCs.²⁴⁻²⁶ Previous studies reported that LPS-stimulated tolerogenic hmoDCs highly express genes involved in energetic metabolism, including genes related to glycolytic and mitochondrial catabolic pathways.²⁴ We observed an increased expression of TCA cycle and OXPHOS genes in Mannan-tolDCs from non-atopic controls and allergic patients. It has been described that many OXPHOS genes are highly expressed in tolerogenic hmoDCs, indicating the potential for a higher mitochondrial activity, with higher production of ROS than immature and mature hmoDCs.^{24-26, 40} In agreement with our data, it has also been demonstrated that tolerogenic hmoDCs display an increased expression of most of the glycolytic genes relative to mature hmoDCs.²⁴ We only observed lower gene expression for LDHA enzyme in Mannan-tolDCs, which is also aligned with the detected reduction of lactate production. We previously showed that allergoid-mannan conjugates directly activate

glycolysis accompanied with ROS production in hmoDCs, which was essential for FOXP3⁺ Treg generation and impaired by alum.¹⁶ Herein, we show that Mannan-tolDCs also display enhanced glycolysis with a clear shift towards OXPHOS and ROS production, suggesting that although certain levels of lactate are needed, a mitochondrial shift and ROS production are indispensable to generate tolerogenic DCs during monocyte differentiation. Collectively, our data indicate that allergoid-mannan conjugates not only imprint tolerogenic features in already differentiated DCs but also reprogram monocyte differentiation into Mannan-tolDCs by imprinting a glucose metabolic status typically associated with tolerogenic DCs.

Epigenetics plays a crucial role in regulating gene expression during monocyte differentiation into DCs and in the generation of stable transcriptional programs in immunogenic vs tolerogenic DCs.²⁰ Our ChIP data revealed that the permissive chromatin marks H3K4me3 and H3K27ac are more enriched near genes of tolerogenic molecules such as *IL10*, *PDL1*, *SOCS1* and *SOCS3* in Mannan-tolDCs than conventional DCs, which might well justify the stable tolerogenic profile observed for Mannan-tolDCs. Accordingly, the inhibition of histone methylation with MTA in LPS-stimulated Mannan-tolDCs significantly reduced the production of IL-10, indicating that its increased production might be regulated by histone methylation as shown with the higher H3K4me3. We did not observe changes in TNF α production, suggesting other alternative mechanisms involved in the regulation of this pro-inflammatory cytokine.

Mannan-tolDCs from both non-atopic donors and allergic patients display a lower expression of all the HDACs quantified isoforms (HDCA-1 to 11) compared to conventional DCs counterparts. It has been previously shown that HDAC activity is a crucial driver of allergic inflammation and that HDAC inhibition reduces allergic inflammation, thus being proposed as a potential anti-inflammatory strategy for the treatment of allergic airway diseases.⁴¹⁻⁴³ HDAC inhibitors also increase the number and function of naturally occurring Treg cells, regulating several DC functions through the induction of IDO,⁴⁴ an enzyme which is upregulated at the mRNA level and less

associated to the inhibitory histone mark H3K27me3 on Mannan-tolDCs than conventional DCs. In the same line, it has been previously reported that the nuclear receptor co-repressor 1 (NCoR1) is a master negative regulator of tolerogenesis in murine conventional DCs and hematopoietic stem cell precursors by mechanisms partially depending on the recruitment of HDACs to promote compact close chromatin within tolerogenic genes.^{45, 46} Overall, our data suggest that allergoid-mannan conjugates imprint a global epigenetic reprogramming that ensure histone acetylation and the subsequent open chromatin status within the specifically regulated tolerogenic genes in Mannan-tolDCs, thus favouring a stable tolerogenic DC phenotype. miRNAs also play a crucial role in the generation of human tolerogenic DCs during monocyte differentiation.^{47, 48} The upregulation of miRNA-155 during inflammatory processes has been correlated with the hyperactivation of myeloid cells, while inhibition of miRNA-155 increases the expression of SOCS3.⁴⁹ In line with this data, the generated Mannan-tolDCs from non-atopic and allergic patients display lower expression of miRNA-155 and higher SOCS3 expression than conventional DCs. In addition, anti-inflammatory miRNA-146, especially miRNA-146a, is significantly increased in non-atopic and allergic Mannan-tolDCs, indicating that allergoid-mannan conjugates induce a miRNA signature in Mannan-tolDCs that contribute to reinforce the imprinted transcriptional tolerogenic program during monocyte differentiation.

In summary, we uncover novel molecular mechanisms by which allergoid-mannan vaccines might contribute to restore tolerance to allergens during AIT. Our findings showing that allergoid-mannan conjugates reprogram monocytes from non-atopic donors and allergic patients into Mannan-tolDCs via epigenetic and metabolic reprogramming significantly enhance our knowledge on the way of action of AIT vaccines aimed at inducing tolerance to allergens. Our results elucidating novel epigenetic and metabolic reprogramming mechanisms induced by allergoid-mannan conjugates during monocyte differentiation could help to pave the way for the identification of novel biomarkers for the management of allergic patients undergoing

477 this AIT and for the design of alternative antigen-specific DC-based strategies for
478 allergy and other inflammatory diseases.

Journal Pre-proof

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FIGURE LEGENDS

Figure 1. Mannan-tolDCs display tolerogenic features. **A**, Mannan-tolDCs generation protocol and viability of the generated cells. **B and C**, Cytokine (B) or cytokine ratios (C) produced in the presence of medium (Ctrl -) or LPS of conventional hmoDCs or Mannan-tolDCs from non-atopic donors. **D**, Heatmap analysis of mRNA expression of tolerogenic molecules by freshly isolated hmoDCs or Mannan-tolDCs. **E and F**, Cytokines (E) or cytokine ratios (F) produced by allogeneic naïve CD4⁺ T cells primed by unstimulated or LPS-stimulated hmoDCs or Mannan-tolDCs from non-atopic donors after 5 days. **G**, Percentage and representative dot plots of induced CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells by allogeneic unstimulated hmoDCs or Mannan-tolDCs after 5 days (gating in lymphocytes). **H**, Proliferation of CFSE-labelled PBMCs gated on CD4⁺ T cells after 5 days of coculture with autologous purified Treg cells and non-Treg cells generated by allogeneic unstimulated Mannan-tolDCs (ratio 1:1). The percentage of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition is shown. Results are mean ± s.e.m. of 8-10 (B and C), 6-7 (E and F), 6 (G) and 5 (H) independent experiments. Wilcoxon test, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Figure 2. Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation. **A**, Quantification of the lactate content in cell-free supernatants with a lactate assay kit and the induced Warburg effect measured as 1/OD570nm of wells from unstimulated (Ctrl -) or LPS-stimulated hmoDCs and Mannan-tolDCs after 18 hours relative to unstimulated hmoDCs. **B**, Metabolic rate induced after stimulation during 18 hours with media or LPS of conventional hmoDCs or Mannan-tolDCs. **C**, Fluorescence intensity of cells stained with MitoTracker Green (mitochondrial mass) or MitoTracker Red (mitochondrial membrane potential) relative to unstimulated hmoDCs. **D**, Intracellular ROS, NADH and ATP quantification. For NADH measures, well volume is 160 µl. **E**,

