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Normal development and function of dendritic cells in mice lacking IDO-1 expression

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ABSTRACT

Dendritic cells (DCs) have been shown to express the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO-1), a protein presently thought to exert dual and possibly contrasting effects on the immune response. Depletion of tryptophan and release of tryptophan catabolites have been shown to exert a tolerogenic influence on T cell responses, while the IDO enzymatic activity has been recently suggested to promote DC maturation. In this report, we have explored the putative role of IDO-1 in regulating DC biology by analyzing DC development and function from IDO-1 deficient mice. In keeping with previous observations, lack of IDO-1 expression was found to affect in vitro DC generation from bone mouse precursors cultured in the presence of GM-CSF. However, change in growth factor (Flt3L) and/or culture conditions (low-adherence vessels) abolished the difference observed between wt (wild type) and IDO-1-deficient, in vitro generated DCs. Moreover, IDO-1-deficient mice displayed a normal DC compartment in vivo, suggesting that IDO-1 does not play a significant role in DC development and function in vivo. Collectively, these observations suggest that despite a possible role for IDO-1 expression in regulating DC differentiation in vitro under commonly used culture conditions, IDO-1 is largely dispensable for DC development and function in vivo.

1. Introduction

Dendritic cells represent a heterogeneous population known to play a central role in both innate and adaptive immunity. These cells were originally characterized by their unique ability to activate naive T cells and hence initiate an adaptive immune response. Immature DCs are present in peripheral tissues, where they continuously capture and present exogenous antigens in association with MHC-encoded molecules. In response to a microbial or inflammatory stimulus, DCs undergo a complex differentiation program (termed maturation) characterized by the ability to relocate to T cell rich areas of lymphoid organs and to express co-stimulatory molecules required for optimal activation of naive T cells [1–3].

The role of DCs in the regulation of adaptive immunity appears however more complex than previously anticipated. It has been indeed recently recognized that in addition to promote immune responses, DCs can also play a role in tolerance induction [4]. In keeping with the notion that TCR engagement without adequate co-stimulation delivers a tolerogenic signal to naive T cells, resting immature DC have often been found to induce T-cell deletion and/or unresponsiveness, suggesting a "default" tolerogenic program by DCs that acquire antigen in the absence of infection and/or injury [5–7]. Moreover, a series of recent observations indicate that DC can also respond to tolerogenic signals and differentiate into antigen-presenting cells able to actively downregulate an ongoing immune response [8]. Immunosuppressive mechanisms displayed by tolerogenic DCs comprise secretion of anti-inflammatory cytokines such as IL-10, and expression of cell surface molecules of the B7 family able to interact with T-cell borne receptors (such as CTLA4 or PD-1) known to negatively affect antigen responsiveness [8–10].

A recent mechanism thought to be exploited by DCs to downregulate T-cell responsiveness involves indoleamine 2,3-dioxygenase (IDO-1), a tryptophan-catabolizing enzyme [11–13]. This enzyme is ubiquitously expressed in non-hepatic tissues, including lymphoid organs. Expression of IDO-1 has been mostly found in cells of the innate immune system, such as in macrophages and dendritic cells [11]. Depending on the cell type, IDO-1 expression can be constitutive, or highly induced by pro-inflammatory

Abbreviation: BMDC, bone marrow-derived dendritic cells.

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soluble factors (IFN-γ, TNF-α or PGE_2), TLR ligands (poly-I:C, LPS or CpG DNA) or co-stimulatory ligands such as CTLA4 [11,14,15]. The observation that IDO-1 expression is induced upon infection is compatible with the proposed role of this enzyme in limiting proliferation of pathogens by depleting an essential amino acid from their microenvironment [16–18]. A similar biostatic effect has been invoked to explain the immunoregulatory role of IDO-1 expressing cells during pregnancy and inflammation [19,20]. Both animal and human studies have demonstrated the immunosuppressive role of IDO-1-expressing cells in diverting T lymphocyte responses toward tolerance [21,22]. Several independent studies also suggested that in addition to its ability to deplete tryptophan from the extracellular milieu, IDO-1 expressing cells can also suppress immune responses by secreting/releasing several tryptophan metabolites (including kynurenine, 3-hydroxy-kynurenine, 3-hydroxyanthranilic and quinolinic acid), able to induce T-cell apoptosis in vitro [23–26]. Increased IDO-1 expression during inflammation is therefore thought to represent both a defense mechanism against pathogen proliferation, and a regulatory mechanism contributing to the resolution of inflammation through the active inhibition of T-cell responses.

Two recent reports examining the role of IDO-1 on human dendritic cells have revealed an additional role for this enzyme in regulating DC maturation in response to selected TLR ligands [27,28]. Both studies independently demonstrated the ability of 1-methyl tryptophan (1MT), a pharmacological competitive IDO inhibitor, to inhibit DC maturation in vitro after LPS stimulation. A positive role for IDO in DC maturation was also confirmed in one study using an additional IDO inhibitor and an IDO-1 knock-down approach [28]. Furthermore, tryptophan metabolites were found to increase maturation of LPS-treated DCs, providing a plausible mechanism whereby IDO-1 may promote DC maturation. Although pharmacological studies should be considered with caution, these reports suggest that IDO-1 expression by antigen-presenting cells may serve to both negatively regulate T-cell responses and promote DC maturation.

To directly evaluate the putative role of IDO-1 in regulating DC differentiation and function, we undertook a detailed analysis of DCs isolated from wild type and IDO-$1^{-/-}$ mice. We demonstrate in this study that although lack of IDO-1 expression may affect in vitro DC generation under conventional culture conditions, endogenous IDO-1 expression is dispensable for DC differentiation and maturation in vivo.

