Generation of Dendritic Cells With Regulatory Properties

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ABSTRACT

Dendritic cells (DCs) are professional antigen presenting cells with the ability to induce and regulate an immune response. DCs that capture and present antigen under noninflammatory conditions maintain an immature phenotype and acquire tolerogenic properties. These DCs generate regulatory T lymphocytes that potentiate tolerogenic responses. Here we developed a method for the generation of immature murine DCs able to process and present a specific antigen in a tolerogenic context. Immature DCs were prepared from bone marrow precursors after differentiation with granulocyte-macrophage colony-stimulating factor (GM-CSF) in the presence of vitamin D_3 and characterized by their low expression of major histocompatibility complex class (MHC) II and CD86 molecules. Purified phagosomes containing either MHC II molecules or ovalbumin were used to deliver antigens to immature DCs. More than 80% of the DCs captured the phagosomes, while maintaining a low expression of maturation markers and showing basal levels of secretion of activating cytokines such as interleukin (IL)-2 and IL-12. Treatment of the immature DCs with lipopolysaccharides (LPS) increased IL-10 secretion, in agreement with their anti-inflammatory and immune regulatory properties. Cocultures of transgenic OT-II T lymphocytes with the immature DCs carrying OVA-phagosomes succeeded in generating a subpopulation of regulatory T lymphocytes characterized by the expression of CD4, CD25, CD62L, and Foxp3. Taken together, our results suggest that vitamin D_3 generates immune tolerance through the modulation of DC phenotype and could be useful to induce tolerance to allotransplants.

DENDRITIC CELLS (DCs) are antigen presenting cells (APC) capable of generating an immune response to foreign antigens or inducing tolerance to self-antigens. The maturation stage of DCs determines if it promotes or suppresses a T-lymphocyte immune response.[1] Immune stimulation is induced when DCs capture and present antigens to T cells in an inflammatory environment. On the other hand, tolerance is induced when this process occurs in a noninflammatory environment in which DCs remain at an immature state, expressing low costimulatory molecules and secreting mainly inhibitory cytokines such as interleukin (IL)-10. In this stage, DCs can generate regulatory T lymphocytes that increase the tolerogenic response by suppressing effector T-lymphocyte responses.[2] Regulatory T cells are characterized by their expression of CD25 (IL-2R α-chain), CD62L (L-selectin), and the transcription factor Foxp3, a key gene in the development and function of these cells.[3]

The strong immune response generated via direct presentation by donor APC of major histocompatibility complex (MHC)/peptide complex to recipient T lymphocytes and indirect presentation of donor MHC II/peptide complex by recipient APC are the main mechanisms causing allograft rejection.[4] An increasing number of researchers are interested in developing new systems to promote specific tolerance to transplanted organs or to autoreactive T lymphocytes provoking autoimmune disease. Therefore, the production ex vivo or in vivo of immature DCs with specific tolerogenic properties may be useful in future

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immunological interventions such as allograft transplantations.[5]

On the other hand, DCs internalize large particles and external antigens in an endocytic compartment—the phagosome—that contains plasma membrane proteins.[6] Recently, the use of low-density latex beads has been used for the isolation of these organelles by flotation on a simple sucrose gradient.[7] Phagosomes isolated from DCs contain, among other molecules MHC II antigens and can be used as molecular vehicles carrying antigens to cells endowed with phagocytic activity such as immature DCs.

The aim of this work was to establish experimental conditions to induce antigen-specific tolerance in a model that could be useful to induce tolerance to allotransplants. For this, we generated murine immature DCs with granulocyte-macrophage colony-stimulating factor (GM-CSF) and vitamin D_3 in vitro. These cells were capable of processing and presenting antigens delivered by purified phagosomes. We demonstrate that phagosome-charged immature DCs generate a population of regulatory T cells. The tolerogenic properties of these DCs are presently being studied in mice skin and heart transplant experiments.

MATERIALS AND METHODS

Mice and Reagents

We used 6- to 8-week-old C57BL/6 (H-2^b), BALB/c (H-2^d), and OT-II mice (TCR transgenic recognizing ovalbumin 323–339 peptides in an H-2^b context). Vitamin D_3 (1α,25-dihydroxy vitamin D_3), deep blue dyed latex beads, and purified lipopolysaccharide (LPS) were purchased from Sigma (St Louis, Mo, USA).

