Inhibins regulate peripheral regulatory T cell induction through modulation of dendritic cell function

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Keywords
dendritic cells; inhibins; peripheral tolerance; Tregs

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(Received 11 October 2018, revised 13 November 2018, accepted 16 November 2018)
doi:10.1002/2211-5463.12555

We have previously reported that the absence of inhibins results in impaired dendritic cell (DC) maturation and function, leading to decreased T cell activation and diminished delayed-type hypersensitivity responses. Here, we investigated the role of inhibins in peripheral regulatory T cell (Treg) induction in vitro and in vivo. Inhibin deficient (Inhα−/−) mice showed an increased percentage of peripherally induced Tregs in colonic lamina propria and mesenteric lymph nodes, compared to Inhα+/+ mice, which correlated with increased expression of PD-L1 in CD103+ and CD8α+ DCs. Lipopolysaccharide-stimulated bone marrow-derived and ex vivo spleen- and lymph node-purified CD11c+ Inhα−/− DCs induced higher Tregs in vitro. Moreover, in vivo anti-DEC205-ovalbumin (OVA) DC targeting of mice with adoptively transferred OVA-specific T cells showed enhanced induced peripheral Treg conversion in Inhα−/− mice. These data identify inhibins as key regulators of peripheral T cell tolerance.

Regulatory T cells (Tregs) play a key role in central and peripheral T cell tolerance by preventing the development of autoimmunity and restraining inflammatory immune responses to pathogens that may result in immunopathology. The balance between effector and regulatory T cells is critical for the maintenance of homeostasis (reviewed in [1]).

Tregs are a subset of CD4+ T cells characterized by a high expression level of CD25 (interleukin (IL)-2α chain receptor) and forkhead box P3 (FoxP3), a transcription factor considered the master regulator of Treg development and function [2]. Their ability to suppress several immune cell responses has become increasingly relevant to understanding and treating several diseases and inflammatory responses [3]. Two major Treg subsets have been identified, those originating in the thymus, referred to as thymic Tregs (tTregs), and those induced in peripheral tissues from naïve T cells, referred to as peripheral Tregs (pTregs) (reviewed in [4]). Both populations share some

Abbreviations
BMDC, bone marrow-derived dendritic cell; BMP, bone morphogenetic protein; cDC, conventional dendritic cell; CT, cholera toxin; DC, dendritic cell; dLN, draining lymph node; FoxP3, forkhead box P3; FACS, fluorescence-activated cell sorting; IL, interleukin; LP, lamina propria; LPS, lipopolysaccharide; mDC, migratory dendritic cell; MFI, mean fluorescence intensity; MHC-II, major histocompatibility complex class II; MLN, mesenteric lymph node; OVA, ovalbumin; PLN, peripheral lymph node; pTreg, peripherally induced Treg; RA, retinoic acid; rDC, resident dendritic cell; TCR, T cell receptor; TGFβ, transforming growth factor β; Th3, T helper 3; Tr1, type 1 regulatory T; Treg, regulatory T cell; tTreg, thymic regulatory T cell; WT, wild-type.
phenotypic markers including FoxP3, CD25, GITR and CTLA-4, although other markers, such as neuropilin 1, CD73 and Helios, have been proposed as specific for tTregs [5,6]. In addition, the signaling events needed to induce pTregs are different from those required for Treg differentiation; transforming growth factor β (TGFβ) is a required cytokine for FoxP3 induction in pTregs, as well as low levels of T cell receptor (TCR) activation and low costimulatory signals. In contrast, tTregs require strong TCR and costimulatory signals and the presence of γ chain cytokines, such as IL-2 and/or IL-7. These different requirements are associated with the transcriptional regulation of the FoxP3 gene in tTregs versus pTregs [7]. Concerning the functional relevance of Treg subpopulations, tTregs have been shown to play a crucial role in the control of autoimmune diseases [8], while pTregs appear to be more relevant in restraining immunopathology after an immune response and in the context of intestinal homeostasis [9] (reviewed in [10]). However, both tTregs and pTregs have been shown to be necessary to prevent colitis, showing a non-redundant role in the maintenance of peripheral tolerance [11].