2. Results

2.1. Normal lymphoid compartment in IDO-1 deficient mice

Aged and sex matched wild type and IDO-1 knock-out mice were analyzed by flow cytometry to evaluate the composition of the major lymphoid organs including the spleen, thymus and lymph nodes. No effect of IDO-1 expression was found on T or B cells development, as judged by the expression of T (TcR, CD4 and CD8) and B (CD19, sIgD and sIgM) cell associated markers (Fig. 1). Notably, lack of IDO-1 did not influence nTregs development, a cell population characterized by the constitutive expression of CD25 and FoxP3 and

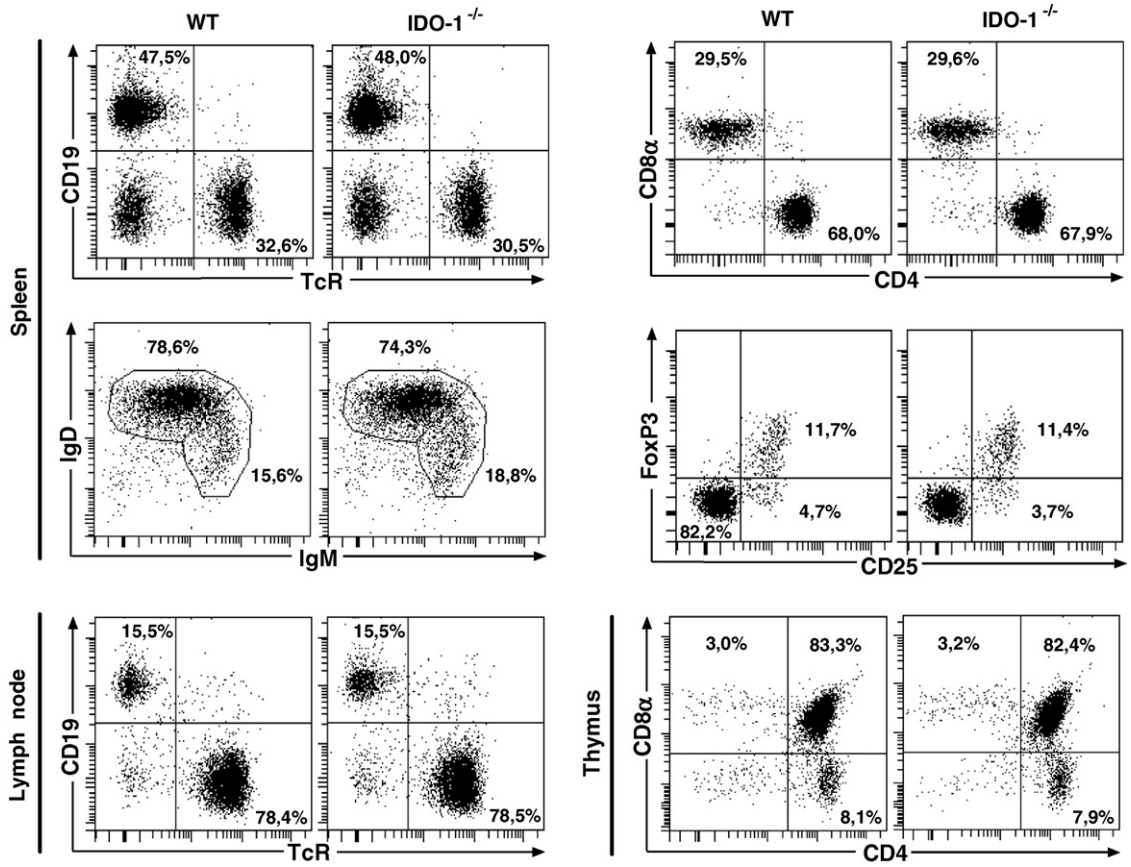

Fig. 1. Normal lymphoid development in mice with IDO-1 deficiency. Cell suspensions from spleens, lymph nodes and thymus were analyzed by multi colour flow cytometry using antibodies to T and B cell markers as indicated. Cells were gated on the basis of forward and side scatter, and data are expressed as percentage of cells expressing a given marker. This experiment is representative of three independent experiments in which a total of nine wt and nine IDO-$1^{-/-}$ mice were analyzed.

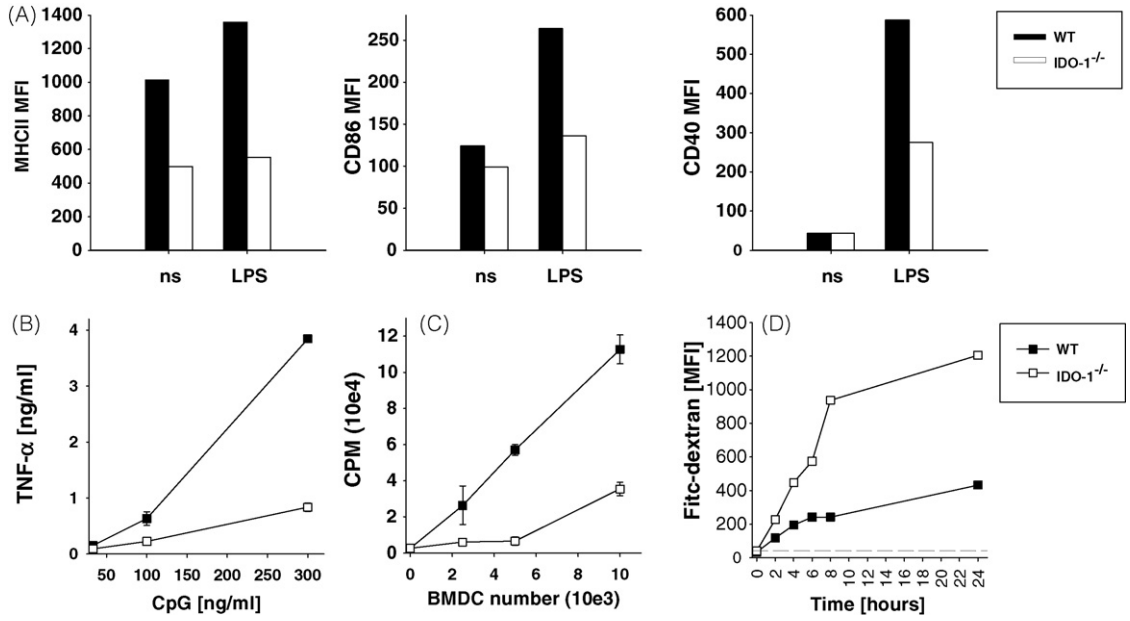

Fig. 2. IDO-1 expression is required for optimal generation of bone marrow-derived DC under standard culture conditions. (A) Bone marrow precursors were incubated in conventional culture vessels containing GM-CSF-supplemented media and analyzed by flow cytometry after 6 days. CD11c-positive cells were counterstained using antibodies to MHC II (I-E^d), CD86 and CD40 before and after exposure to LPS (1 μg/mL), and results are expressed as MFI (mean fluorescence intensity) of each marker as indicate on the y axis. GM-CSF-derived cells were assayed for their ability to produce cytokines (B), to stimulate naive T cells from OVA Tg D0.11.10 mice to proliferation in the presence of OVA (C), and capacity to internalize Fitc-Dextran (D) as indicated in Section 4. (A) is representative of more than 10 independent experiments while (B, C and D) are representative of three independent experiments.