Heatmap of mRNA expression levels of genes in hmoDCs and Mannan-tolDCs treated with LPS for 4 hours. Results are mean $\pm$ s.e.m. of 5-10 (A), 7 (B), 6 (C) and 4-8 (D) independent experiments. Paired Student *t* test or Wilcoxon test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

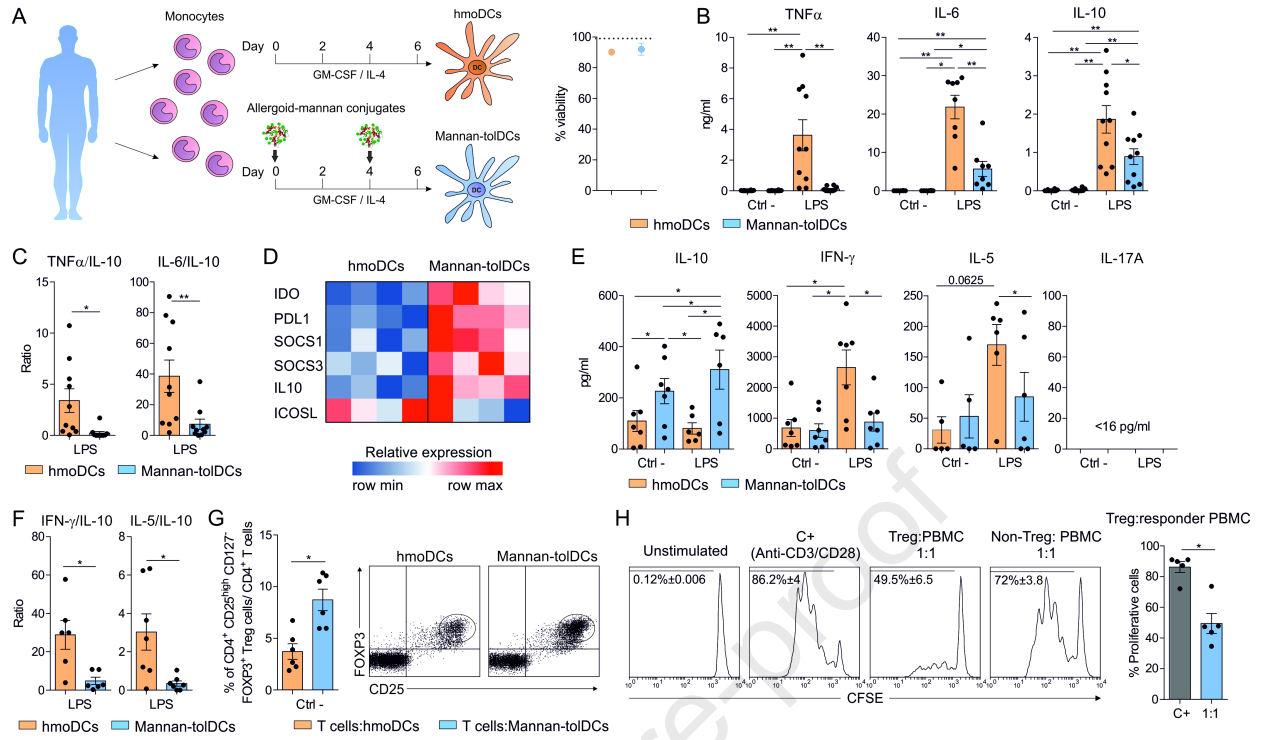
Figure 3. Mannan-tolDCs display epigenetic reprogramming involving specific histone marks associated with the activation of a tolerogenic transcriptional program. Chromatin immunoprecipitation data of freshly hmoDCs and Mannan-tolDCs to analyse the presence of H3K4me3, H3K27ac and H3K27me3 histone modifications associated to the indicated molecules and cytokines. Results are mean $\pm$ s.e.m. of 6 independent experiments normalized to positive control gene B2M (for H3K4me3 and H3K27ac) or to negative control gene MYOD1 (for H3K27me3). Mann-Whitney test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Figure 4. Mannan-tolDCs epigenetic reprogramming is associated with tolerogenicity. **A**, Cytokine production by unstimulated or LPS-activated Mannan-tolDCs in the presence of 5'-methylthioadenosine (MTA) or pargyline as inhibitors of histone methyltransferases and demethylases, respectively. **B**, Heatmap analysis of mRNA expression of histone deacetylases (HDACs) by unstimulated hmoDCs or Mannan-tolDCs. **C**, miRNA expression of hmoDCs and Mannan-tolDCs stimulated with medium (Ctrl -) or LPS. Results are mean $\pm$ s.e.m. of 4 (A) and 7-8 (C) independent experiments. Paired Student *t* test or Wilcoxon test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Figure 5. Allergoids conjugated to mannan also reprogram the differentiation of monocytes from allergic patients into tolerogenic DCs. **A and B**, Cytokine (A) or cytokine ratios (B) produced after stimulation with medium (Ctrl -) or LPS of conventional hmoDCs or Mannan-tolDCs from allergic patients. **C**, Heatmap analysis of mRNA expression of tolerogenic molecules by freshly isolated hmoDCs or Mannan-tolDCs from allergic patients. **D and E**, Cytokines (D) or cytokine ratios (E) produced by

allogeneic naïve CD4⁺ T cells primed by unstimulated or LPS-stimulated hmoDCs or Mannan-toIDCs from allergic patients after 5 days. **F**, Proliferation of CFSE-labelled PBMCs gated on CD4⁺ T cells after 5 days of coculture with autologous purified Treg cells and non-Treg cells generated by allogeneic unstimulated Mannan-toIDCs (ratio 1:1). The percentage of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition is shown. Results are mean $\pm$ s.e.m. of 6 (A, B, D and E) and 3 (F) independent experiments. Wilcoxon test, * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 6. Mannan-toIDCs from allergic patients also display epigenetic and metabolic rewiring. **A**, Lactate content in cell-free supernatants from LPS-stimulated hmoDCs and Mannan-toIDCs from allergic patients after 18 hours relative to LPS-stimulated hmoDCs. **B**, Fluorescence intensity of cells from allergic patients stained with MitoTracker Green (mitochondrial mass) or MitoTracker Red (mitochondrial membrane potential) relative to LPS-stimulated hmoDCs. **C**, Heatmap of mRNA expression levels of metabolic genes in hmoDCs and Mannan-toIDCs from allergic patients treated with LPS for 4 hours. **D**, Heatmap analysis of mRNA expression of histone deacetylases by unstimulated hmoDCs or Mannan-toIDCs from allergic patients. **E**, miRNA expression of hmoDCs and Mannan-toIDCs from allergic patients stimulated with medium or LPS. Results are mean $\pm$ s.e.m. of 4 (A), 4-6 (B) and 4 (E) independent experiments.