In addition to FoxP3+ Tregs, other regulatory T cell subsets can be induced from naive T cells, such as type 1 regulatory T (Tr1) cells and T helper 3 (Th3) cells (reviewed in [12]). Compared with Tregs, Tr1 and Th3 cells normally do not express CD25 or FoxP3 [13,14]. Tr1 cells are characterized by the expression of CD49b, LAG3 and the production of IL-10; their differentiation is favored under suboptimal antigen stimulation in the presence of IL-10 [15,16]. On the other hand, Th3 cells are characterized by the production of TGFβ1 and the expression of CD69+ and LAP+ [14,17].

Dendritic cells (DCs) are a heterogeneous group of professional antigen presenting cells that originate in the bone marrow, principally from myeloid progenitors that differentiate into Pre-DCs. Pre-DCs seed peripheral tissues, where they complete their differentiation to DCs in the lymph node, where they are known as resident DCs (rDCs), or in non-lymphoid tissues, where they are known as migratory DCs (mDCs) [18,19]. Both conventional DC (cDC) subsets can be identified in lymph nodes as CD11c+MHC-IImed and CD11c+MHC-IIghi for rDCs or mDCs, respectively [19]. DCs play an important role in peripheral tolerance through several mechanisms including clonal deletion, anergy and regulation. In homeostasis, DCs capture self-antigens and present them to naïve T cells, preventing the activation of self-reactive clones and favoring the induction of Tregs and T cell anergy. In this context, murine cDCs can be subdivided into two main subtypes that are considered independent cDC lineages: type 1 DCs (cDC1) for CD8α+ rDCs and CD103+ mDCs, and type 2 DCs (cDC2) for CD4+CD11b+ rDCs and CD11b+ mDCs (reviewed in [20]). CD103+ mDCs in mesenteric lymph node (MLN) are considered as tolerogenic DCs due to their low levels of costimulatory molecules (CD40, CD80 and CD86), high levels of coinhibitory molecules (PD-L1 and PD-L2) and the expression of IL-10, retinoic acid (RA) and TGFβ, which can lead to Tr1 and FoxP3+ pTreg induction [21,22]. In addition, CD8α+ rDCs have also shown tolerogenic potential through TGFβ production, and targeting antigen to CD205 (DEC205), leading to clonal deletion [23] and Treg differentiation [24].

The TGFβ family comprises several structurally related proteins, including TGFβ, bone morphogenetic proteins (BMPs), activins and inhibins [25]. Inhibins and activins were first characterized as hormones [26] and are currently known to be involved in several immunological processes [27]. The canonical signaling pathway of this family is highly conserved and is shared among TGFβ, BMPs and activins. Briefly, dimeric ligands bind their serine/threonine kinase receptors (type I and II) and lead to phosphorylation of receptor SMADs, which heterodimerize with the common SMAD and translocate to the nucleus thereby regulating gene expression [28]. Several mechanisms have been proposed to explain the antagonistic effect of inhibins on activin-mediated functions (reviewed in [29]); inhibins are known to bind type II receptors through their β subunit and TGFβ type III coreceptor (TβRIII) through their α subunit, thus inhibiting the recruitment of type I receptor to the tertiary complex, interfering with SMAD-dependent signaling. Consequently, inhibins were considered non-signaling molecules; however, several reports support the possibility that inhibins may signal through a different receptor, which has not been identified to date (reviewed in [30]). This is supported by evidence showing that inhibins do not always antagonize activin functions. Specifically, inhibins and activins were shown to similarly control specific checkpoints during T cell development [31]; in addition, our group has shown that inhibins can regulate tTreg cell differentiation by controlling medullary/cortical thymic epithelial cell differentiation and DC maturation within the thymus [32]. Moreover, in recent work, we have demonstrated that the absence of inhibins in DCs results in an impaired maturation, characterized by low expression of major histocompatibility complex class II (MHC-II) and costimulatory molecules, as well as alterations in migration and, more importantly,
diminished ability to initiate T cell responses, such as in vitro proliferation of allogeneic CD4+ T cells and delayed-type hypersensitivity responses [33].