known to be activated in the periphery in an IDO-1 dependent fashion [28–30]. Collectively, these observations suggest a limited role for IDO-1 during immune cell development.

2.2. Culture conditions affect the DC development from IDO-1-deficient bone marrow precursors

To directly evaluate the effect of IDO-1 deficiency on dendritic cell differentiation, bone marrow cells from wt and IDO-$1^{-/-}$ mice were cultured in vitro in the presence of recombinant GM-CSF. This well established culture procedure led to the in vitro differentiation of bone marrow-derived dendritic cells (BMDC) from both wt and IDO-$1^{-/-}$ precursors. Although similar numbers of CD11c-positive cells were derived from both mouse strain cultures, cells derived from IDO-$1^{-/-}$ displayed an immature phenotype when compared to wt BMDCs. In particular, IDO-$1^{-/-}$ BMDCs were characterized by reduced expression of maturation-associated markers such as MHC class II, CD86 and CD40 (Fig. 2A), suggesting a putative role for IDO-1 in regulating in vitro DC differentiation. Furthermore, IDO-$1^{-/-}$ BMDCs failed to adequately upregulate expression of these markers in response to LPS, in keeping with a putative role of IDO in regulating DC maturation in vitro (Fig. 2A). Similarly, IDO-$1^{-/-}$ BMDC failed to adequately respond to CpG by TNF-α secretion (Fig. 2B). IDO-$1^{-/-}$ BMDCs displayed in fact a typical immature phenotype, characterized by a low capacity to activate naive T-cell proliferation (Fig. 2C), associated with increased phagocytic activity (Fig. 2D).

Since bone marrow-derived cells that differentiate in the presence of GM-CSF are thought to represent a subset of monocyte-derived inflammatory cells [1,31], we repeated these experiments using Flt3L as a growth factor in vitro, a culture procedure known to generate a broader spectrum of dendritic cell populations. Much to our surprise, culture of wt and IDO-$1^{-/-}$ derived bone marrow precursors in the presence of Flt3L led to the development of comparable numbers of CD11c-positive cells without major phenotypical differences. As shown in Fig. 3B, Flt3L led to the in vitro differentiation of an equivalent number of immature $CD11c^+$ MHC II^+ BMDC, irrespectively of IDO-1 expression. As previously shown, GM-CSF-derived BMDC from IDO-1 deficient precursors were characterized by high numbers of $CD11c^+$ lacking MHC II expression (compare Fig. 3A and B). Notably, these GM-CSF-derived, IDO-1 deficient DCs displayed a more rounded phenotype and reduced adherence to the substrate (not shown), in keeping with a recent report suggesting a role for IDO-1 in regulating cell adherence [32]. To evaluate the putative role of the plastic surface in regulating BMDC differentiation in vitro, we repeated these experiments using low-adherence culture vessels (see Section 4). Surprisingly, no phenotypical differences were observed between wt and IDO-$1^{-/-}$ bone marrow derived DCs in the presence of both GM-CSF and Flt3L in this experimental setting (Fig. 3C and D). DCs derived from cultures performed under limited plastic adherence displayed a more immature phenotype, independently of IDO-1 expression and culture media compositions. Collectively, these experiments concur with previous observations suggesting a potential role for IDO-1 in regulating dendritic cell differentiation and function under selected culture conditions, but cast some doubts on the physiological role of IDO-1 in regulating DCs development and function in vivo.

2.3. Lack of IDO-1 expression does not impair DC development and maturation in vivo

To evaluate the role of IDO-1 in DCs differentiation in a more physiological setting, a detailed analysis of in vivo resident splenic DCs subpopulations was performed. No major differences were found in cell numbers and subpopulations among CD11c-expressing cells from wt and IDO-$1^{-/-}$ mice. When whole spleen cells from naive mice were analyzed, a similar number of CD11c-positive cells expressing high or low levels of CD11b was found in both wt and IDO-$1^{-/-}$ mice (Fig. 4). Analysis of low-density cells revealed a similar percentage of classically defined myeloid ($CD11c^{high}$ PDCA-1^-) and plasmacytoid ($CD11c^{low}$ PDCA-1^+) dendritic cells. Finally, no statistically significant differences were

