Video Article

# Development and Functional Characterization of Murine Tolerogenic Dendritic Cells

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## Abstract

The immune system operates by maintaining a tight balance between coordinating responses against foreign antigens and maintaining an unresponsive state against self-antigens as well as antigens derived from commensal organisms. The disruption of this immune homeostasis can lead to chronic inflammation and to the development of autoimmunity. Dendritic cells (DCs) are the professional antigen-presenting cells of the innate immune system involved in activating naïve T cells to initiate immune responses against foreign antigens. However, DCs can also be differentiated into TolDCs that act to maintain and promote T cell tolerance and to suppress effector cells contributing to the development of either autoimmune or chronic inflammation conditions. The recent advancement in our understanding of TolDCs suggests that DC tolerance can be achieved by modulating their differentiation conditions. This phenomenon has led to tremendous growth in developing TolDC therapies for numerous immune disorders caused due to break in immune tolerance. Successful studies in preclinical autoimmunity murine models have further validated the immunotherapeutic utility of TolDCs in the treatment of autoimmune disorders. Today, TolDCs have become a promising immunotherapeutic tool in the clinic for reinstating immune tolerance in various immune disorders by targeting pathogenic autoimmune responses while leaving protective immunity intact. Although an array of strategies has been proposed by multiple labs to induce TolDCs, there is no consistency in characterizing the cellular and functional phenotype of these cells. This protocol provides a step-by-step guide for the development of bone marrow-derived DCs in large numbers, a unique method used to differentiate them into TolDCs with a synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-difluoro-propyl-amide (CDDO-DFPA), and the techniques used to confirm their phenotype, including analyses of essential molecular signatures of TolDCs. Finally, we show a method to assess TolDC function by testing their immunosuppressive response *in vitro* and *in vivo* in a preclinical model of multiple sclerosis.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57637/

## Introduction

Dendritic cells (DCs) are an integral part of the innate immune system and were first discovered and characterized by Ralph Steinman and Zanvil Cohn in 1973 as primary professional antigen presenting cells[1]. DCs have been shown to play an important role in immune activation by presenting processed antigens to T cells and B cells via major histocompatibility complexes (MHC) in secondary lymphoid organs to link the innate and adaptive immune systems[2]. In the mammalian immune system, there are at least two categories of DCs which have been described as myeloid DCs and plasmacytoid DCs (pDCs)[3]. Myeloid DCs, also known as conventional DCs (cDCs), are characterized by the expression of CD11c and can be differentiated as immature DCs (iDCs) *in vitro* from bone marrow progenitor cells or peripheral blood monocytes using granulocyte-macrophage-colony-stimulating factor (GM-CSF) and IL-4 in murine or human species, respectively[4].

Activating 'danger' signals, such as pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP), will drive iDCs maturation toward immunogenic DCs as mature DCs (mDCs) via binding various pattern recognition receptors on the DC surface[5]. Immunogenic DCs further prime naïve T cell proliferation and differentiation through upregulation of MHCII[2], costimulatory ligands (CD80, CD86, and CD40)[6], cytokines, and other soluble mediators[7]. A cascade of pro-inflammatory mediator production from Immunogenic DCs is essential for cytokine-mediated T cell differentiation. For example, both IFN-γ and IL-12 are necessary for Th1 differentiation[8] and IL-1, IL-6, and IL-23 are critical for naïve T cell polarization towards Th17 cells[9]. Although mature DCs react to foreign antigens, uncontrolled DC activation by self-antigens may cause tolerance ablation and foster the development of autoimmune diseases by generating autoreactive T cells whose activation leads to tissue destruction[10].

Recent reports have provided clear evidence of DC plasticity, exemplified by their ability to interact with different cues within their tissue microenvironment and to differentiate into distinct effector/suppressor DC subsets. The anti-inflammatory mediators, such as IL-10[11], TGF-β[12], and HO-1[13] have been shown to play an important role in immune suppression by inducing tolerogenic DCs (TolDCs). These TolDCs acquire

 

regulatory functions and suppress T cell proliferation[14]. Moreover, the lack of co-stimulation by DCs and the production of anti-inflammatory mediators from TolDCs both contribute to the induction of regulatory T cells (Tregs) and also effectively inhibit both Th1 and Th17 differentiation and expansion[15]. In past two decades, the therapeutic potential of TolDCs has been reported by several investigators. In these studies, the administration of *ex-vivo* generated TolDCs not only ameliorated pathological symptoms in different preclinical models of autoimmune diseases[16] but also led to the development of immune tolerance in patients[17,18]. Interestingly, today the TolDCs therapy has been considered as an alternative or adjunctive approach for autoimmune diseases in several clinical trials, including type 1 diabetes mellitus[19], rheumatoid arthritis[20,21], multiple sclerosis (MS)[22,23,24], and Crohn's disease[25].

There are a variety of protocols that have been employed to develop TolDCs and several laboratories have reported methods for generation and phenotypic characterization of TolDCs. These methods can be used to reproducibly generate TolDCs *in vitro* from hematopoietic progenitors and to stably maintain them in a tolerogenic state *in vivo*[26,27,28,29]. The iDCs can be converted into TolDCs by exposure to various immunomodulatory pharmacological agents or anti-inflammatory cytokines. For example, Vitamin D3 is a well-known pharmacological agent known to augment IL-10 production and suppress IL-12 secretion from DCs and to thereby boost their immunosuppressive function[30]. Moreover, when DCs are exposed to potent inflammatory stimuli, such as lipopolysaccharides (LPS), several pharmacological agents such as dexamethasone[31], rapamycin[32], and corticosteroids[33] have been shown to induce the TolDC phenotype by reducing DC surface expression of CD40, CD80, CD86, and MHCII[34]. IL-10 and TGF-β are the most-studied anti-inflammatory cytokines to induce DC tolerance[35] and the concomitant exposure to both of these cytokines have been shown to induce a tolerogenic phenotype in DCs[36].