DC Generation and Analysis

DCs were generated from bone marrow precursor cells extracted from tibias and femurs of C57BL/6 mice. Red blood cells were lysed in a NH_4Cl solution and the cells were cultured in a RPMI plus 10% FCS medium, 50 μmol/L 2-mercaptoethanol, 50 μg/mL gentamycin, and 3% vol/vol of a supernatant obtained from the J558L cell line transfected with the GM-CSF murine gene (kindly provided by Dr A.M. Lennon-Duménil, Institut Curie, Paris, France). On days 2, 4, 6, and 8, 1 nmol/L vitamin D_3 was added. On days 4 and 8, the cultures were fed with complete fresh medium. After 12 days, most of the cells stayed adherent and were trypsinized for subsequent analysis and experiments. Nonadherent cells were eliminated at this step. The DC phenotype was evaluated by flow cytometry through the expression of CD86, CD11c, and MHC II molecules using a FACScan flow cytometer and CellQuest software (BD Biosciences).

Preparation and Analysis of Phagosomes

Phagosomes were prepared from BALB/c bone marrow-derived DCs cultured with 30% of J558L supernatant for 12 days. Adherent and nonadherent cells were used to obtain phagosomes as previously described.[7] Phagocytosis of latex beads by DCs was carried out for 1 hour at 37°C and stopped by adding cold phosphate buffered saline (PBS). Cells were lysed mechanically by passing them 10 times through a 22G needle. Phagosomes were obtained from lysed cells after ultracentrifugation at 100,000*g* for 1 hour at 4°C at the 25% to 8% interface of a discontinuous sucrose gradient. Size and shape were determined via electron microscopy after fixing with 8% paraformaldehyde and 2% glutaraldehyde as previously described.[8] To prepare phagosomes conjugated with OVA protein, latex beads were previously incubated for 12 hours at 4°C with 1 mg/mL OVA before purification as indicated above.

Phagosomes prepared from DCs were evaluated for the presence of MHC II molecules by Western blot (SDS-PAGE 12%) using a rat anti-mouse IA-IE monoclonal antibody (BD Biosciences) as the primary antibody, followed by incubation with a mouse anti-rat Ig (Biosource), and finally with a goat anti-mouse HRP (Dako) as the detection antibody. Proteins transferred to the nitrocellulose membranes were visualized using the ECL plus kit (Amersham Biosciences AB, UK) following the manufacturer's instructions.

OVA phagosomes were analyzed for their ability to activate OT-II TCR transgenic T lymphocytes after phagocytosis and presentation by DCs.

Phagocytosis Assays

Phagosome uptake by DCs was determined after 24 hours using flow cytometry and fluorescent microscopy. The phagosomes were previously labeled with 100 μmol/L DiIC[18] for 20 minutes at 37°C and added to vitamin D_3-treated DCs at 37°C. Control experiments were done at 4°C.

Cytokine Measurement

IL-2, IL-10, and IL-12 were detected under different conditions using a capture ELISA system (BD OptEIA mouse ELISA kit, BD Biosciences) following the manufacturer's instructions.

T-Cell Preparation and Coculture Experiments

Naïve T lymphocytes were obtained from splenocytes of OT-II transgenic mice and depleted of APCs by using an anti-I-A^b antibody (KH74 clone) and MACS anti-mouse IgG immunomagnetic beads (Miltenyi Biotec, Germany).

Naïve T cells (2×10^5 cells/well) were cocultured with vitamin D_3-treated DCs loaded with OVA phagosomes (4×10^5 cells/well) for 4 days. CD4 T cells were analyzed by flow cytometry for the expression of activation markers such as CD25, CD62L, CD44, and CD69.

Reverse Transcriptase-Polymerase Chain Reaction for Foxp3

Total RNA was extracted from 4×10^6 transgenic OT-II T cells cocultured for 4 days with vitamin D_3-treated DCs. cDNA was synthesized at 42°C for 1 hour from 2 μg of total RNA using 500 ng of oligo (dT), 2 U MMLV-RT buffer, 15 U RNAsin, and 0.5 mmol/L dNTP. Foxp3 cDNA was amplified using 2 μL of reverse transcriptase (RT) reaction with the following polymerase chain reaction (PCR) protocol: 2.5 minutes at 42°C, followed by 31 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C, and 1 cycle of 10 minutes at 72°C. The primers used for the detection of Foxp3 were: 5′-CAGCTGCCTACAGTGCCCCTAG-3′ (forward) and 5′-CATTTGCCAGCAGTGGGTAG-3′ (reverse) as described by Hori et al.[3] The β-actin gene was used to control the amount of cDNA used and was amplified by employing the following protocol: 5 minutes at 94°C followed by 14 cycles of 30 seconds at 94°C, 45 seconds at 58°C, and 90 seconds at 72°C, and 1 cycle of 10 minutes at 72°C. The following primers were used: 5′-AAATCGTGCGTGACATTAAGG-3′ (forward) and 5′-CCGATCCACACGGAGTACTT-3′ (reverse). The PCR products were visualized in a 1.2% agarose gel.