**Materials and methods**

**Mice**

Inhibin α heterozygous mice (Inhα+/−) in C57BL/6 background were donated by M. Matzuk (Baylor College of Medicine, Houston, TX, USA) and have been previously described [34]. FoxP3EGFP knock-in mice (B6.Cg-Foxp3tm2Tch/J), CD45.1 and OT-II transgenic mice in C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were intercrossed to generate Inhα+/−FoxP3EGFP, Inhα−/−FoxP3EGFP and CD45.1+OT-II+ mice. Mice were bred and maintained in the animal facility of the Instituto de Investigaciones Biomédicas (IIB, UNAM, México), in specific pathogen free conditions, according to ethics guidelines. The study was approved by the Comité para el Cuidado y Uso de Animales de Laboratorio (CICUAL) of the IIB. For all experiments, 4-week-old female mice were used.

**Preparation of lymphocyte suspensions from colonic lamina propria, mesenteric lymph node, peripheral lymph nodes or spleen**

Lymphocytes from colonic lamina propria (LP) were isolated using modified methods previously described [35]. Briefly, the gut was flushed with PBS, opened longitudinally and colon was cut into 5 mm pieces. The tissue was incubated in calcium- and magnesium-free HBSS containing 2 mM EDTA and 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. The remaining tissue was washed with PBS, and incubated for 30 min more at 37 °C in RPMI supplemented with 100 U·mL−1 penicillin and 100 μg·mL−1 streptomycin, and differentiated with granulocyte-monocyte colony-stimulating factor. After 5 days of culture, mature BMDCs (mBMDCs) were obtained after stimulation with 1 μg·mL−1 Escherichia coli 0111:B4 LPS for 24 h. At day 6, non-adherent cells were harvested, and CD11c+ cells were purified by magnetic-activated cell sorting and used for further experiments.

**Preparation of DCs from MLN, PLN or spleen**

DCs were obtained after collagenase digestion from MLN, PLN and spleen, as previously described [33]. Cells were resuspended in FACS buffer for phenotype analysis. CD11c+ magnetic-activated cell sorting-enriched DCs, lipopolysaccharide (LPS)-stimulated (mCD11c+) or not (iCD11c+), were used in the functional assays.

**Flow cytometry**

For phenotypic analysis, single cell suspensions were stained as previously described [36]. For ex vivo Treg cell analysis, anti-CD25-PECy5, anti-Helios-FITC, anti-CD8-PE (from Biolegend, San Diego, CA, USA), anti-CD4-APC-AF750 (from Thermo Fisher Scientific), and anti-FoxP3-APC (from eBiosciences, San Diego, CA, USA) were used. For in vitro induced Treg analysis, Zombie Aqua fixable dye, anti-CD4-APC and anti-CD25-PECy5 from Biolegend were used. For ex vivo DC analysis, cells were blocked with purified anti-CD16/32, followed by staining with Zombie Aqua, anti-I-A/I-E-AF488, anti-CD11c-PECy7, anti-CD80-PECy5 (from Biolegend), anti-CD3-PE, anti-TER119-PE, anti-CD11b-VF450, anti-CD86-APC, anti-CD8-PECy7 (from Tonbo Biosciences, San Diego, CA, USA), anti-CD19-PE, anti-CD49b-PE, streptavidin-APCCy7 (from BD Biosciences, San Jose, CA, USA), anti-CD103-biotin and anti-PD-L1-PerCP-eFluor710 (from eBiosciences) were used.