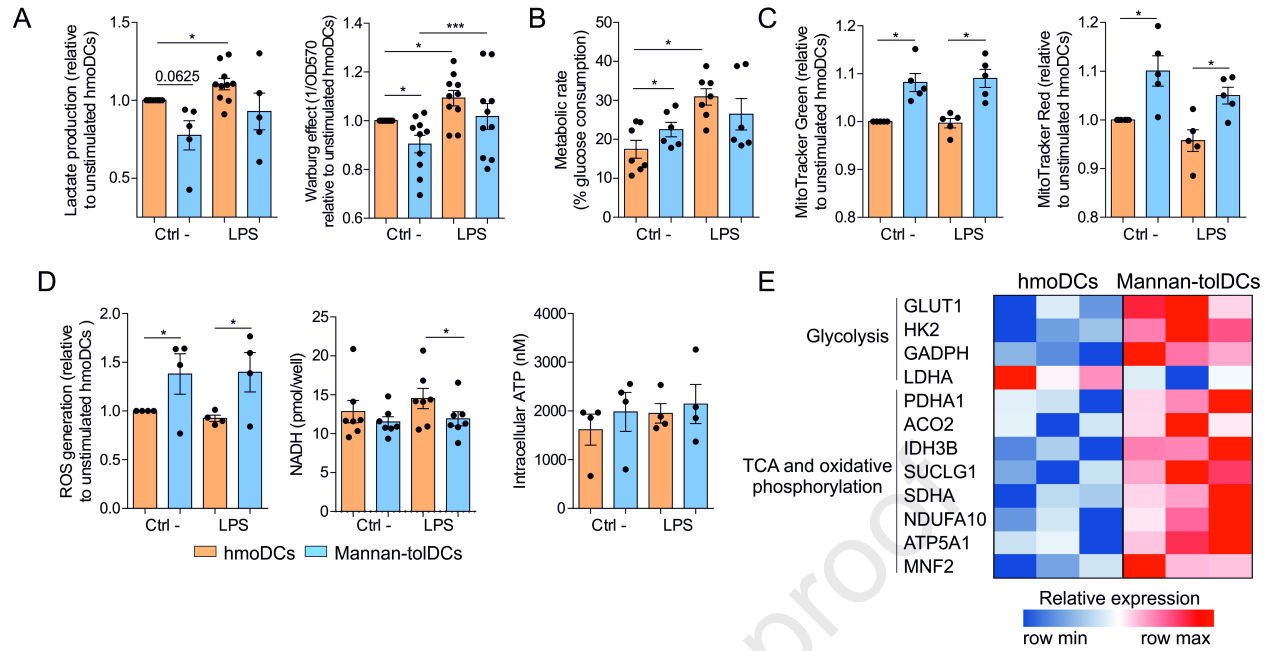


Figure 2. Benito-Villalvilla et al.