Since the tolerogenic DC is defined by functional characteristics rather than by phenotypic markers, there is a great need to develop a consistent method for cellular and functional characterization of TolDCs. Moreover, a rigorous and consistent protocol must be established for the consistent evaluation and characterization of the tolerogenic DC phenotype if we are to effectively and reproducibly compare the ability of new agents to induce the TolDC phenotype in the laboratory. Here we provide a detailed protocol with step-by-step methods to isolate iDCs from hematopoietic progenitors of mice and to subsequently analyze the efficacy of new agents under evaluation for their capacity to convert iDCs into TolDCs, providing a robust functional and phenotypic characterization of TolDCs both *in vitro* and *in vivo*. This description includes an elaborate method to characterize the TolDCs by their surface ligands, cytokine profile, and immunosuppressive functions *in vitro*. We also provide an example of a method to explore the potential therapeutic application of these TolDCs in a pre-clinical model of MS, experimental autoimmune encephalomyelitis (EAE). This established protocol will help investigators to evaluate the capacity of new agents to promote the induction of TolDCs and will facilitate the effort to broaden the scope of TolDC therapeutic development.

## Protocol

All studies were performed in compliance with procedures approved by the Case Western Reserve University School of Medicine's Institutional Animal Care and Use Committee.

# 1. Prepare Bone Marrow-derived Dendritic Cells (BMDCs)

1. Sterilize all the surgical instruments via autoclaving and perform the experiment in class II biological safety cabinet with appropriated safety procedures.
2. Euthanize C57BL/6 mice 8 - 10 weeks of age by using $CO_2$ chamber. Place the mouse on a dissecting board and rinse with 70% ethanol. Excise tibia-fibula and femur bones by using a surgical scissor and place them with 70% ethanol in a 10 cm culture dish.
3. Use surgical blades and forceps to dissect tissue away as much as possible on the lid of the 10 cm culture dish and isolate tibias and femurs to place them with 70% ethanol in a 6 cm culture dish.
4. Use surgical blades to trim both ends of tibias and femurs. Use 3 mL PBS in 3 ml syringe with a 23G needle to flush the contents of marrow from one end of bones to a conical tube containing 12 ml PBS. Repeat this step 3x for each end of bones.
5. Centrifuge the cell suspension at 300 x g for 5 min.
6. Remove the supernatant and resuspend the cell pellet with 1 ml ACK lysing buffer for 5 mins to remove red blood cells.
7. Add 9 ml PBS to dilute ACK lysing buffer and centrifuge at 300 x g for 5 mins.
8. Remove the supernatant and resuspend with 10 ml culture medium (RPMI-1640 plus L-glutamine, 10% FBS, 1% non-essential amino acid (100x), 10 mM HEPES, 50 nM β-mercaptoethanol, and 5% penicillin/streptomycin).
   NOTE: Endotoxin level has to be less than 0.1 EU/ml in FBS.
9. Pass the cell suspension through a 40 µm cell strainer and take 20 µl of cell suspension and mix with 80 µl of trypan blue to count the number of live cells by using cytometer.
10. Adjust the cell number to 1 x $10^6$ cells/ml with 15 ng/ml GM-CSF and 10 ng/ml IL-4.
11. Plate 3 ml of 1 x $10^6$ cells/ml in each well of 6-well plate and incubate the cells at 37°C, 5% $CO_2$, and 95% humidity in the $CO_2$ incubator.
    NOTE: The bone marrow cells from one mouse is around 3 - 5 x $10^7$ cells, indicating that it can be placed from 2 to 3 6-well plates.
12. On day 3, remove all 3 ml culture medium from each well, add 2 ml fresh PBS in each well, and then gently swirl the plate to ensure removing all the non-adherent cells.
13. Replace 3 ml fresh culture medium with 15 ng/ml GM-CSF and 10 ng/ml IL-4 in each well. Incubate the cells at 37 °C, 5% $CO_2$, and 95% humidity in the $CO_2$ incubator.
14. On day 5, directly add another 3 ml fresh culture medium with 15 ng/ml GM-CSF and 10 ng/ml IL-4 in each well. Incubate the cells at 37°C, 5% $CO_2$, and 95% humidity in the $CO_2$ incubator.
    NOTE: The total volume in each well is now 6 ml.
15. On day 7, put the entire plate on ice for 10 mins. Gently pipette the culture medium in each well to dislodge the loosely-adherent BMDCs as iDCs into suspension.
    NOTE: The adherent macrophages are still attached to the plate. Low temperature and gently procedures are the key to avoid DC activation to affect further experiments.
16. Centrifuge the cell suspension at 300 x g for 5 mins and resuspend with fresh culture medium for further experiments.

NOTE: BMDCs can be identified with fluorescence-labeled CD11c antibody by flow cytometry and we showed our gating strategy of BMDCs for further experiments in **Figure 1**.

## 2. Characterize TolDC Gene and Protein Profile

1. Plate 2 ml of 1 x $10^6$ BMDCs/ml per well in 6 well-plate with culture medium in the presence or absence of 100-400 nM CDDO-DFPA for incubating 1 h at 37°C, 5% $CO_2$ and 95% humidity in the $CO_2$ incubator.
   NOTE: CDDO-DFPA, a synthetic triterpenoid, is a nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2) inducer and nuclear factor-κB (NF-κB) inhibitor. Apply other agents for induction of TolDCs from iDCs, such as IL-10, vitamin D3, dexamethasone or BAY 11-7085, and modify this step to an optimal condition for each agent.
2. Add 10 or 100 ng/ml of LPS for incubating 4-24 hrs to induce mDCs (incubation time is different due to mRNA or protein measurement).
3. Gently pipette the culture medium in each well and harvest the cell suspension by using 1 ml pipette. Centrifuge at 300 x g for 5 mins to collect the cells and supernatant, respectively.
4. Analyze the surface ligands from the cell pellets by flow cytometry, such as stimulatory ligands: CD40, CD80, CD86, MHC-II, OX40L, ICOSL, or inhibitory ligands: PD-L1, PD-L2, ILT3, ILT4.
5. Isolate the RNA from the cell pellets and analyze the RNA and supernatant samples for the cytokine profile and gene and protein levels by quantitative real-time PCR (qRT-PCR) and ELISA, respectively.
   NOTE: For example, inflammatory cytokines: TNF-α, IFNγ, EDN-1, IL-6, IL-12, and IL-23, or anti-inflammatory cytokines: IL-4, IL-10, IL-15, TGF-β1, and HO-1.