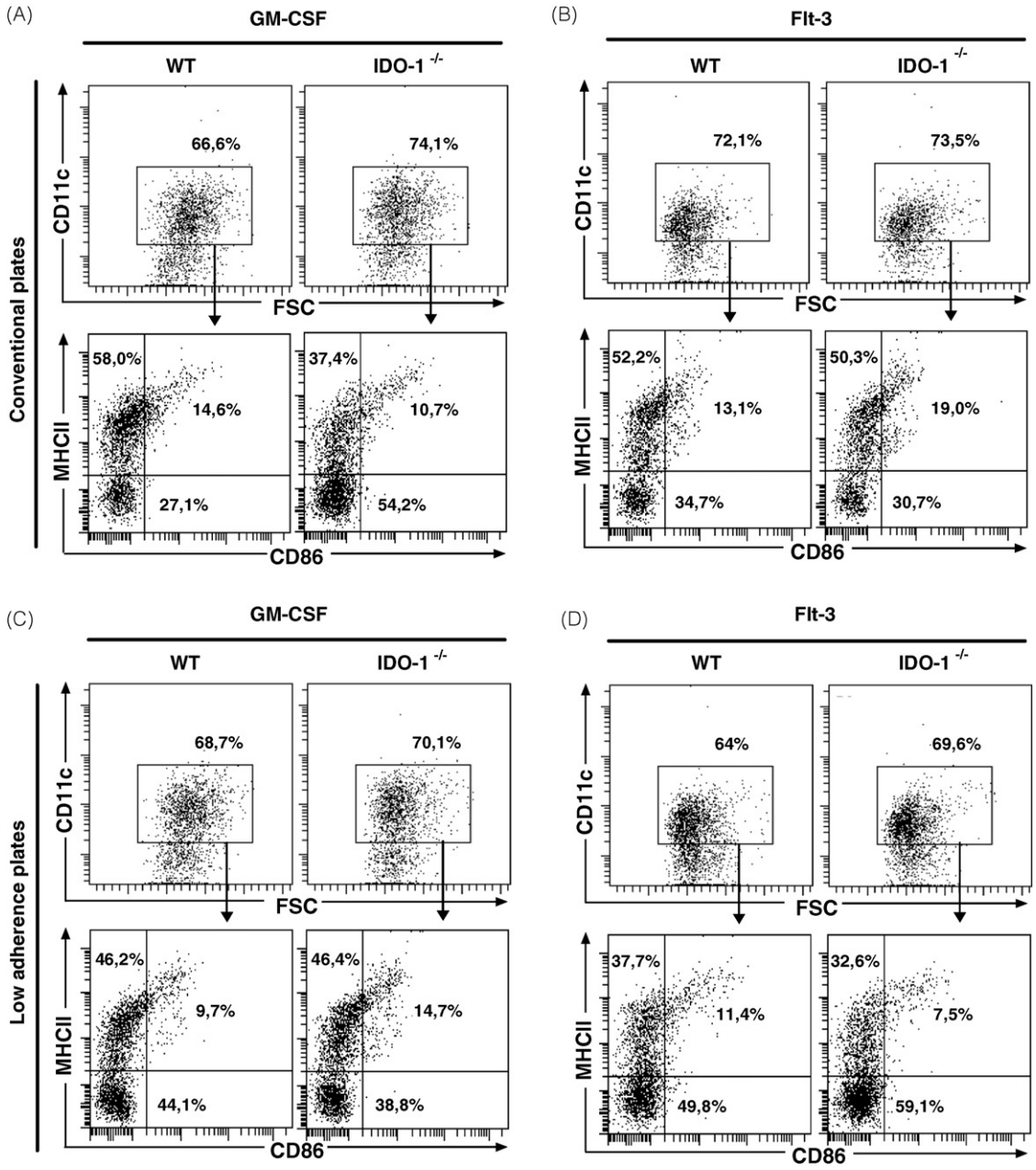

Fig. 3. Culture conditions affect the generation of DCs from IDO-deficient bone marrow precursors. Bone marrow precursors were incubated in conventional (A and B) or low-adherence (C and D) culture vessels in GM-CSF (A and C) or Flt3L (B and D) –supplemented medium. Cells were analyzed by flow cytometry after 6 days on the basis of CD11c expression as indicated. CD11c-positive cells were further counterstained using antibodies to MHC II ($I\text{-}E^d$) and CD86.

found when subsets of splenic resident $CD11c^{high}$ cells were analyzed using the CD4 and CD8α markers. In agreement with phenotypical analysis, IDO-1-deficiency did not appear to affect the ability of splenic DCs to stimulate naive T cells proliferation, as shown in Fig. 4B. To exclude a possible compensatory expression of IDO-2, a novel gene related to IDO-1 and encoding for an enzyme displaying a similar enzymatic activity, wt and IDO-$1^{-/-}$ splenic DCs were assayed for IDO-2 expression by qRT-PCR. Neither wt nor IDO-$1^{-/-}$ spleen DCs expressed detectable levels of this novel tryptophan catabolizing enzyme, while high levels of IDO-2 expression were found in the liver, as expected from previous reports (Fig. 4C) [33].

To circumvent any possible in vitro artefact, the ability of splenic dendritic cells to undergo maturation in response to LPS was assayed in vivo. Mice were injected with diluent or LPS, and low-density cells analyzed 6 h post-treatment for the expression of maturation-associated markers. Lack of IDO-1 did not affect in vivo DCs maturation as shown by the equivalent increase in MHC class II, CD80, CD86 and CD40 expression observed in both mouse strains upon in vivo LPS administration (Fig. 5A). Furthermore, TNF-α serum levels were assayed to evaluate the overall innate response to LPS. As shown in Fig. 5B, both mouse strains displayed a similar systemic inflammatory response, further strengthening the conclusion that IDO-1 deficiency has limited influence on the development of a competent innate immune system.

Finally, based on the commonly accepted view that in vitro GM-CSF-derived DC could represent a subset of monocyte-derived cells that differentiate into dendritic cells in an inflammatory