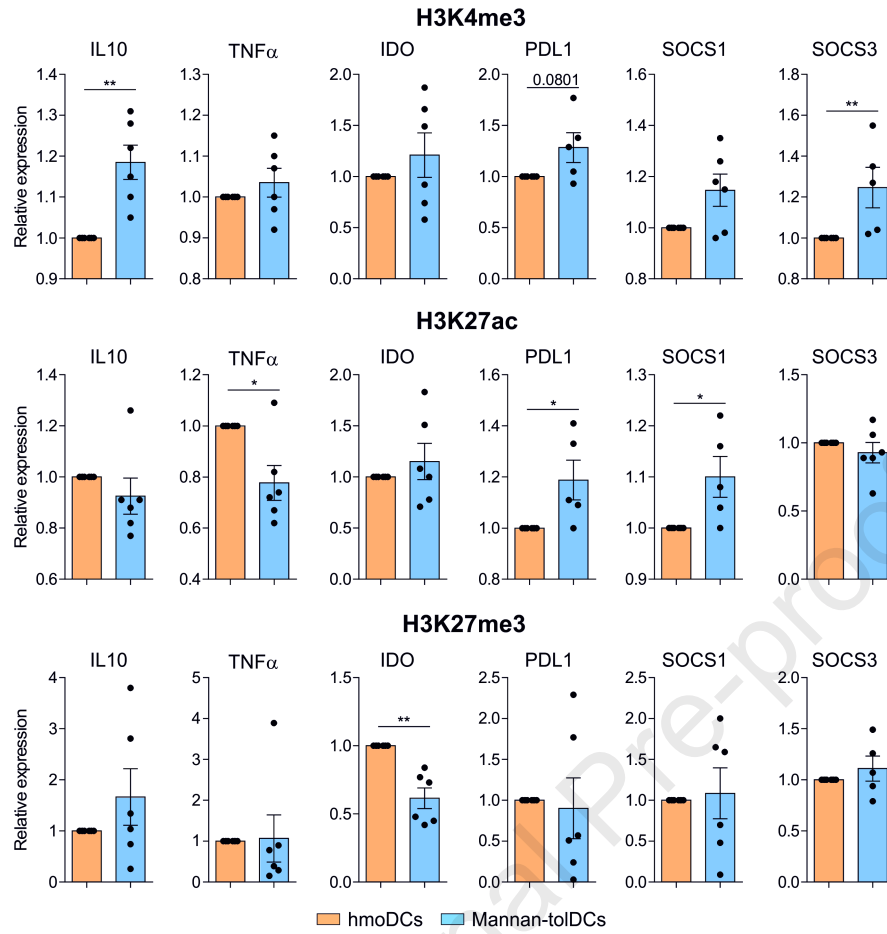


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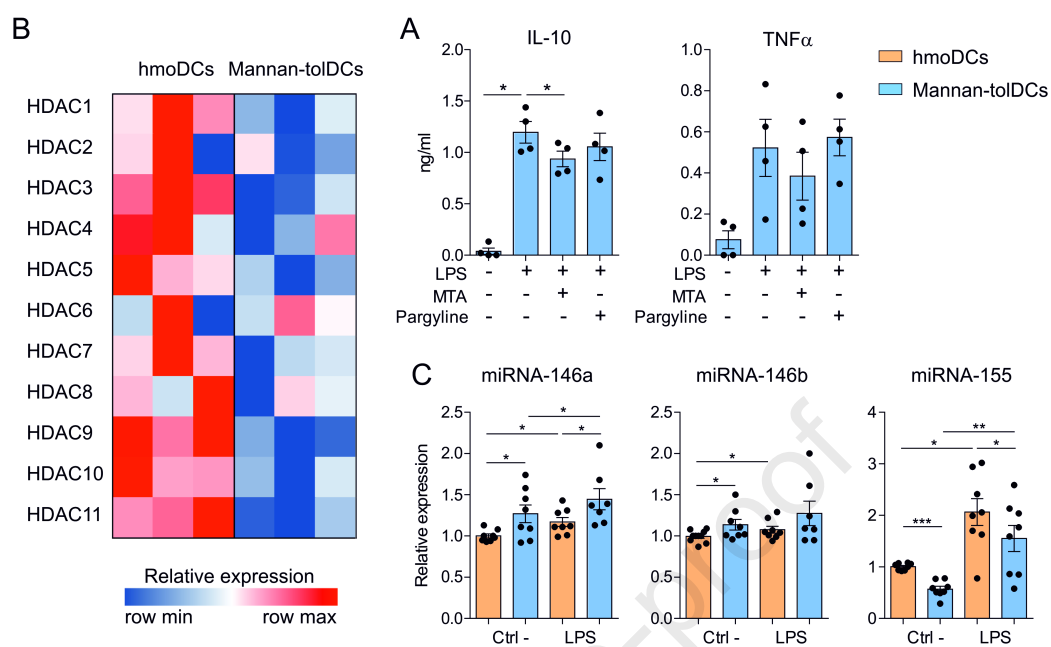


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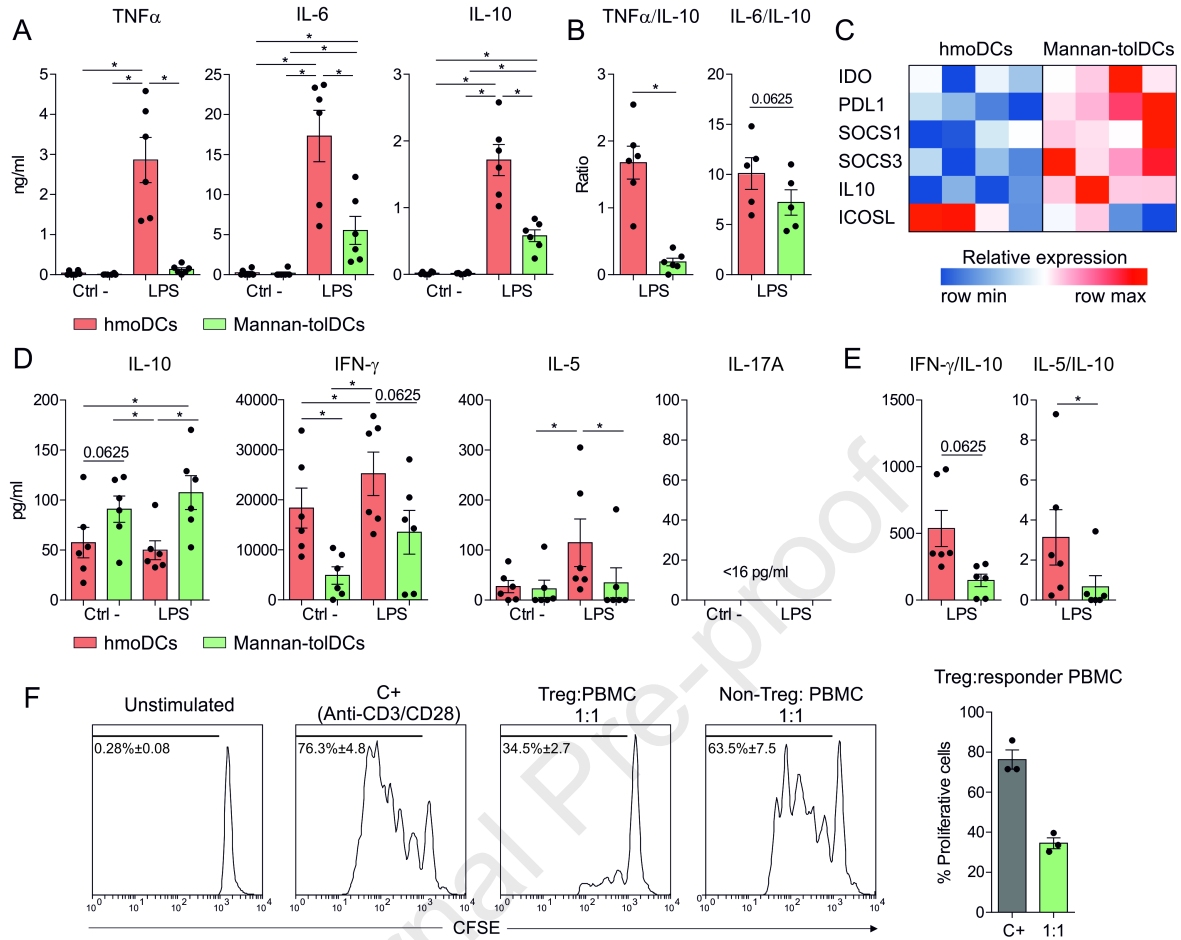


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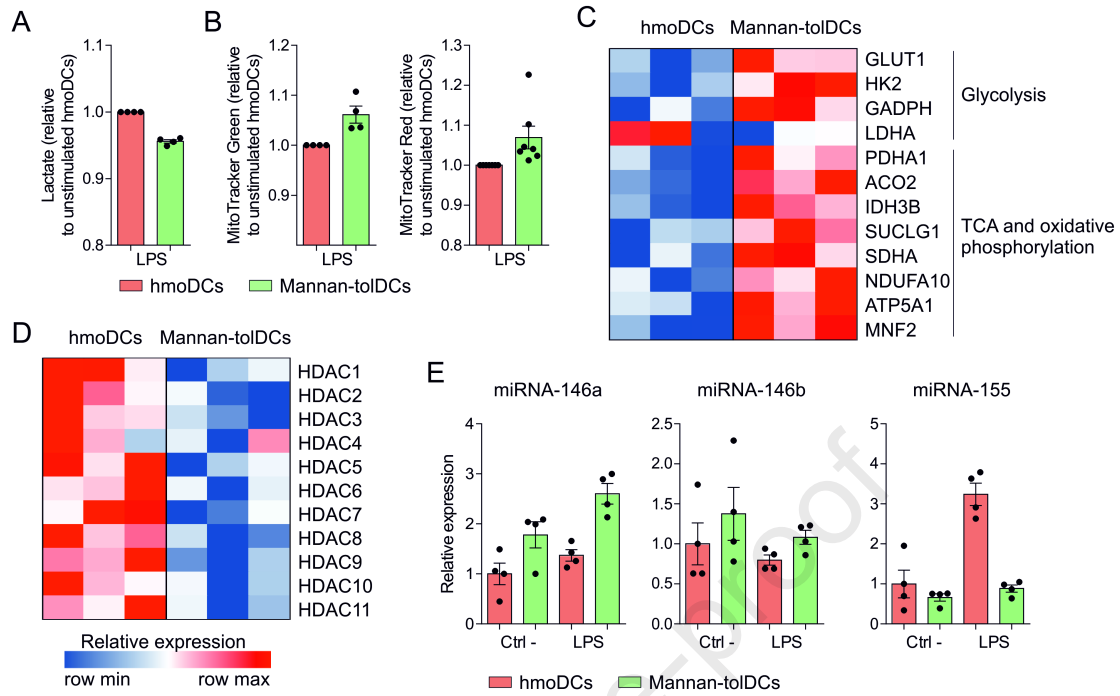