## 3. Evaluate the Function of TolDCs *In Vitro* and *In Vivo*

1. **T-cell Syngeneic Proliferation Assay**
   1. Sterilize all the surgical instruments via autoclaving and perform the experiment in class II biological safety cabinet with appropriated safety procedures.
   2. Prepare MACS buffer using 0.5% bovine serum albumin (BSA) and 2 mM EDTA in 500 ml PBS. Sterilize the buffer by filtering through a 0.2 μm filter.
      NOTE: Keep the buffer on the ice during the following experiment.
   3. To obtain $CD4^+$ T cells, euthanize OT-II T-cell receptor (TCR) transgenic mice 8-10 weeks of age by using $CO_2$ chamber. Place the mouse on a dissecting board and rinse with 70% ethanol. Isolate the spleen from the left side of the abdomen by using surgical scissors and forceps.
   4. Put the spleen with 2 ml PBS in a 6 cm culture dish and use the back of push-stick of 3 ml syringe to mince the spleen by passing a 40 μm cell strainer.
   5. Collect the cell suspension and centrifuge at 300 x g for 5 mins.
   6. Resuspend the cell pellet in 400 μl of MACS buffer.
   7. Add 100 μl of $CD4^+$ T Cell Biotin-Antibody Cocktail at 4°C for 5 mins.
      NOTE: The antibody cocktail binds on other cell types except $CD4^+$ T cells, such as CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, Anti-MHC class II, Ter-119, and TCRγ/δ.
   8. Add 300 μl of MACS buffer and 200 μl of Anti-Biotin beads (**Table of Materials**) at 4°C for 10 mins.
   9. Place the column composed of ferromagnetic spheres (**Table of Materials**) and Pre-Separation Filter together in the magnetic field and rinse it with 3 ml of MACS buffer.
   10. Add 9 ml of MACS buffer in the cells and centrifuge at 300 x g for 5 mins.
   11. Resuspend the cell pellet in 3 ml of MACS buffer and apply onto the column. Collect flow-through containing $CD4^+$ T cells.
   12. Wash column with another 3 ml of MACS buffer and also collect the flow-through.
   13. To obtain splenic pan DCs, euthanize C57BL/6 mice 8-10 weeks of age by using $CO_2$ chamber. Place the mouse on a dissecting board and rinse with 70% ethanol. Isolate the spleen from the left side of the abdomen by using surgical scissors and forceps. Put the spleen in a 6cm culture dish containing 2 ml of collagenase D solution (2 mg/ml collagenase D dissolved in HBSS containing calcium, magnesium).
   14. Inject 1 ml of collagenase D solution to the spleen two times with a 1 ml syringe and a 25G needle. Cut the spleen into small pieces with small scissors.
   15. Shake and incubate at room temperature for 25 mins.
   16. Add 500 μl of 0.5 M EDTA at room temperature for 5 mins.
       NOTE: The steps from 3.1.13-3.1.14 are critical for increasing the yield of DCs.
   17. Now use the back of push-stick of 3 ml syringe to mince the spleen slurry by passing a 40 μm cell strainer and collect the cell suspension by centrifuging at 300 x g for 5 mins.
   18. Resuspend the cell pellet in 350 μl of MACS buffer, 50 μl of FcR Blocking Reagent, and 100 μl of Pan Dendritic Cell Biotin-Antibody Cocktail at 4°C for 10 mins.
       NOTE: The antibody cocktail against antigens that are not expressed by DCs.
   19. Wash the cells by adding 9 ml of MACS buffer and centrifuge at 300 x g for 5 mins.
   20. Resuspend the cell pellet in 800 μl of MACS buffer and add 200 μl of Anti-Biotin beads at 4°C for 10 mins.
   21. Repeat the steps from 3.1.9-3.1.11 to collect DCs.
   22. Wash column with another 3 ml of MACS buffer two times and also collect the flow-through.
   23. Treat the 2 x $10^5$ DCs/ml in the presence or absence of 100-400 nM CDDO-DFPA at 37°C for 1 hr. Seperately, label the $CD4^+$ T cells (1 x $10^7$/ml) with 1 μM CFSE at 37°C for 15 mins, wash with PBS, and readjust the volume to get final concentration at 2 x $10^6$ T cells/ml.
   24. Next, in a 96-well plate, co-culture 100 μl of the treated dendritic cells with the same volume of CFSE-labeled $CD4^+$ T cells, both collected from the above steps to get 1:10 ratio. Now add 100 ng/mL ovalbumin (OVA) peptide 323-329 per well and measure the CFSE intensity of the T cells by flow cytometry after 2-3 days of incubation.
       NOTE: The cell numbers and ratio of DCs and T cells have been optimized from our previous work[37].

2. **Induce Passive EAE by Injecting BMDCs Pulsed with Myelin Oligodendrocyte Glycoprotein (MOG) (35-55)**
    1. Plate 2 ml of 1 x $10^6$ BMDCs/ml per well in 6 well-plate with culture medium in the presence or absence of 100-400 nM CDDO-DFPA for incubating 1 hr.
    2. Add 10 ng/ml of LPS for incubating 24 hrs.
    3. Add 100 µg/ml of MOG (35-55) for incubating 4 hrs.
    4. Harvest the cell suspension and centrifuge at 300 x g for 5 mins.
    5. Resuspend the cell pellet with PBS and count the cells.
    6. Subcutaneously inject 200 µl of 2 x $10^6$ cells to 8-10 weeks old female C57BL/6 mice (100 µl into each hind leg).
    7. On the day of BMDC injection and 48 hrs. later, inject 200 ng of pertussis toxin (PTX) in each mouse.
    8. Repeat steps 3.2.6-3.2.7 once each week for 4 consecutive weeks.
    9. Evaluate the clinical symptoms of EAE daily by using standard criteria[37] (0.5-limp tail end, 1-limp tail, 2-moderate hind limb weakness, 3-severe hind limb weakness, 4-complete hind limb paralysis, 5-quadriplegia or moribund state, 6-death).