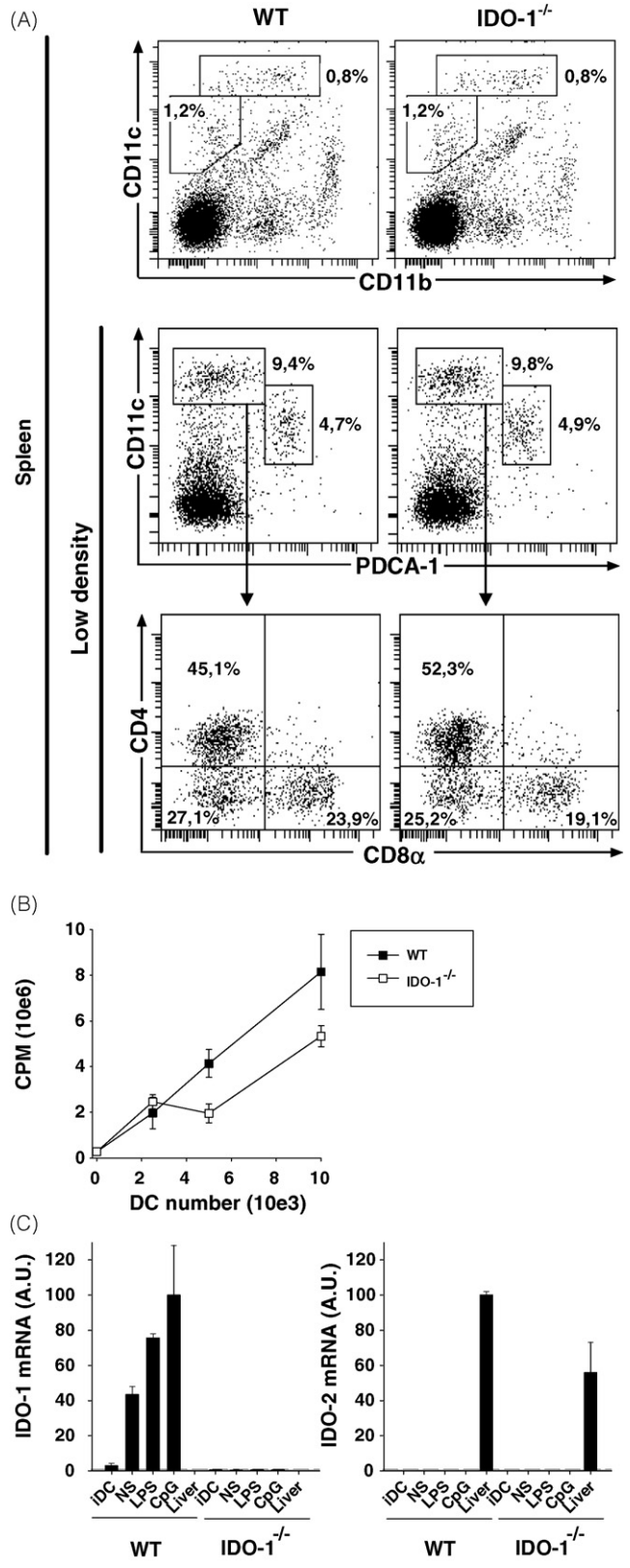

Fig. 4. (A) Development, phenotype and function of splenic DC are not affected by the lack of IDO-1 expression. (A) Total spleen cell suspensions or low-density cells from wt and IDO-1$^{-/-}$ mice were analyzed by flow cytometry for the expression of the DC-associated markers CD11c, CD11b and PCDA-1, as indicated. CD11c-positive cells were further counterstained with CD4 and CD8α-specific antibodies to identify selected DC subpopulations. Data are representative of four independent experiments, (B) graded numbers of splenic DCs were assayed for their ability to stimulate naive T from OVA Tg D0.11.10 mice to proliferation. Data are representative of two independent experiments. (C) Immature DC (iDC, purified ex-vivo as indicated in the methods section) and DC stimulated overnight in the presence of CpG (300 ng/mL) or LPS (1 μg/mL) were analyzed by qRT-PCR for IDO-1 and IDO-2 expression. Data were normalized to those of RPL32 mRNA and expressed as arbitrary units. Data for IDO-1 and IDO-2 are respectively representative of four and two independent experiments.

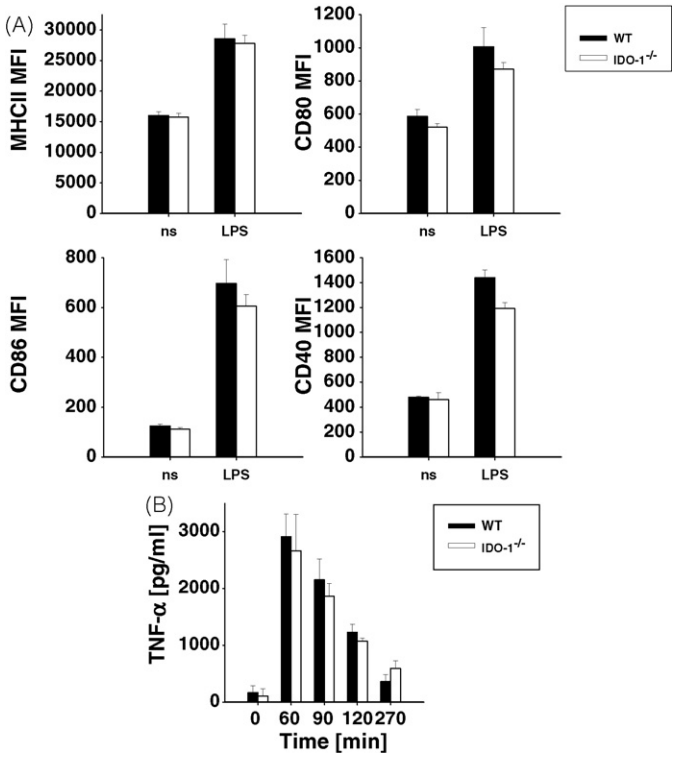

Fig. 5. IDO-independent in vivo DC maturation. IDO-1$^{-/-}$ and wt mice were injected intravenously with 25 μg of LPS or PBS. (A) 6 h after injection, low-density spleen cells were purified and analyzed by flow cytometry. DCs were identified as CD11c-positive cells, and further counterstained using the maturation-associated markers MHC II, CD80, CD86 and CD40. Results represent the mean of three wt and three IDO-1$^{-/-}$ mice analyzed individually and are expressed as the mean fluorescent intensity associated to each marker (B) A distinct group of mice were injected with 50 μg LPS and TNF-α serum levels determined by ELISA at the indicated time points. Results represent the mean and S.D. response from six mice assayed individually. These data are representative of four independent experiments.

environment [1,31,34], naive wt and IDO-1$^{-/-}$ mice were challenged subcutaneously with 10^6 metacyclic promastigotes of *Leishmania major*. As previously described, infection by this parasite led to the accumulation of MHC class II and Ly6C positive cells co-expressing the CD11c and CD11b markers [34], a hallmark of a subset of dendritic cells that differentiate in situ during an inflammatory response. As shown in Fig. 6, equivalent numbers of CD11c$^+$ CD11b$^+$ cells (expressing the MCH class II and Ly6C markers, not shown) accumulated in the draining lymph nodes of infected mice from both strains, suggesting that IDO-1 does not regulate in vivo development of this novel inflammatory DC subset.