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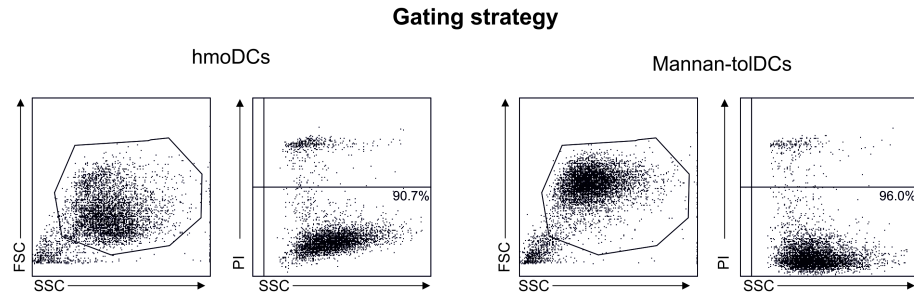


Figure E1. Benito-Villalvilla et al.

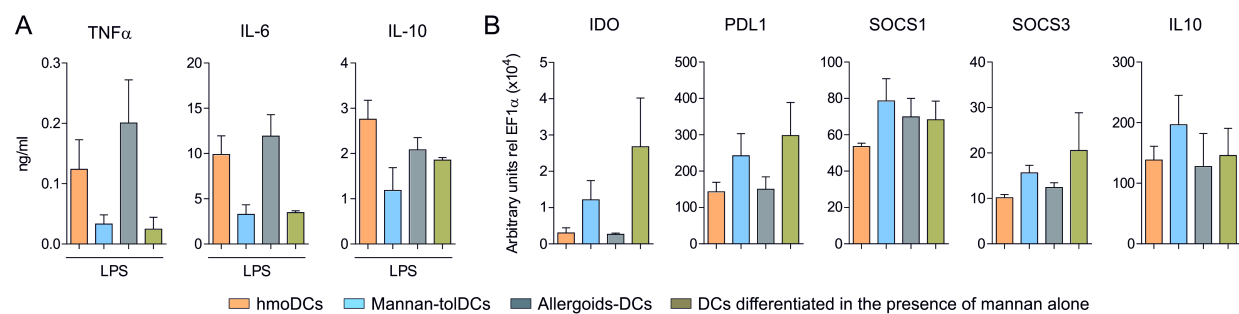


Figure E2. Benito-Villalvilla et al.