## Representative Results

**The differentiation and selection of BMDCs:**

Bone marrow progenitor cells were cultured in complete RPMI medium in the presence of GM-CSF and IL-4 to differentiate into iDCs for 7 days (**Figure 1A**). On day 1, cells were small in size and showed spherical morphology. Washing with PBS before the replacement of fresh medium on Day 3 helped cells to form clusters and also increased the population of $CD11c^+$ cells. On day 4, BMDCs were enlarged in size and initiated cluster formation. Adhered macrophages were also converted and observed at the bottom of the plate with an elongated shape. On day 5, large sized clusters of BMDCs are formed. On day 6, a large number of semi-adherent and floating BMDCs were also observed. BMDCs were harvested on day 7 and analyzed by flow cytometry for CD11c expression as a specific marker of murine DCs. As shown in a representative flow cytometry plot in **Figure 1B**, around 83.6% of BMDCs expressing CD11c were obtained by this method.

**Induction and genetic characterization of TolDCs:**

Some of the agents known to induce TolDCs, such as vitamin D3[38] and dexamethasone[39], are known to down-regulate the expression of DC surface ligands, including MHC II and costimulatory molecules, CD40, CD80, and CD86. By contrast, in the context of either LPS or CD40L-induced maturation of DCs, calcineurin inhibitors cyclosporin A and FK506 showed no effect on the expression of CD83, CD80, CD86, and MHC II[33]. As demonstrated by flow cytometry analysis, the induction of TolDC by CDDO-DFPA had no significant effect on the LPS-induced surface ligand expression of DCs, including CD80, CD86, MHC II, PD-L1, and CD40. (**Figure 2**).

Moreover, the comparative transcriptome and cytokine analysis of BMDCs treated with or without CDDO-DFPA in presence or absence of LPS depicted modulated inflammatory gene profile. CDDO-DFPA treatment significantly reduced LPS induced pro-inflammatory cytokine genes such as IFN-γ, IL-12, EDN1, TNFα, IL-6, and IL-23 (**Figure 3A-3F**). These are known cytokines for Th1 (IFN-γ and IL-12) and Th17 (IL-6 and IL-23) cell differentiation. Furthermore, CDDO-DFPA treated BMDCs showed enhanced expression of anti-inflammatory cytokine genes such as IL-4, IL-10, TGF-β, and HO-1 (**Figure 4A-4D**). These anti-inflammatory genes are known autoimmunity modulator by promoting Th2 (IL-4) and Treg (IL-10 and TGF-β) cell differentiation.[40] Here it is important to note that the distinctive $IL\text{-}12^-$;$IL\text{-}10^+$ cytokine production[36], the induction of HO-1 expression[13], and the inhibition of EDN-1[41] induced by CDDO-DFPA treatment, are all known to authenticate DCs tolerogenic function.

**Cellular and functional characterization of TolDCs *in vitro***

DC-mediated T cell proliferation is known to be triggered by the engagement of costimulatory surface ligand as well as the secretion of cytokines and soluble mediators[7]. However, TolDCs inhibit T cell responses through several mechanisms: by inducing T cell anergy, by actively promoting the deletion of autoreactive cells and by promoting the polarization of naïve T cells to Tregs[42]. We have already reported the functional characterization of CDDO-DFPA induced TolDCs[37]. T cell anergy and deletion of autoreactive T cells can be analyzed independently by checking surface markers or Annexin V/7-AAD staining. However, we indirectly confirmed this phenotype by measuring T cell proliferation index. For this, we utilized C57BL/6 OTII transgenic mice T cells and exposed them to syngeneic DCs extracted from C57BL/6 mice and treated with or without CDDO-DFPA. These DCs were washed and co-cultured with CFSE stained T cells with OVA peptide. We observed a significant reduction in T cell proliferation by CDDO-DFPA pre-treated DCs, suggesting their tolerogenic phenotype (**Figure 5**).

***In vivo* functional characterization of TolDCs in a preclinical model of autoimmunity**

Finally, the functionality of DFPA-induced TolDCs can be tested in any of a number of preclinical *in vivo* models characterized autoimmune or inflammatory features. Since, EAE is a widely accepted preclinical murine model of the clinical immune-mediated central nervous system disease, MS[43], we utilized this in our current study. In order to further examine the *in vivo* functionality of CDDO-DFPA induced TolDCs, we challenged them in a preclinical murine model of passive EAE. For this, BMDCs were cultured with or without CDDO-DFPA in presence of LPS and MOG (35-55). These cells were then injected repeatedly as scheduled protocol depicted in **Figure 6A** and mice were routinely observed for the manifestation of clinical signs. As expected, LPS and MOG (35-55) pulsed BMDCs showed clinical symptoms of EAE, however; CDDO-DFPA primed BMDCs treated group showed delayed onset of disease with significantly reduced clinical symptoms (**Figure 6B**). These results validate the immunosuppressive phenotype of TolDCs induced by CDDO-DFPA treatment.