RESULTS

Vitamin D_3 Treated DCs Have an Immature Phenotype

DCs were obtained from bone marrow-derived precursors differentiated in the presence of GM-CSF with or without 1 nmol/L vitamin D_3. Cells obtained in the presence of vitamin D_3 showed a homogeneous phenotype expressing high levels of the DC marker CD11c and lower levels of MHC II and costimulatory molecule CD86 compared with DCs produced in the absence of vitamin D_3 (Fig 1A). In addition, no detectable amounts of IL-2, IL-10, or IL-12 were secreted by vitamin D_3-generated DCs and control DCs as determined by capture ELISA. However, upon activation with LPS, vitamin D_3-treated DCs secreted higher levels of the inhibitory cytokine IL-10 and low levels of activating IL-2 and IL-12 cytokines (Fig 1B) compared with control DCs.

Loading of DCs With Allogeneic Phagosomes

Phagosomes generated from bone marrow-derived DCs from BALB/c mice are approximately 1 μm in diameter and spherical, as seen under the electron microscope (Fig 2A). The presence of MHC II molecules on DC phagosomes has been reported previously.[9] The SDS stable MHC II (about 50 kDa) and the heavy chain of this complex (34 kDa) were detected by Western blot (Fig 2B). When these phagosomes were added to vitamin D_3-treated DCs from C57BL/6 and cultured for 24 hours at 37°C, more-than 80% of allogeneic phagosomes were taken up (Fig 2C,D). As expected, no phagocytosis was seen at 4°C. Under this condition DCs remained in an immature state, as seen by their low expression of MHC II and costimulatory molecules CD86 (Fig 2D). Moreover, DCs that had taken up allogeneic phagosomes maintained basal levels of cytokine secretion and only after LPS activation showed secretion of the inhibitory cytokine IL-10 (Fig 2E).

Induction of Regulatory T Lymphocytes (Treg) by Immature DCs

To evaluate the effect of vitamin D_3-treated-DCs on T lymphocytes, we evaluated the phenotype of the T cells after OT-II CD4+ transgenic naïve T lymphocytes were cocultured for 4 days with vitamin D_3-treated DCs preloaded with OVA phagosomes. Figure 3A shows that OT-II