3. Discussion

Elucidation of the regulatory pathways governing dendritic cell maturation is of fundamental and clinical relevance. In particular, the well described ability of DCs to activate naive T cells in vivo has led to the development of cell-based vaccines for cancer immunotherapy [35]. Optimal efficacy of these vaccines relies on the ability of antigen-loaded DCs to efficiently migrate to the

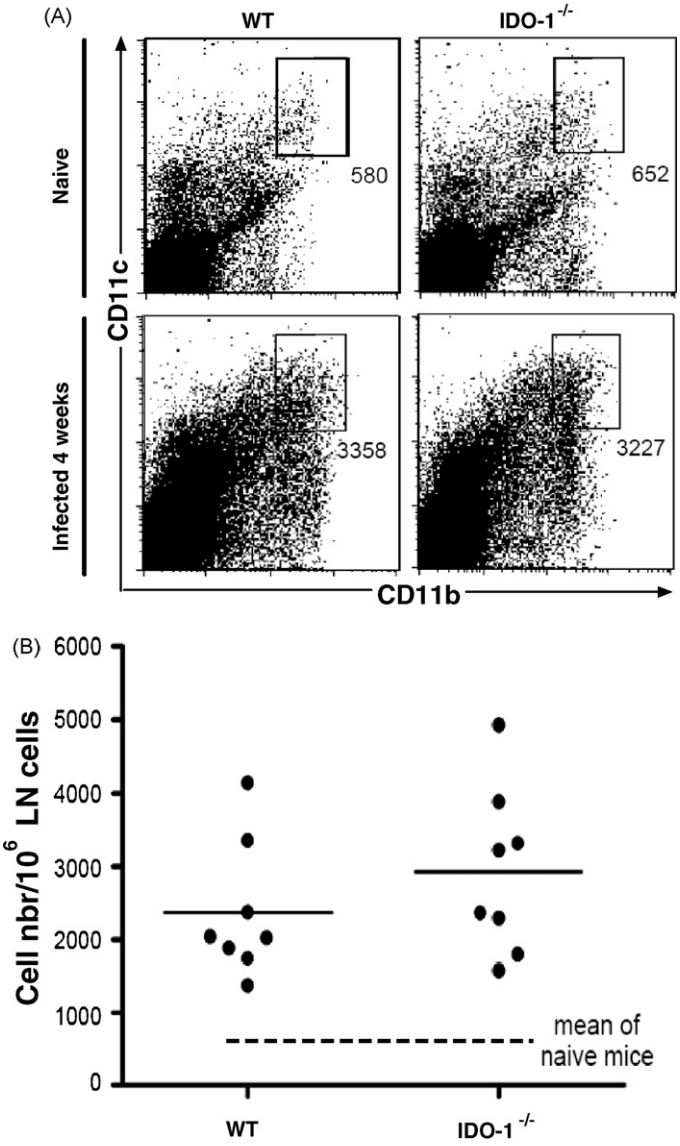

Fig. 6. Normal in vivo development of inflammatory DCs in mice lacking IDO-1 expression. wt and IDO1$^{-/-}$ mice were left untreated or challenged subcutaneously with 10^6 metacyclic promastigotes of L. major and lymph nodes suspensions analyzed by flow cytometry 4 weeks post-infection. The numbers of CD11c$^+$ CD11b$^+$ cells were determined based on the gate shown in (A) and were shown to significantly increase in infected vs naive animals. Individual determinations of CD11c$^+$ CD11b$^+$ cells present in draining lymph nodes are shown in (B), where the dotted line represent the levels of inflammatory DC in lymph nodes from naive animals. Data are representative of two independent experiments in which eight wt and eight IDO-1$^{-/-}$ mice were infected.

draining lymph node and to deliver adequate activation signals to naive T cells. Numerous studies have demonstrated that the quality of signals delivered to naive T cells depends on both the maturation status and the dendritic cell subset considered [1,3]. IDO-1, one of the rate-limiting enzymes initiating tryptophan catabolism is thought to play a central role in regulation of the activation vs tolerogenic properties of antigen-presenting cells [36]. IDO-1 is indeed expressed by cells of the innate immune system, including dendritic cells [11]. Expression of IDO-1 is upregulated in response to microbial stimulation, an observation that is easy to rationalize with a possible role of this "tryptophan-depleting" enzyme in limiting pathogen proliferation [16–18]. However, subsequent studies have suggested that IDO-1 expression may in fact represent a T-cell specific, tolerogenic mechanism used by DCs to selectively inactivate and/or deplete antigen-specific T cells [12,19–26]. This conclusion was based on the finding that T cells that require adequate level of extracellular tryptophan are sensitive to the pro-apoptotic activity of several tryptophan metabolites produced/released by IDO-1 expressing cells [23–26]. Despite some early support, the role of IDO-1 expressing DCs in mediating tolerance in a physiological setting remains to be firmly established. In particular, the level of expression and enzymatic activity of IDO-1 in several DC subsets is still a matter of debate. Human studies using in vitro generated DCs suggest that the majority of DCs express IDO-1 upon maturation [12,28], while studies performed in mouse models have led to the conclusion that IDO-1 expression is confined to a selected DC subpopulation expressing the CD19 marker [37].

The high expression of IDO-1 induced by TLR agonists is also compatible with a possible role for this enzyme in promoting DC maturation. In this scenario, IDO-1 expression would not represent a tolerogenic signature, but rather constitute an additional mechanism to promote acquisition of T-cell stimulatory capacity by dendritic cells. Support for this novel, "positive" role for IDO-1 in DC biology stems from several reports that have demonstrated the ability of pharmacological inhibitors of IDO-1 to downregulate human DC maturation in vitro [27,28,38]. The availability of IDO-1$^{-/-}$ mice led us to evaluate the possible role of this enzyme in regulating DC development and maturation both in vitro and in vivo.