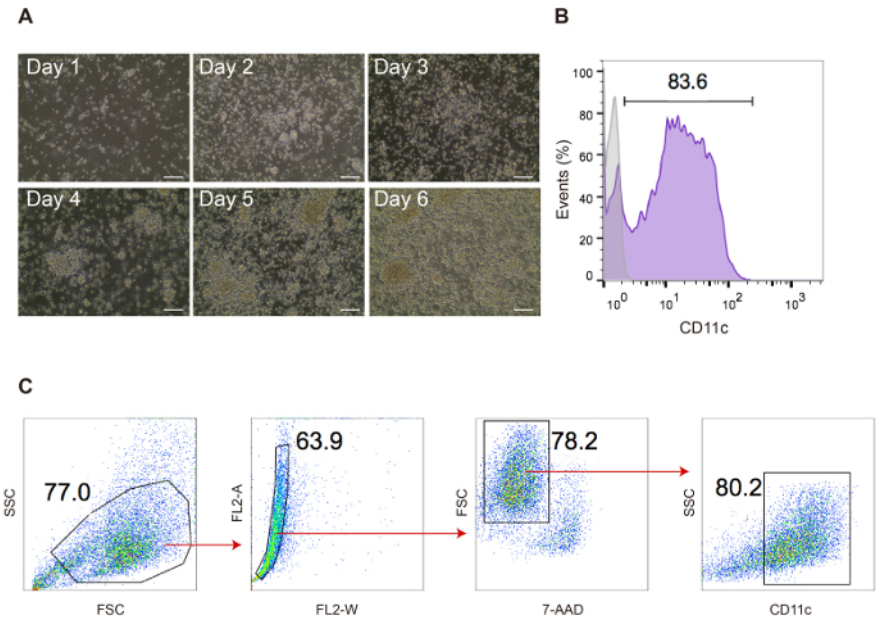


**Figure 1: Development and phenotypic characterization of BMDCs:** (A) Bone marrow cells were flushed out from tibia and femur bones of C57BL/6 mice and further differentiated to BMDCs. An appropriate cell cluster formation was tracked by light microscopy during the entire period of differentiation (scale bars, 100µm). (B) The BMDCs differentiation was confirmed by the CD11c surface expression, as analyzed by FACS. Graphs depict the percentage of the expanded $CD11c^+$ cell population. (C) Gating strategy of BMDCs. Cells were first gated to exclude debris (FSC vs. SSC). A doublet exclusion gate was utilized to gate on singlets cells (FL2-W vs. FL2-A). Within the singlet cells, live cells were gated on 7-AAD plot (7-AAD vs. FSC). Then the cells were further analyzed and gated on CD11c expression. Please click here to view a larger version of this figure.

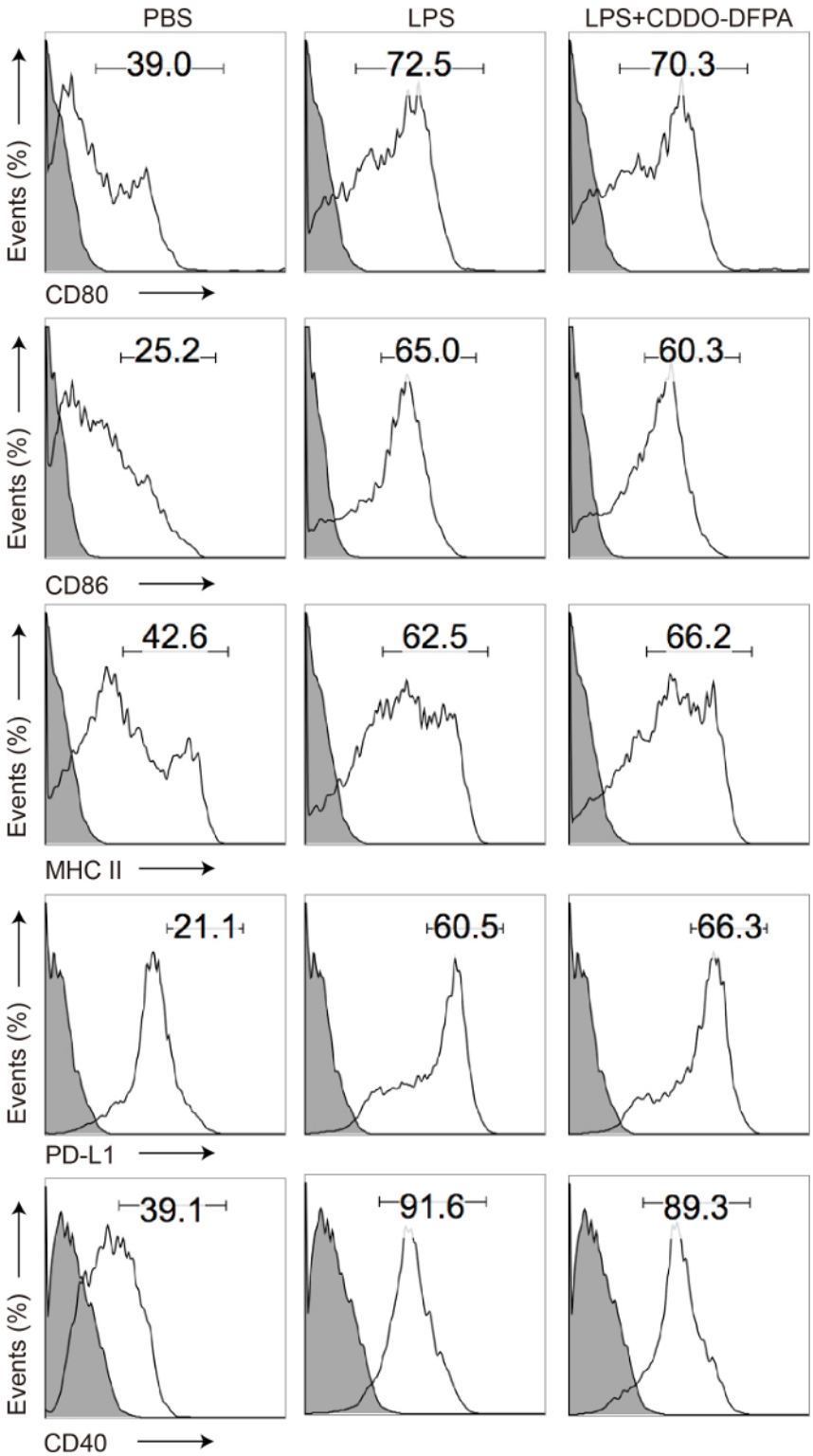


**Figure 2: DC cell surface ligand expression is unaltered by CDDO-DFPA.** Cells were pre-treated in either the presence or absence of CDDO-DFPA (200 nM) for 1 hour prior to stimulation with LPS (100 ng/ml) for 24 hrs. Cell surface expression of CD80, CD86, MHC II, PD-L1, and CD40 was analyzed by flow cytometry. This figure has been modified from *Scientific Reports* 7, Article number: 9886 (2017), doi:10.1038/s41598-017-06907-4. Reproduced and republished with copyright permission. Please click here to view a larger version of this figure.