For in vivo transfer experiments, anti-CD45.1-PECy7, anti-CD4-ITC, anti-CD25-PECy5, streptavidin-BV605 (from Biolegend), anti-Vβ5-biotin (from BD Biosciences), and anti-FoxP3-APC (from eBiosciences) were used for staining ovalbumin (OVA)-specific T cells.

Samples were acquired in an Attune Acoustic Focusing Flow Cytometer (Thermo Fisher Scientific) and analyzed using FLOWJO 10.0 software (Tree Star Inc., Ashland, OR, USA).

**Generation of bone marrow-derived DCs**

Bone marrow derived DCs (BMDCs) were obtained from femurs and tibias of mice, as previously described [33]. Cells were resuspended in RPMI supplemented with 10% FBS, 100 U·mL−1 penicillin and 100 μg·mL−1 streptomycin, and differentiated with granulocyte-monocyte colony-stimulating factor. After 5 days of culture, mature BMDCs (mBMDCs) were obtained after stimulation with 1 μg·mL−1 Escherichia coli 0111:B4 LPS for 24 h. At day 6, non-adherent cells were harvested, and CD11c+ cells were purified by magnetic-activated cell sorting and used for further experiments.

**Treg cell induction**

For in vitro cultures, naïve CD4+CD25−CD44lowCD62Lbi-FoxP3-GFP+ T cells were sorted from spleen and PLN from FoxP3EGFP mice and cocultured with either CD11c+ BMDCs or spleen and PLN CD11c+ DCs, at different DC : Tnaïve ratios (1 : 1, 1 : 2, 1 : 4, 1 : 10, 1 : 20). Cultures were stimulated with 0.1 μg·mL−1 anti-CD3 (Tonbo) and 0.25 ng·mL−1 TGFβ (R&D systems, Minneapolis, MN, USA). Expression of FoxP3 and CD25 was evaluated after 5 days by flow cytometry.

For in vivo peripheral Treg induction, CD4+CD25+ T cells were sorted from PLN and spleen of OT-II × CD45.1
mice; 4 × 10^6 cells were transferred intravenously to CD45.2 Inhα^+/+ or Inhα^−/− mice. After 24 h, intradermal immunization with anti-DEC205-OVA, anti-DEC205-OVA+cholera toxin (CT), OVA or OVA+CT was performed in the mouse ears. Seven days after immunization, pTregs were analyzed as CD25^+FoxP3^+ within the population of transferred OT-II cells (CD4^+CD45.1^+VP5^−) in single cell suspensions obtained from draining lymph nodes (dLN).

**Statistical analysis**

Data are presented as means ± SEM. The significance of results was calculated by paired or unpaired, one or two-tailed Student’s t test, utilizing Prism 6 statistical software (GraphPad Software, La Jolla, CA, USA). P values < 0.05 were considered as statistically significant. P values > 0.05 and < 0.1 were considered as trends.

**Results and discussion**

Peripheral Tregs are increased in the absence of inhibins

To investigate whether inhibins play a role in the induction of Tregs in the periphery, we first evaluated Treg cell subpopulations from the Inhα^−/− or Inhα^+/+ mice. Inhα^−/− is an α subunit null mouse where neither inhibin A nor inhibin B can be synthesized [34]. As shown in Fig. 1, in the absence of inhibins, the numbers of CD25^+FoxP3^+ Tregs were significantly increased in PLN, specifically those Tregs expressing Helios, which correlates with our previous report showing enhanced tTreg development in Inhα^−/− mice [32]. However, when we evaluated Treg subpopulations in MLN and colonic LP, we found an increased frequency of CD25^+FoxP3^+Helios^− Tregs which, under homeostatic conditions, are considered pTregs [37]. These data suggest that inhibins regulate de novo generation, maintenance or recruitment of pTregs in the gut mucosa under homeostatic conditions. As the gut microenvironment provides a continuous stimulation from commensal bacteria and dietary antigens, this mucosa is particularly prone to tolerance induction by means of production of anti-inflammatory cytokines (IL-10, TGFβ), which promote Tr1 and pTreg conversion, while the production of RA by CD103^+ DCs induced FoxP3 expression and gut homing molecules CCR9 and α4β7 integrins, which retain Tregs in the intestinal mucosa [10]. Indeed, experiments using 'depletion of regulatory T cell' (DEREG) mice revealed that the constitutive presence of Tregs is required for the prevention of autoimmune inflammation and colitis [38].