The major conclusion from the present study is that lack of IDO-1 expression does not significantly impair DC development and maturation in vivo. IDO-1$^{-/-}$ mice displayed similar numbers of splenic dendritic cells at the steady state (see Fig. 4A and B). When stimulated in vivo by LPS according to a previously described protocol, both IDO-1-sufficient and IDO-1 defective cells acquire a mature phenotype characterized by upregulation of membrane-associated markers known to promote T-cell activation (MHC class II, CD80, CD86 and CD40). No evidence of a defective innate immune response was found in vivo, as suggested by their ability to produce TNF-α in vivo in response to LPS (see Fig. 5B).

Although the findings reported herein do not support a physiological role for IDO-1 in dendritic cell biology, they do not completely contradict previous publications performed in vitro using IDO specific pharmacological inhibitors [27,28,38]. In fact, our study extends those findings to a mouse model, and may shed some light on the process of dendritic cell development in vitro. Culture of bone marrow precursor cells lacking IDO-1 expression under conventional conditions and using GM-CSF-supplemented media, led to the accumulation of a typical "immature", CD11c-expressing, dendritic cell population. In keeping with this conclusion, these cells expressed low levels of T cell-stimulatory markers and retained a high phagocytic activity. In contrast, IDO-1-sufficient precursors led to the differentiation of cells expressing higher levels of MHC class II and decreased capacity to internalize antigen. Although these observations appeared to support a role for IDO-1 in regulating DC maturation, further examination of cultures led us to consider a possible interference between IDO-1 expression and plastic adherence, in keeping with a previously published study [32]. Much to our surprise, DCs derived using culture conditions minimizing cell adherence displayed identical phenotype and functional characteristics between wt and IDO-1$^{-/-}$ cells. Both cell populations displayed a similar, low adherent phenotype, and responded equally well to TLR agonists in vitro (data not shown). Moreover, no role for IDO-1-expression was found when DC was derived in the presence of Flt3L, further stressing the conclusion that the role of IDO-1 in DC development is strongly dependent upon culture conditions.

In vivo studies were compatible with the conclusions drawn from in vitro analysis performed using low adherent culture substrates and strongly suggested that IDO-1 does not play a major role in DC development and maturation. In particular (i) no differences were observed when wt and IDO-1$^{-/-}$ DC were derived in Flt3L-supplemented media, under both adherent and non-adherent culture conditions (see Fig. 3B and D); (ii) mice lacking IDO-1 expression display an adequate numbers of resident, splenic dendritic cells comprising all major DC subsets identified to date (see Fig. 4A); (iii) the in vivo maturation response to LPS appears as IDO-1 independent (see Fig. 5A) and finally (iv) in vivo infection of both wt and IDO-1$^{-/-}$ mice led to the similar accumulation of TNF-α-producing DCs (see Fig. 6B), an inflammatory DC subset thought to represent the in vivo counterpart of the in vitro, GM-CSF derived, DC subset [1,31,34].

The observations that IDO-1$^{-/-}$ cells display an altered in vitro development recapitulate the observations described by several independent laboratories indicating a possible role for the enzymatic activity of IDO-1 in regulating human DC maturation in vitro [27,28,38]. Collectively, these studies and ours suggest a possible role for IDO-1 in regulating DC maturation in vitro through interference with cell adhesion, an observation that may be of relevance for the development of in vitro culture conditions leading to the expansion and accumulation of high numbers of monocyte-derived DC suitable for immunotherapy. The present study reinforces however the notion that caution should be taken in interpreting in vitro studies and observations obtained using pharmacological inhibitors.

Finally, the concept that IDO-1 expression by tumour cells may represent an immune escape mechanism has received experimental support [39–41]. Accordingly, in vivo administration of pharmacological IDO-1 inhibitors has been shown to enhance immune therapy against established tumours in mouse models [39], and this strategy is presently considered of clinical relevance. The observation that IDO-1 expression is not required for adequate DC maturation in vivo (this study) strongly suggest that this enzyme may represent a valuable target for enhancing in vivo immune responses without interfering with the DC maturation process.

4. Materials and methods

4.1. Mice and in vivo treatments

Balb/C mice were purchased form Harlan (Horst, Nederland). IDO$^{-/-}$ mice in the Balb/C background were generated as previously described [42] and kindly provided by A. Mellor (Medical College of Georgia, GA). All mice, including OVA-specific TcR Tg DO11.10 mice, were bred in our pathogen-free facility and used at 6–9 weeks of age. All experiments were performed in compliance with the relevant laws and institutional guidelines and have been approved by the local committee from the Institut de Biologie et Médecine Moléculaires from the Université Libre de Bruxelles (Gosselies, Belgium). When indicated, mice were infected subcutaneously in the rear left hind footpad with 1×10^6 stationary-phase promastigotes of *L. major* in a final volume of 25 μl as previously described [43].

4.2. Dendritic cell culture

BM cells were isolated by flushing femurs with PBS and resuspended in Tris-ammonium chloride at room temperature for 1 min to lyse RBC. Two distinct protocols were used to generate DCs from bone marrow precursors. Flt3L-derived DCs were obtained by culturing BM cells (10^6 cells/mL, at 37 °C in a humidified atmosphere containing 10% CO_2) for 9 days in IMDM supplemented with essential and non-essential amino acids, 1 mmol/L sodium pyruvate, 2.5 mmol/L HEPES buffer (pH 7.4), 5.5×10^{-5} mol/2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.3 mg/mL L-glutamine (PSG), 10% FCS and 200 ng/mL of recombinant murine Flt3L as previously described [44]. Alternatively, BM cells were cultured at 3×10^5 cells/mL (at 37 °C in a humidified atmosphere containing 5% CO_2) for 6 days in RPMI 1640, 10% FCS, 1% L-glutamine, non-essential amino acids, 1 mmol/L sodium pyruvate in the presence of 20 ng/mL GM-CSF (kindly provided by K. Thielemans, VUB, Belgium). Cells were harvested at the end of the culture period after removal of non-adherent cells.