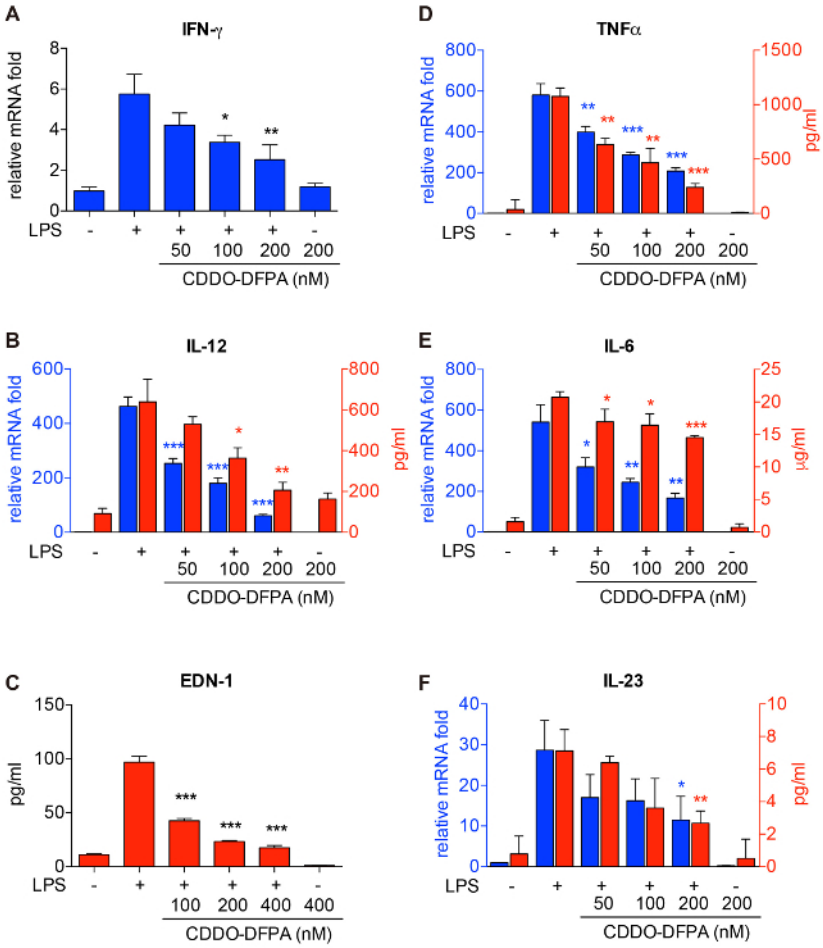


**Figure 3: CDDO-DFPA altered the genetic and protein phenotype of immunogenic DCs. BMDCs** were pre-treated in either the presence or absence of CDDO-DFPA (50-400 nM) for 1 hour prior to addition of LPS (100 ng/ml), and either harvested for RNA extraction (4 hrs.) or allowed to condition culture medium for 24 hrs. prior to collection for cytokine analyses. The levels of IL-12 (B), TNFα (D), IL-6 (E), and IL-23 (F) were measured by qRT-PCR and ELISA. Whereas the level of IFN-γ (A) was measured by qRT-PCR and EDN-1 (C) by ELISA alone. The results are expressed as mean ± S.D. of three experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the LPS-treated groups. Unpaired student t-test. This figure has been modified from *Scientific Reports* 7, Article number: 9886 (2017), doi:10.1038/s41598-017-06907-4. Reproduced and republished with copyright permission." Please click here to view a larger version of this figure.

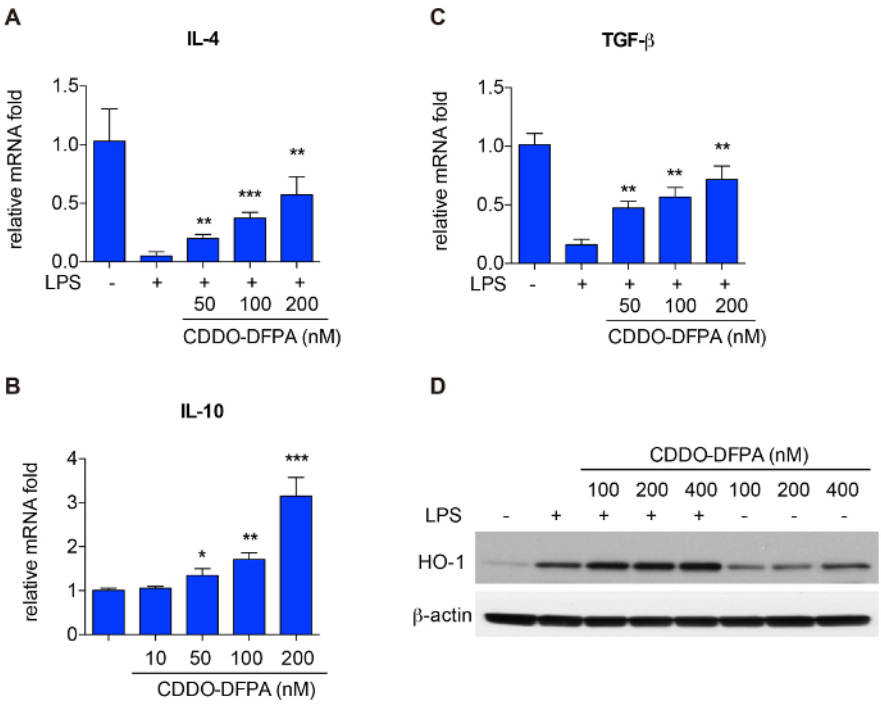


**Figure 4: CDDO-DFPA induced TolDCs phenotype confirmed by gene and protein expression.** BMDCs were pre-treated in the presence or absence of CDDO-DFPA (10-400 nM) for 1 hour prior to addition of LPS (100 ng/ml), and cells were harvested for RNA extraction after 24 hrs. The levels of IL-4 (A), IL-10 (B), and TGF-β (C) were measured by qRT-PCR. (D) Cell protein lysates were collected at 12 hrs. for analyses and the levels of HO-1, and β-actin expression was determined by Western blotting. The results are expressed as mean ± S.D. of three experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the LPS-treated groups. Unpaired student t-test. Please click here to view a larger version of this figure.