Mesenteric Inhα^−/− CD103^+ DC display increased levels of PD-L1

We have recently reported that, in the absence of inhibins, DCs showed impaired maturation after in vitro LPS stimulation, which correlated with reduced capacity to induce CD4^+ T cell proliferation in vitro and lower delayed-type hypersensitivity responses in vivo [33]. This ‘semi-mature’ phenotype has been associated with the ability of DCs to promote tolerogenic responses including FoxP3^+ Treg generation [39]. To understand whether the increased pTregs observed in MLN and LP of Inhα^−/− mice were related to differences in MLN DC subpopulations, we analyzed the frequency and phenotype of DC subpopulations as shown in Fig. S1. We analyzed resident and mDCs, based on their expression of MHC-II and CD11c, as CD11c^hiMHC-II^hi and CD11c^loMHC-II^bi, respectively. To further analyze DC subsets, we used CD8α to discriminate CD8α^+ and CD8α^− rDCs, and for mDCs we used CD11b and CD103 to discriminate the following subpopulations: CD103^+CD11b^+, CD103^−CD11b^+ and CD11b^−CD103^+. A minor subpopulation, CD11b^−CD103^−, can also be observed; however, this subset has not been further characterized [40]. Frequency and numbers of DC subsets analyzed were not altered in the absence of inhibins (not shown); however, Inhα^−/− DCs in MLN showed a diminished expression of MHC-II in all DC subsets (Fig. 2A, upper graphs), similarly to our previous report showing lower MHC-II expression on Inhα^−/− epidermal Langerhans cells [33]. Interestingly, when we evaluated the expression of costimulatory/inhibitory molecules in MLN DC subsets we found a significantly increased expression of the co-inhibitory molecule PD-L1 in CD8α^+ rDCs and in CD103^+CD11b^- mDCs and a trend towards an increase of PD-L1 in CD103^−CD11b^− mDCs from Inhα^−/− mice. These CD103^+ DC subpopulations have been reported to play a key role in tolerance induction in the gut, as they produce high levels of RA and TGFβ, which are key mediators of FoxP3 induction in the intestinal microenvironment [41,42]. In fact, it has been previously shown that CD103^+CD11b^+PD-L1^hi DC are high inducers of pTregs [43], in agreement with the reported effect of PD-L1 during Treg conversion from naïve T cells by immature DCs in vitro [44]. Despite the lower expression of MHC-II, we observed an increase in CD80 and CD86 in CD8α^+ rDCs and CD103^−CD11b^- mDCs. This context, CD80 and CD86 do not exclusively act as costimulatory molecules, as they can bind coinhibitory receptors such as CTLA-4 and PD-L1 with high affinity,
favoring tolerance induction, by competing with costimulatory receptors (CD28) for T cell activation and inhibiting T cell proliferation [45,46]. Interestingly, a recent report has shown that expression of PD-L1 can bind CD80 in cis on the same cell, blocking the binding of CD80 to its ligand [47]. Therefore, coexpression of these molecules in vivo could promote a tolerogenic response.