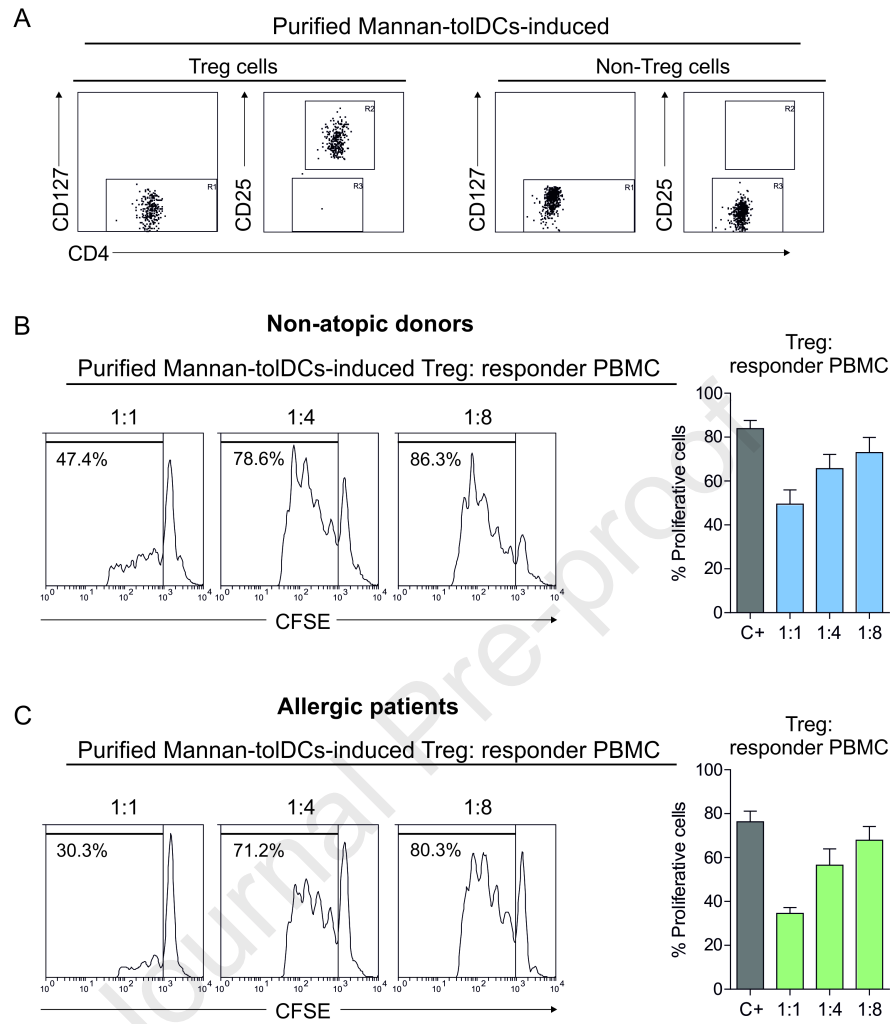


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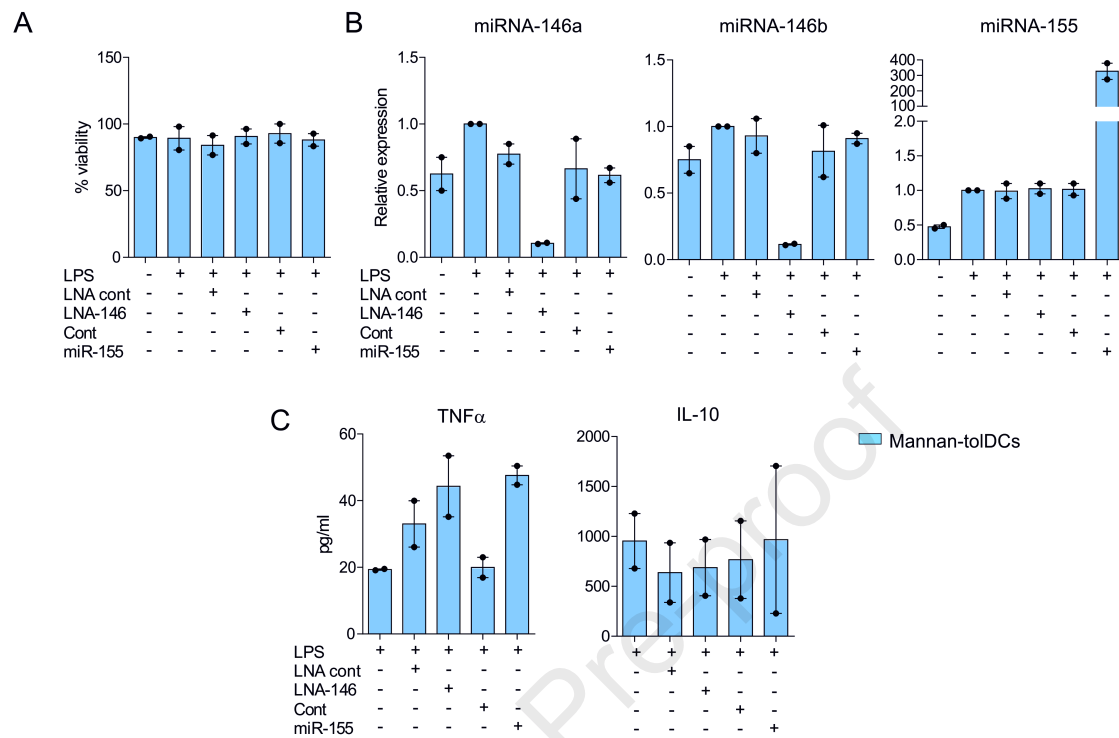


Figure E4. Benito-Villalvilla et al.

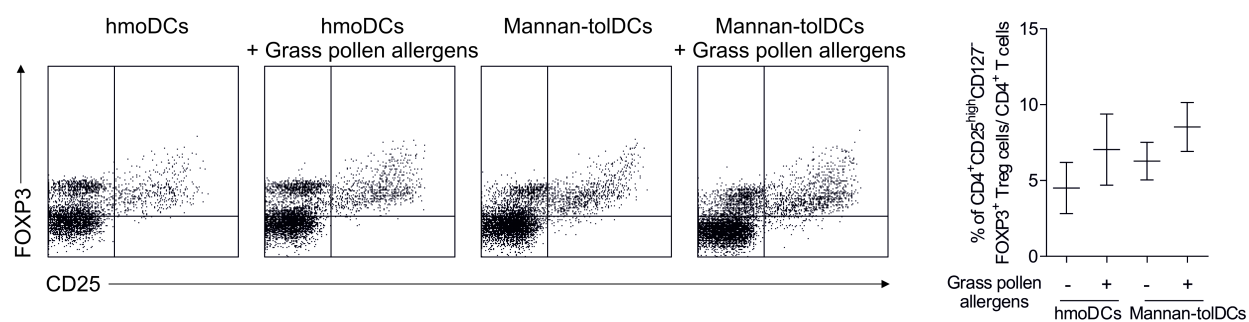


Figure E5. Benito-Villalvilla et al.

Allergoid-mannan conjugates reprogram monocytes into tolerogenic DCs via epigenetic and metabolic rewiring

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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). LPS (0127:B8) and inhibitors for histone methyltransferase 5'-methylthioadenosine (MTA) and histone demethylase (Pargyline) (Sigma-Aldrich, St Louis, Mo) were used. Allergoid-mannan conjugates (Immunotek) 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). Mannan (Sigma, 50µg/ml) was used to differentiate monocytes into dendritic cells for comparing experiments.

HmoDCs generation and naive CD4⁺ T cells purification

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of non-atopic controls or allergic patients by Ficoll density gradient centrifugation (800 g, 20 minutes). Immature hmoDCs were generated from blood monocytes obtained from total PBMCs using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured for 6 days with RPMI medium containing 100 ng/mL of IL-4 and GM-CSF (PeproTeck, Rocky Hill, NJ). To generate Mannan-tolDCs, allergoid-mannan conjugates (50 µg/mL) were added on days 0 and 4 of the differentiation. To generate

Allergoids-DCs, mannan-free glutaraldehyde-polymerized grass pollen allergoids (50 $\mu\text{g/mL}$) were added on days 0 and 4 of the differentiation. The purity and phenotype of monocytes and generated immature hmoDCs were analysed by flow cytometry with lineage-specific markers. Peripheral blood naïve CD4^+ T cells were isolated from buffy coats of non-atopic controls using the “Naive CD4^+ T Cell Isolation Kit” (Miltenyi Biotec), according to manufacturer’s protocol. In all the cases, viable cells were counted using Trypan blue with an optical microscope and analyzed by propidium iodide by flow cytometry.

Cell cultures

HmoDCs, Mannan-tolDCs or Allergoids-DCs from non-atopic controls or allergic patients were treated with medium or LPS (100 ng/mL) for 18 hours. Cell pellets were used to analyze their phenotype by qPCR and cell-free supernatants to quantify IL-6, $\text{TNF}\alpha$ and IL-10 by ELISA. For coculture experiments, hmoDCs or Mannan-tolDCs treated with medium (Ctrl -) or LPS (100 ng/mL) were cocultured with purified allogeneic naïve CD4^+ T cells (DC/T cell ratio of 1:5) for 5 days. IL-17A, IFN- γ , IL-5 and IL-10 were quantified in cell-free supernatants by ELISA. To analyze the induction of allergen-specific Treg cells, hmoDCs or Mannan-tolDCs from allergic patients were treated with medium or native grass pollen allergens (50 $\mu\text{g/mL}$) for 18 hours and then were cocultured with autologous peripheral blood lymphocytes (PBLs) at a DC/PBL ratio of 1:5 to analyze FOXP3^+ Treg cells.

For inhibition experiments, Mannan-tolDCs were preincubated for 1 hour with MTA (50 μM) or Pargyline (5 μM) (or their vehicle control, DMSO) prior to activation. Then, cells were stimulated with LPS for 18 hours in the presence of the corresponding inhibitors to quantify $\text{TNF}\alpha$ and IL-10 by ELISA. Cell viability was analysed in all the cases by trypan blue exclusion with a light microscope.