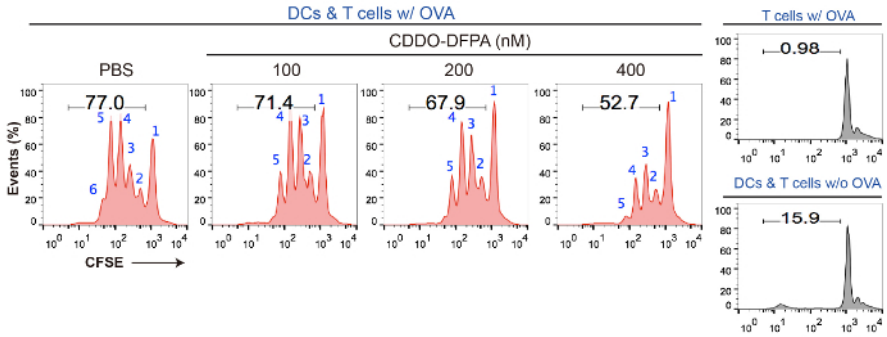


**Figure 5: CDDO-DFPA exposed DCs suppress T cell proliferation.** DCs were pre-treated with CDDO-DFPA (100-400 nM) for 1 hour only, then washed and co-cultured with CFSE stained T cells at a 1:10 ratio. (A) Splenic T cells and DCs were isolated from C57BL/6 OTII transgenic mice and C57BL/6 mice, respectively. CDDO-DFPA pretreated DCs were co-cultured with CFSE stained T cells with (w/) or without (w/o) OVA addition during incubation. T cell proliferation was determined by flow cytometry at day 2. Graphs depict the percentage of dividing T cells relative to numbers T cell division. The data is a representation of 3 independent experiments. This figure has been modified from *Scientific Reports* 7, Article number: 9886 (2017), doi:10.1038/s41598-017-06907-4. Reproduced and republished with copyright permission. Please click here to view a larger version of this figure.

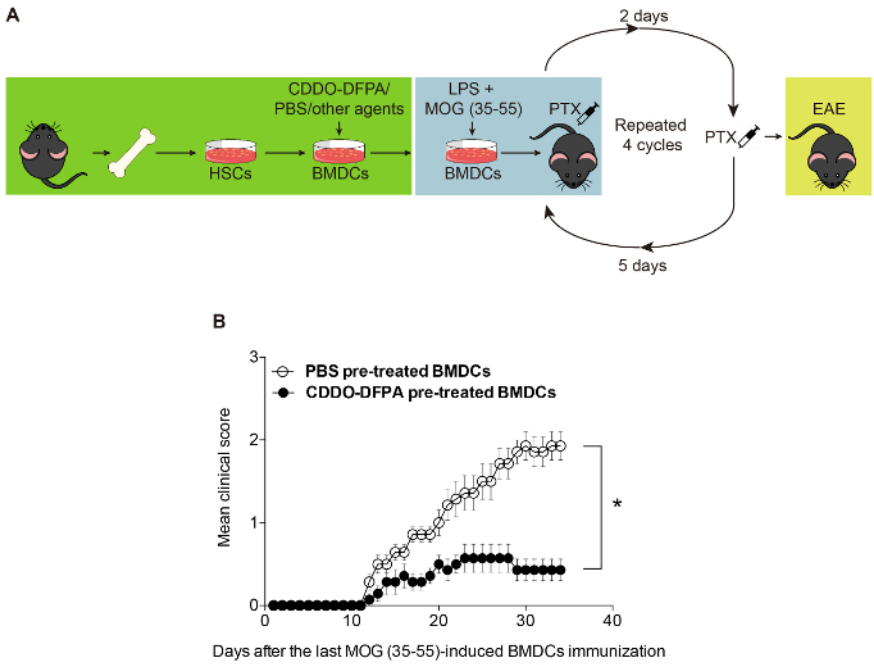


**Figure 6: MOG primed DC-induced passive EAE is abrogated by TolDCs.** (A) EAE induction by MOG (35-55)-pulsed BMDCs. Mature BMDCs were treated in the presence or absence of CDDO-DFPA (400 nM) and subsequently pulsed with MOG (35-55) for 4 hrs. A total of 200 µl of 2 × $10^6$ cells was administered by subcutaneous injection into the flank region of C57BL/6 mice once each week for a total of four injections. Each time, PTX was administered by i.p. injection immediately and again 2 days later (day 0 on 4th cycle). (B) Clinical scores were recorded after all four injections, using standard criteria. All data were presented as the mean ± S.E.M. *P < 0.05. Multiple t-tests with Holm-Sidak analysis (n= 7 mice in each group). This figure has been modified from *Scientific Reports* 7, Article number: 9886 (2017), doi:10.1038/s41598-017-06907-4. Reproduced and republished with copyright permission. Please click here to view a larger version of this figure.

## Discussion

This paper describes an efficient protocol that may be used to reproducibly to generate iDCs and to subsequently differentiate them into TolDCs, and we propose that this may be applied to evaluate the capacity of new molecular target agents to induce the TolDC phenotype. As described in this report, we followed a sequence in which we first analyzed TolDC expression of surface ligands by flow cytometry, followed by an assessment of the DC cytokine profile as measured by qRT-PCR and ELISA. Finally, the immunoregulatory function of TolDCs was confirmed by demonstrating their capacity to reduce T cell proliferation *in vitro* and their efficacy to suppress autoimmunity in vivo, using the preclinical murine EAE model of MS.