4.3. In vitro bone marrow-derived dendritic cell and dendritic cell maturation

After 6 days culture for BMDC cultured in GM-CSF and 9 days for BMDC derived in Flt3L, cells were collected, centrifuged and resuspended in fresh medium. Cultures were performed in conventional 24-well tissue culture plates (experiments shown in Figs. 2 and 3) or 24-well Ultra Low Attachment plates (Costar 3473, Corning, depicted in Fig. 3) as indicated. Cells were induced to mature by overnight culture at 5×10^5 cells/mL in the presence of 1 μg/mL LPS or 300 ng/mL CpG-ODN 1826 (Eurogentec, Seraing, Belgium). DC maturation was evaluated by analyzing the cell surface expression level of MHC II and CD86 on CD11c positive cells by flow cytometry.

4.4. Cells isolation

CD4$^+$ T cells were purified from naive animals by magnetic depletion of B cells, macrophages, dendritic cells, NK cells, granulocytes, erythroid precursors and CD8$^+$ T cells, as previously described [45]. The percentage of purified cell fractions in all experiments ranged between 90% and 98%, as estimated by flow cytometry.

CD11c$^+$ dendritic cells were purified from naive animals by magnetic positive selection. Briefly, spleen cells were digested with 200 U/mL collagenase type 3 (Worthington Biochemicals, New Jersey, USA) in HBSS with Ca^{2+} and Mg^{2+} for 30 min at 37 °C. The spleens were then mashed and further dissociated in Ca^{2+}-free medium in the presence of 2 mmol/L EDTA. Cells were separated into low- and high-density fraction on a Nycodenz gradient (Nycomed, Oslo, Norway). Immature splenic DCs were further purified by positive selection according to CD11c expression by incubation with anti-CD11c-coupled microbeads followed by one passage over a MACS column (Miltenyi Biotec, Bergisch-Gladbach, Germany). The CD11c-positive cells were cultured in RPMI 1640 containing 2% Ultroser HY (Life Technologies, Paisley, Scotland). The percentage of purified cell fractions in all experiments ranged between 90% and 98%, as estimated by flow cytometry.

4.5. FITC-dextran phagocytosis

Fluorescein isothiocyanate (FITC)-dextran (Sigma) was added to a final concentration of 1 mg/mL. Endocytosis of the tracer was halted at the indicated time points by rapid cooling of the cells on ice. The cells were then washed twice with ice-cold HBSS. The fluorescence intensity of the cells was analyzed by flow cytometry. Incubation of cells with the endocytic tracer on ice was used as a background control. The mean fluorescence intensity (MFI) represented the amount of incorporated tracer by CD11c positive cells.

4.6. Antibodies and cytofluorometric analysis

Single-cell suspensions were incubated for 30 min at 4 °C with saturating doses of 2.4G2 (a rat anti-mouse Fc receptor MAb; ATCC) and specific antibodies in staining buffer (0.5% BSA in PBS/NaN3)

before analysis on a FacsCanto II flow cytometer (Becton Dickinson, Mountain View, CA). Intracellular staining was performed in accordance with the manufacturer's protocol (eBioscience). The following antibodies were used for staining: FITC-conjugated CD8α (53–6.7), IgD (11–26), CD11b (M1/70), MHC II (14.4.4) and CD25 (PC61.5); PE-conjugated IgM (eB121-15F9), CD86 (GL-1), Foxp3 (FJK-16s); biotinylated CD19 (MB19-1), PDCA1 (440c7); APC-conjugated TcRβ (H57-597), CD11c (N418), CD4 (GK1.5) and PeCy7-conjugated streptavidin, all purchased from eBioscience, except PDCA1 purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany). The cells were gated based on characteristic forward and light scatter to eliminate dead cells and debris from analysis.

4.7. Measurement of $CD4^+$ T-cell priming capacities by BMDC and DC in vitro

DCs and BMDCs were purified and generated as described above. For proliferation assay, naive T cells from the TcR Tg DO11.10 mouse strain at 5×10^4 cells/well were seeded into 96-U-well plate and cultured with varying numbers of DCs or BMDCs in RPMI 1640 supplemented with additive in presence of OT-II OVA peptide (chichen OVA peptide 323–339 ISQAVHAAHAEINEAGR) purchased from Neosystem, Strasbourg, France. Cultures were maintained for 48 h at 37 °C. The proliferation was assayed by pulsing the cells with [^{3}H] thymidine overnight and harvested the next day.

4.8. Quantitative RT-PCR

Immature splenic dendritic cells were induced to mature by overnight culture at 5×10^5 cells/mL in the presence of 1 μg/mL LPS or 300 ng/mL CpG-ODN 1826. Total RNA was extracted using TRIzol reagent (Invitrogen, MD) and was primed with oligo (dT) for first-strand cDNA synthesis (M-MLV Reverse transcriptase, Invitrogen, MD) according to the manufacturer's instructions. QPCR core kit for SYBR Green (Eurogentec, Belgium) and GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, CA) were used for real-time PCR. The following primer pairs were used: *RPL32*, forward 5′-GGCACCAGT CAGACCGATAT-3′, and reverse 5′-CAGGATCTGGCCCTTGAAC-3′; *IDO-1*, forward 5′-AGATGAAGATGTGGGCTTTGCT-3′, and reverse 5′-GGCAGATTTCT AGCCACAAGGA-3′; *IDO-2*, forward 5′-CAAAGTCAGAGCATGA-CGCTG-3′, and reverse 5′-CGCTGCTCACGGTAACTCTTTA-3′.

4.9. In vivo dendritic cell maturation and cytokine production

Mice were injected intravenously (i.v.) with 25 μg of Ultra Pure *Escherichia coli* LPS (strain 0111:B4, InvivoGen, San Diego, CA) in 200 μl pyrogen-free PBS or with the same PBS volume for control animals. After 6 h mice were sacrificed and dendritic cell maturation was evaluated by analyzing the surface expression level of MHC II and CD86 on CD11c positive cells by flow cytometry. Serum TNF-α levels in LPS-treated mice were determined in duplicate by routine TNF-α sandwich ELISA according the manufacturer's protocol (eBioscience).

Conflict of interest

The authors declare no conflict of interest.

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