Cytokine quantification

Concentrations of IL-6, TNF α , IL-10, IFN- γ , and IL-5 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, CA). IL-17A levels were quantified by quantikine Elisa Kit (RD Systems). 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 pair primers are in **Table E1**. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems, Foster City, Calif). Data were normalized to EF1 α 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 EF1 α . For miRNA analysis, RNA isolation Qiazol (Qiagen, Germany) and Total RNAzol out Mini kit (A&A Biotechnology, Poland) were used according to the manufacturer's instructions. To analyze miRNA expression, TaqMan $^{\circledR}$ MicroRNA Assays (Life Technologies, California, USA) and 5 $\times$ HOT FIREPol $^{\circledR}$ Probe qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) were used according to the manufacturer's protocols. Each PCR reaction was performed using a ViiA 7 Real-Time PCR system (Life Technologies, Carlsbad, California, USA). For normalization let-7a and $\Delta\Delta Ct$ calculation were used.

Flow cytometry

were determined 18 hours after stimulation by using the Glucose (GO) Assay Kit (Sigma-Aldrich). The metabolic rate was derived mathematically in percentage of medium without DCs (glucose concentration in RPMI 1640 = 2 mg/mL). To measure mitochondrial mass and mitochondrial membrane potential, MitoTracker Green FM (25 nM) or MitoTracker Red CMXRos (250 nM) were used, respectively (Thermo Fisher Scientific). Arbitrary fluorescence values were obtained in a FLUOstar Omega microplate reader (Ex/Em = 490/516 nm for Mitotracker Green and Ex/Em = 579/599 nm for Mitotracker Red CMXRos). Reactive oxygen species were measured by DCFDA Cellular ROS Detection Assay Kit (Abcam) following manufacturer's instructions. Briefly, total ROS levels were measured using the cell permeant reagent 2',7' - dichlorofluorescein diacetate (DCFDA). Cells were stained with DCFDA for 30 minutes at 37°C after 18 hours of treatment with the corresponding stimuli. Fluorescence was measured in a FLUOstar Omega microplate reader (Ex/Em = 485/535 nm). Total NADH concentrations in lysates of DCs were determined by using NAD/NADH Assay Kit (Abcam), according to the manufacturers' recommendations. For NADH measures, well volume is 160 µL. ATP concentrations in lysates of hmoDCs were determined by using ATP Determination Kit (InvitroGen), according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP)

hmoDCs and Mannan-toIDCs were fixed after differentiation. Cells were subjected to lysis and sonication using the Bioruptor UCD-200 sonicator (Diagenode) to obtain chromatin fragments of 200–400 bp. Sheared chromatin was immunoprecipitated with 2 µL of anti-H3K4me3 antibody (Millipore, 07-473), 2 µL of anti-H3K27me3 antibody (Millipore, 07-449), 2 µL of anti-H3K27ac antibody (Abcam, ab4729) or 2 µL of anti-IgG antibody (Millipore, 12-370) and 3 µL of Dynabeads M-280 magnetic beads (Sheep Anti-Mouse IgG, Sheep Anti-Rabbit IgG, Life Technologies) using the SX-8G IP-Star Automated System (Diagenode). ChIP samples were decrosslinked at 65°C for 4 hours. After incubation, the samples were treated with 0.2 mg/mL RNaseA (RNase

Cocktail Enzyme Mix, Ambion, Life Technologies) and 0.4 mg/mL Proteinase K (Thermo Scientific). DNA was purified with the DNA Clean kit & Concentrator TM-5 (Zymo Research) according to the manufacturer's protocol. Real-time quantitative PCR was performed on the purified ChIP and INPUT DNAs by using FastStart Universal SYBR Green Master. Sequences of the used pair primers are in **Table E1**. Data from H3K4me3 and H3K27ac marks were normalized to B2M positive control and data from H3K27me3 mark were normalized to MYOD1 negative control.

Transfection experiments

Transfection with NickFect71 was used for Figure 4D and E2 and was performed as previously described¹. Briefly, NickFect71 was incubated with miRNA mimic or lock nucleic acid (LNA) as inhibitor at a 17:1 CPP:miRNA molar ratio (MR) in MQ-water in 1/10 of final treatment volume at room temperature (RT) for 1 hour to form CPP:miRNA complexes; mixed with media and added to the cells. After 24 hours, Mannan-toIDCs were stimulated with 100 ng/mL of LPS for 24 hours or left unstimulated. Transfections in dendritic cells were performed at 100 nM of miRNA mimic negative control, miR-155-5p (from Life Technologies) and miRCURY LNA™ inhibitors (microRNA Power inhibitor hsa-miR-146 and Negative control A, Exiqon, Vedbaek, Denmark). Cells were harvested in Qiazol and kept at -80 °C until RNA isolation.

ELISA of serum specific-IgE from allergic patients to native grass pollen allergens

Microtiter plates (Corning Inc, Corning, NY) were coated with 10 µg of N in 100 µl of PBS overnight at 4°C. Plates were washed 3 times with PBS and 0.1% vol/vol Tween-20 and blocked for 1 hour with PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. Then plates were incubated for 2 hours with individual serum from patients with well-defined grass pollen allergy diluted in PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. After 4 washes, bound IgE antibodies were detected by incubating for

2 hours with goat anti-human IgE-biotin (1:2000 diluted) (ThermoFisher), followed by 1 hour with horseradish peroxidase-coupled streptavidin (diluted 1:500). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% H₂O₂ and 0.63 mg/mL o-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0), and the reaction was stopped with 3 N H₂SO₄. OD was measured at 492 nm in an ELISA reader.

SUPPLEMENTARY FIGURES

Figure E1. The presence of allergoid-mannan conjugates during monocyte differentiation did not affect cell viability. Gating strategy for propidium iodide (PI) labelling analysis with representative histograms of conventional hmoDCs and Mannan-tolDCs viability.

Figure E2. The presence of mannan in the conjugate structure is essential for the induction of tolerogenic DCs. **A**, Cytokines produced after unstimulation (Ctrl -) or LPS stimulation of hmoDCs, Mannan-tolDCs, hmoDCs differentiated in the presence of Allergoids (Allergoids-DCs) or hmoDCs differentiated in the presence of mannan alone (n=3-4). **B**, mRNA expression levels of genes in freshly isolated hmoDCs, Mannan-tolDCs, Allergoids-DCs and DCs differentiated in the presence of mannan alone. Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4 , with ΔCT defined as the difference between the CT value for the gene of interest and EF1 α (n=3).