In our protocol, iDCs were generated and differentiated from murine bone marrow precursors with the combination of GM-CSF and IL-4. Other protocols have used forms-like tyrosine kinase 3 ligands (Flt3L) in the culture medium to generate iDCs[44]. Although the use of Flt3L increases the yield of iDCs, these iDCs usually take 2 more days (9 days) to harvest, compared to GM-CSF/IL-4 addition (7 days)[44]. More importantly, iDC generated from Flt3L often differentiate into both cDCs ($CD8^-$ and $CD8^+$) and pDCs[45] that were functionally and phenotypically equivalents to steady-state DCs in vivo. However, GM-CSF/IL-4 invariably induces the differentiation of iDC toward cDCs ($CD8^-$) only[44] and during maturation, they demonstrated similar characters of inflammatory DCs[46]. It is noteworthy to know that GM-CSF/IL-4 is often used in clinical trials and basic research because of the advantage of differentiating DCs from monocytes or $CD34^+$ progenitors[44]. It has been shown that iDCs generated from these two methods produce morphologically different cells, which also possess different cell surface markers and exhibit distinct cytokine profiles upon their activation. Furthermore, TolDCs induced by these methods vary in their ability to migrate in response to chemotactic factors and to induce antigen-specific T cell responses[44,45]. We understand that $CD8^+$ DCs may exhibit more potential in TolDC induction due to their unique capacity of cross-tolerance[47]. However, it is then needed further cell sorting from BMDCs derived by Flt3L to investigate the specific $CD8^+$ DC subset. In addition, since, GM-CSF/IL-4 induced BMDCs are superior at T cell stimulation and the production of inflammatory mediators following LPS treatment[44,45], we found this strategy gave more reproducible results in our experiments.

BMDCs derived from GM-CSF/IL-4 start to express CD11c around day 4 and to enrich the expression after day 6[48]. This culture procedure can be sustained until day 10-12 with a lower plating density of bone marrow cells and a low dose of GMC-SF on day 8-10[49]. In this protocol, CDDO-DFPA (synthetic triterpenoid) and LPS were added in tandem as agents to induce DC tolerance and maturation, respectively. We recently reported that iDCs harvested from cultures utilizing this method of BMDC generation exhibit a functional TolDC phenotype when pretreated with CDDO-DFPA[37]. It is noteworthy that CDDO-DFPA, which was only added to cultures after iDC harvest (day 7)[37], in contrast to agents like vitamin D3 which must be added to cultures multiple times during iDC differentiation (day 2, 4, 6)[50]. Moreover, this induction of TolDCs by vitamin D3 may depend on diverse mechanisms that must be initiated both during and after iDC differentiation[51]. Our data suggest that adding CDDO-DFPA during iDC differentiation had no impact on the purity of CD11c+ DCs but did dose-dependently lower the yield of iDCs on day 7 (data not shown). Our analyses also suggest that the higher concentration of CDDO-DFPA used in these experiments may be toxic to bone marrow

progenitor cells, even though iDC showed normal viability with this concentration of CDDO-DFPA[37]. It is anticipated that TolDC induction may be optimized by careful analyses of the impact of the timing and length of exposure to agents like CDDO-DFPA.

It is important to consider the alternative methods and mechanisms through which iDCs can be induced to mDCs by others than LPS, such as CD40L, TNF-α, and IFN-γ[52]. DC maturation by LPS through Toll-like receptors 4(TLR4) leads the activation of several transcription factors, including nuclear factor-κB (NF-κB), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase (ERK1/2). The maturation of DCs by CD40L and TNF-α through CD40 and TNF receptors respectively induces the NF-κB pathway. In contrast, IFN-γ stimulates a different pathway including the activation of Janus kinase (JAK), tyrosine kinase (TYK), and the signal transducer and activator of transcription proteins (STATs), and these downstream effects are also complemented by activation of the NF-κB pathway[52]. In addition, while the gene expression profile of CD40L/TNF-α-based DC maturation suggests that these DCs polarize T cells toward a Th2 cell response[53], DC maturation by IFN-γ is strongly biased towards a Th1 cell response[54]. Therefore, these other inducers of DC maturation which are linked to differentiation toward specific T cell subsets are not employed in this protocol.

Several reports have shown that *ex vivo*-differentiated DCs are capable of inducing the full spectrum of EAE clinical symptoms and pathology in mice[55]. The method of passive EAE induction by *ex vivo* activated DCs has been adopted with various modifications by different laboratories. The combination of passive and active EAE induction by administration of MOG primed BMDCs, 7 days before the immunization with MOG and complete Freund's adjuvant (CFA) results in higher clinical symptom scores (peak clinical score: 3.5) compared to the passive EAE induction by the administration of MOG primed BMDCs alone (peak clinical score: 2)[56]. Results with this method combining MOG primed BMDCs with MOG immunization suggests that disease onset happens on day 9 after the immunization with MOG and CFA[56]. However, since it is known that CFA causes significant inflammation at the site of administration[57], we have utilized passive EAE induction by administration of DCs alone in our protocol in order to effectively analyze the effect of TolDCs during EAE. In order to further confirm the functional phenotype of TolDCs, one should also test them in suppressing clinical symptoms of active EAE mice. The administration route of DCs is also critical to the induction of EAE symptoms[58]. As reported by our lab and by others, subcutaneous injection of BMDCs into the flank region of C57BL/6 mice once a week for a total of 3-4 injections induces a peak clinical score of EAE around 2-2.5 with reproducible disease onset on day 11-12[37,58]. We provided a short clip to demonstrate the different clinical symptoms between active and passive EAE induction (**Video 1**).

The recognized limitations of our protocol include the time required by the methods employed and the dependence on a non-physiologically relevant microenvironment for BMDC differentiation. However, while the antibody enriched column separation methods of collection for DCs and T cells from murine spleen may be more time efficient, it suffers from a low yield of the desired DC population. Thus, the advantage of the method of bone marrow-derived DC development is the log scale higher yield of DC populations when compared to antibody column separation from the whole spleen. As noted above, the DCs generated *in vitro* by this method requires a high concentration of cytokine supplementation which is not physiologically relevant to DCs *in vivo* microenvironment[59] and also does not ensure the long-term sustainability of DC. The GM-CSF used in our protocol is also not an essential cytokine for normal DC differentiation *in vivo*[60]. Nevertheless, the proposed BMDC generation protocol reproducibly yields a high fraction of functionally TolDCs and should be viewed as a highly reliable and reproducible method for generating functional DCs with tolerogenic properties.

In conclusion, the TolDCs generated from the protocol described here can be effectively characterized by assessing their gene and protein expression profiles, by determining their capacity to modulate T cell-dependent immune responses *in vitro*, and by assessing their function as an adoptive cell therapy to suppress EAE induction *in vivo*. Our protocol provides a framework for the evaluation of any new agents thought to have the capacity to induce TolDCs and to further accelerate the therapeutic development process for TolDCs from bench to clinic.

## Disclosures

None

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