As we observed an increase in ‘tolerogenic’ DCs in MLN of Inhα−/− mice, we next evaluated whether spleen DCs were prone to differentiate into tolerogenic DCs in the absence of inhibins. As shown in Fig. 2B, LPS-stimulated ex vivo Inhα−/− CD11c+ splenic DCs showed decreased upregulation of MHC-II and CD80 in comparison with Inhα+/− counterparts (Fig. 2B). In summary, the tolerogenic phenotype of Inhα−/− DCs may explain the enhanced pTreg generation in MLN. Alternatively, we cannot exclude an intrinsic effect of inhibins on T cells, since Inhα−/− T cells appear to express different levels of TβRIII compared to Inhα+/+ T cells in response to TCR stimulation (S. Ortega-Francisco, M. de la Fuente-Granada, R. Olguín-Alor, L. C. Bonifaz & G. Soldevila, manuscript in preparation). In this context, TβRIII acts as a coreceptor that potentiates TGFβ-mediated signals [48] and most recently, our group has shown that it promotes Treg induction in vitro [36].

Fig. 1. Tregs are incremented in the periphery in the absence of inhibin. Inhα+/+ and Inhα−/− mice were analyzed for Tregs (CD4+CD25+FoxP3+), thymic (Helios+) or peripheral (Helios−). (A) Gate strategy for Treg analysis. (B) Frequency (top) and number (bottom) in colonic lamina propria (LP) (left), mesenteric lymph node (MLN) (center), and peripheral lymph node (PLN) (right). Mean ± SEM, n = 3–5 mice. Statistical significance was determined by two-tailed unpaired Student’s t test. *P ≤ 0.05.
Inhibins regulate DC-mediated induction of Tregs in vitro

Naïve T cell differentiation towards an effector or regulatory phenotype requires several signals derived from the interaction between the T cell and the antigen presenting cell, including TCR–MHC, costimulation/coinhibition and cytokine mediated signals (reviewed in [21]). Since MHC-II, CD80 and PD-L1 are altered in Inhα+/+ DCs, we next investigated whether inhibin expression by DCs could impact in vitro Treg conversion. In respect to this, we have previously reported that BMDCs express significant levels of inhibin A in response to LPS stimulation [33]. As expected, Inhα+/- did not produce detectable levels of inhibin A in response to LPS stimulation [33].
These differences may be in part explained by the upregulation of PD-L1 and the ‘semi-mature’ phenotype found in LPS-stimulated Inhα−/− BMDCs [33]. The enhanced Treg conversion was accompanied by an increased CD25 and FoxP3 expression (Fig. 3B), suggesting that these induced Tregs might present an increased suppressive function [49]. In this context, we have observed that total FoxP3+ Tregs purified from Inhα−/− mice show increased suppressive activity towards polyclonally activated CD4+ T cells, in correlation with higher CD25 expression (data not shown). Moreover, LPS-stimulated CD11c+ DCs (mCD11c+) from spleen and PLN of Inhα−/− mice cocultured with naive T cells, in the presence of suboptimal anti-CD3 crosslinking and TGFβ, also induced a significantly higher generation of Tregs in vitro compared to their Inhα+/+ counterparts, indicating that Inhα−/− DCs have an intrinsic enhanced capacity to promote peripheral T cell tolerance (Fig. 3C). No differences in the expression levels of CD25 or FoxP3 were observed between in vitro induced FoxP3+ Tregs in the presence of Inhα−/− DCs compared to WT DCs (Fig. 3D).

**Inhα−/− DCs enhance the induction of pTregs in vivo**

To analyze the relevance of these findings in vivo, we used a strategy to directly deliver antigen to DCs, using anti-DEC205-OVA (α-DEC-OVA) DC targeting
and evaluated the response of adoptively transferred OT-II (OVA specific) TCR transgenic T cells. This system has been reported to generate either tolerogenic or immunogenic responses, depending on the adjuvant used during the α-DEC205 targeting [23]. Specifically, the use of CT as adjuvant induces effective Th1 and Th17 responses after intradermal immunization [23], while in the absence of adjuvant, α-DEC205 antigen targeting promotes a tolerogenic response, by a mechanism that involves FoxP3+ Treg generation [51].