Figure E3. Suppression capacity of Treg cells induced by allogeneic Mannan-tolDCs from non-atopic and allergic subjects. **A**, Representative dot plots of the purification of Treg cells by sorting. **B and C**, Proliferation of CFSE-labeled PBMCs gated on CD4⁺ cells after 5 days of coculture with autologous purified Treg cells induced by allogeneic Mannan-tolDCs from non-atopic donors (Fig. E2B) or Mannan-tolDCs from allergic patients (Fig. E2C). The percentages of proliferating responder

PBMCs stimulated with anti-CD3 and anti-CD28 for each condition at different Treg cell/responder PBMC ratios are shown (n = 3).

Figure E4. Inhibition of miRNA-146 and overexpression of miRNA-155 in Mannan-toIDCs. **A**, Viability of unstimulated or LPS-stimulated Mannan-toIDCs after transfection with miRNA-146 inhibitor (LNA-146), miRNA-155 or the corresponding vehicle controls (LNA cont or cont). **B**, Expression of indicated miRNAs in the transfected cells. **C**, TNF α and IL-10 production by Mannan-toIDCs transfected with miRNA-146 inhibitor (LNA-146), miRNA-155 or the corresponding vehicle controls (LNA cont or cont) and stimulated with LPS for 24 hours. Results are mean $\pm$ s.e.m. of 2 independent experiments.

Figure E5. Induction of allergen-specific Treg cells. **A**, Percentage and representative dot plots of induced CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells by autologous unstimulated or stimulated with grass pollen allergens Mannan-toIDCs from allergic patients after 5 days of coculture with peripheral blood lymphocytes (gating in lymphocytes) (n=2).

Gene	Primer sequences	
	Forward	Reverse
PDL1	AAGATGAGGATATTTGCTGTCTTTATATTC	GTCCTTGGGAACCGTGACAGT
IDO	AGAAGTGGGCTTTGCTCTGC	TGGCAAGACCTTACGGACATCTC
SOCS1	CCCTGGTTGTTGTAGCAGCTT	CAACCCCTGGTTTGTGCAA
SOCS3	CCTCAGCATCTCTGTCTGGAAGA	GCATCGTACTGGTCCAGGAAGT
ICOSL	CTCCGCCCCGACCAT	CTACCATCGCTCTGACTTCCTTCT
IL10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT
EF1α	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT
GLUT1	GGCTTCTCCAACTGGACCTC	CCGGAAGCGATCTCATCGAA
HK2	TTCGCACTGAGTTTGACCAG	TCACCAGGATAAGCCTCACC
GADPH	CTGCACCACCAACTGCTTAGC	TCATGTTCTGGAGAGCCCCG
LDHA	ATGGCAACTCTAAAGGATCAGC	CCAACCCCAACAAGTGAATCT
PDHA1	ATGGAATGGGAACGTCTGTTG	CCTCTCGGACGCACAGGATA
ACO2	AATGGATGTAAGTCTGTTGGC	ACAGCCTACTGGTGAAGTCGG
IDH3B	CTGATGCACGCCGTCAAG	GCCATATTCTGCACCTCACTCA
SUCLG1	CCTACACAGCTTCTCGGCAA	CTGTGAAAGGTGCCCTGTTTG
SDHA	CGAACGTCTTCAGGTGCTTT	AAGAACATCGGAAGTGCAGC
NDUFA10	ACAGAACGCAGCAGAGTGATA	GGAAAGTGCTTGAAGCCTAGTT
ATP5A1	AAGACACGCCAGTTTCTTC	TTTGGGTTTCATCTTTTCATTGC
MNF2	TCTGGGACCTTTGCTCATCT	CAACCAACCGGCTTTATTCC
HDAC1	ACTACGACGGGGATGTTGGA	CAG CAT TGG CTT TGT GAG GG
HDAC2	TCTGCTACTACTACGACGGTGA	TCATTTCTTCGGCAGTGGCT
HDAC3	ATCTCTGCAAGGAGCAACCC	AGCCAGAGGCCTCAAACCTC
HDAC4	CCACCTCACTCCCTACCTGA	CCCAGGCCTGTGACGAG
HDAC5	TCCCGTCCGTCTGTCTGTTA	GACCTGACATCCCATCCGAC
HDAC6	CTGGCGGAGTGGAAGAACC	GGGGAACGGCTCCCTTTTTTA
HDAC7	CTCGGAGGCTGACAGTGACC	GAGGGGTCCAGGAGGAGAAT
HDAC8	CAGAAGGTCAGCCAAGAGGG	GGCAGTCATAACCTAGCCCA
HDAC9	GACGTGTGGTGTTGGCTCTA	GTGGCTCCAGCTCATTTCTT
HDAC10	TACCATGAGGACATGACGGC	GGGATACATACTCTGGGCTGTG
HDAC11	CCCGGGATGCTACACACAAC	CAGGCCCATGAAGGTGATGT
IL10 ChIP	TTGCCTGGGAAGTGGGTGCA	AGAAGGCATGCACAGCTCAGC
TNF ChIP	ACCACGCTCTTCTGCCTGCT	TCCATCCCTCCCTATCAGCGCA
PDL1 ChIP	GCTTCCGCCGATTTACCGA	TCGGGAAGCTGCGCAGAACT
IDO ChIP	AGGTTGTGTTTCCGGGCTGCT	ACAGGTGGCCGGAGAAGAACA
SOCS1 ChIP	TCGGCCCTGTTTCCCTCTCT	CCTGGCGGCAGAAAGTGGAA
SOCS3 ChIP	GGTCCCGAATCGAAGTCTCCGT	ACTTCACGGCCGCCAACATCT
B2M ChIP	CTCTCTCGCTCCGTGACT	GCCGAAAGGGGCAAGTAG
MYOD1 ChIP	GTAGGAGAGGCGGGAGAA	TAGGTCTGCGAGGGTCTC

243 **Table E1.** PCR primers used in this paper

244

Patient no.	Age (y)	Sex	Allergic to	Specific IgE ⁽¹⁾	Symptoms	Other pollen allergies
1	28	M	Grasses	3.220	RC	<i>Olea</i> species, <i>Cupressus</i> species
2	26	F	Grasses	0.355	RC	<i>Olea</i> species, <i>Cupressus</i> species
3	30	M	Grasses	0.603	RC	
4	27	F	Grasses	3.500	RC	<i>Olea</i> species, <i>Platanus</i> species
5	57	M	Grasses	0.642	RC-A	<i>Olea</i> species, <i>Cupressus</i> species
6	27	M	Grasses	1.300	RC-A	<i>Platanus</i> species, <i>Betula</i> species

245 A, Asthma; F, Female; M, Male; RC, Rhinoconjunctivitis;

246 (1): Determined in ELISA as OD at 492 nm

247 **Table E2.** Clinical features of the patients with grass pollen allergy included in the
 248 study

249

250 REFERENCES

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