CD4+CD25+OT-II+CD45.1+ naïve T cells were transferred intravenously to Inhα+/− or Inhα+/+ CD45.2+ mice, and 24 h later they were immunized in the ear with soluble OVA or OVA-targeted to DC through DEC205 (α-DEC-OVA), either with or without CT as adjuvant. Analysis of T cell responses in the dLN showed that immunization with OVA+CT resulted in a lower percentage and total numbers of transferred OVA-specific (Vb5+) CD4+ T cells in Inhα−/− recipient mice compared to Inhα+/+ (Fig. 4A,B), suggesting that inhibins may regulate CD4+ T cell expansion, through the modulation of MHC-II and costimulatory/coinhibitory molecules. Furthermore, we found a significant increase in the number of OT-II+CD45.1+FoxP3+ pTregs in Inhα−/− mice immunized with α-DEC-OVA compared to Inhα+/+, while Inhα−/− mice immunized with α-DEC-OVA+CT showed a trend towards an increase in the number of pTregs compared to the Inhα+/+ counterparts, indicating that Inhα−/− DCs are more prone to induce a tolerogenic response in vivo even in the presence of adjuvant.

The fact that PD-L1 is upregulated in the absence of inhibins suggests that they could be a target to prevent tolerance induction in clinical protocols destined to boost the immune response, as PD-1 blockage had been shown effective in anti-tumor immunotherapy (reviewed in [52]). In contrast, engagement of the PD-L1/PD-1 coinhibitory pathway is important for controlling several autoimmune diseases (reviewed in [53]). Therefore, to understand how the expression of this coinhibitory molecule can be regulated is crucial for future clinical approaches.

In summary, our data demonstrate that inhibins regulate peripheral T cell tolerance by directly restraining pTreg generation in vivo through modulation of DC function. Our results are relevant for immunotherapy, identifying inhibins as new potential targets
for immune intervention. By enhancing or blocking their effects, it would be possible to promote immunogenic or tolerogenic responses in different pathological settings.

Acknowledgements

We thank Dr Juliana Idoyaga (Stanford University School of Medicine, Stanford, CA, USA) for providing the α-DEC-OVA antibody. We also thank Oscar Hernandez and Luis Daniel Ferrer for technical assistance in mice genotyping. Finally, we would like to thank Carlos Castellanos, Erick Espindola and Dr Andrea Bedoya from the LABNALCIT-UNAM (CONACYT) for the technical support in the sorting of flow cytometry samples. This work was supported by DGAPA PAPIIT UNAM (IN209615). MF-G, RO-A and SO-F were students of the PhD program ‘Programa de Doctorado en Ciencias Bioquímicas’ UNAM, and supported by fellowships #344606, #339206 and #389761, respectively.

Author contributions

GS conceived and designed the project. MF-G, RO-A and SO-F performed the experiments. GS, LCB and MFG wrote the paper. LB provided reagents. RO, SOF and LB critically reviewed the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Ex vivo analysis of DC subpopulations in MLN. Gating strategy to define DC subsets in MLN. Within the cells suspensions, CD19−CD3−TER119−NK1.1− single live cells were selected for further analysis. The CD11cMHC-II population represents lymphoid rDCs and can be further divided into CD8α+ and CD8α− DCs. CD11cMHC-II population represents mDCs, which can be further divided into CD103+CD11b−, CD103−CD11b+, CD11b−CD103− and CD11b−CD103+.

**Fig. S2.** Inhibin A is produced by wild-type DCs upon LPS stimulation but not by inhibin-deficient (Inhα+/−) DCs. Time course of inhibin A from supernatants of wild-type (Inhα+/+) or Inhα−/− BMDC cultures were quantified by ELISA. Detection limit of the ELISA kit is represented by a blue line.