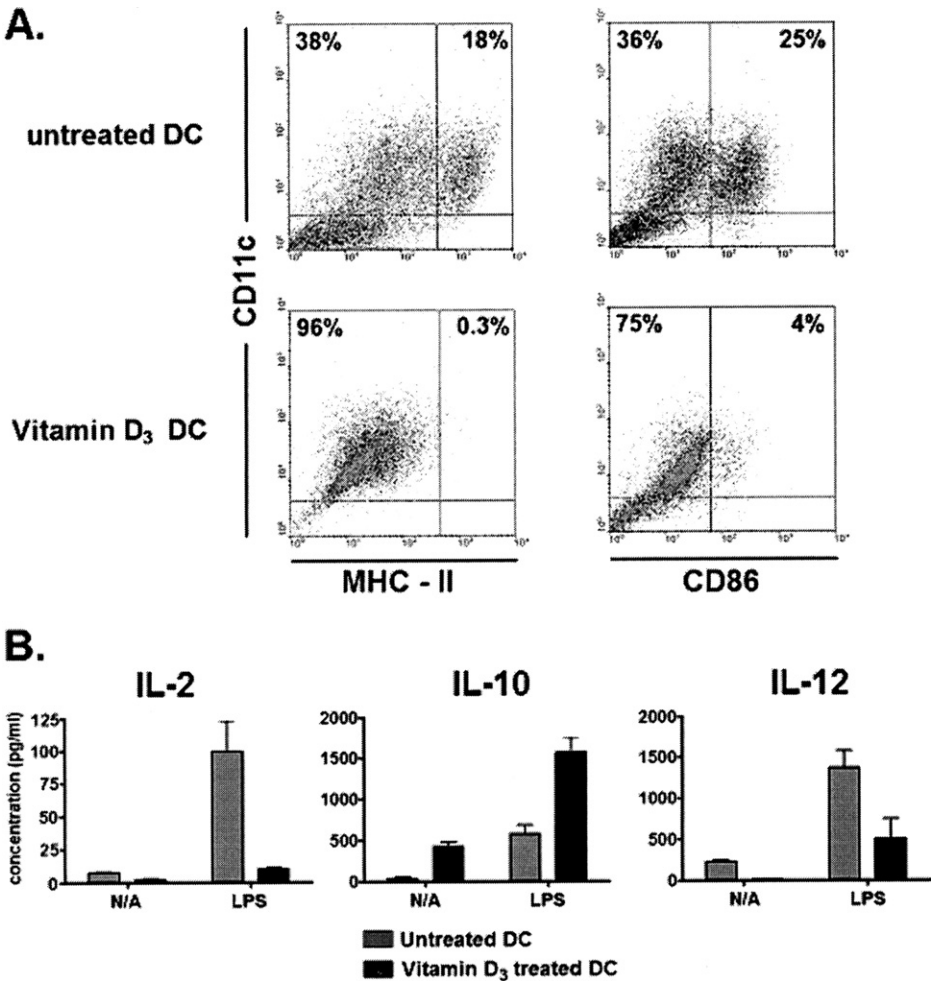

Fig 1. Vitamin D_3-treated dendritic cells (DCs) show an immature phenotype and Th2-type cytokine secretion. **(A)** Flow cytometric analysis of bone marrow-derived DCs cultured with or without vitamin D_3. **(B)** Cytokine secretion measured by capture ELISA of DCs cultured with or without vitamin D_3, and activated or not with LPS. Vitamin D_3-treated DCs showed higher secretion of IL-10 and lower levels of IL-2 and IL-12 compared with untreated DCs. N/A = not activated.

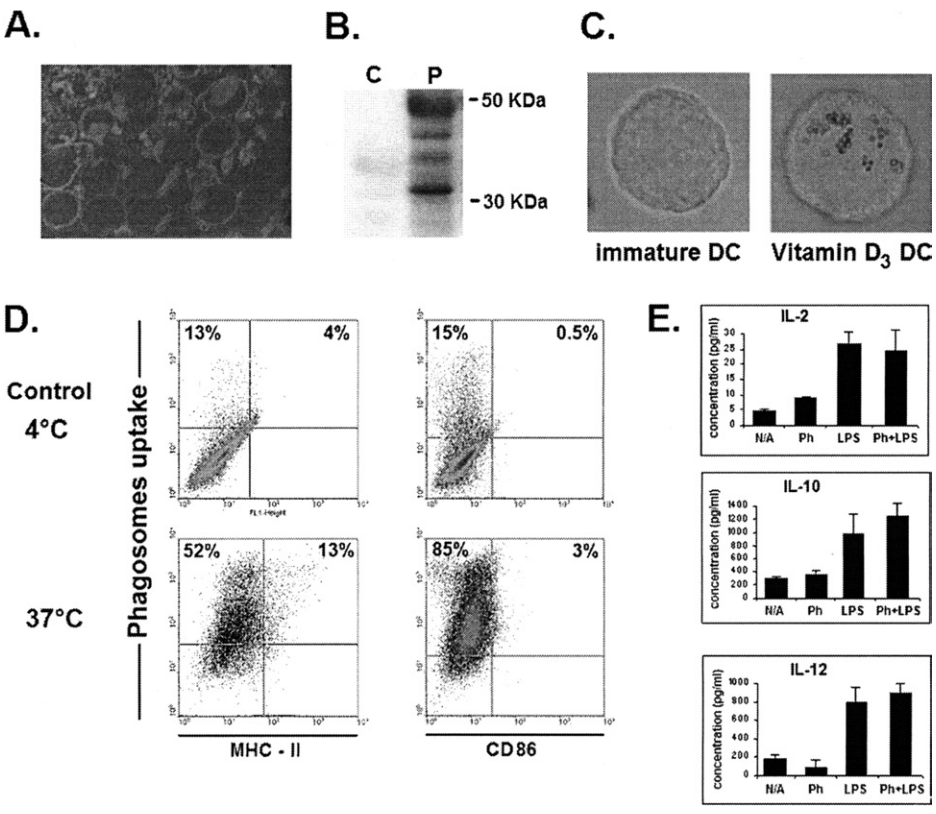

Fig 2. Dendritic cells (DCs) cultured with vitamin D_3 incorporate allogeneic phagosomes. **(A)** Electron microscopic slide of purified phagosomes. **(B)** Western blots of phagosomes containing MHC II molecules. Stable SDS MHC II (about 50 kDa) and α-chain (34 kDa) are labeled. C = control, P = phagosomes. **(C)** Fluorescent microscopy of DCs loaded with $DiIC^{18}$-labeled phagosomes. **(D)** FACs analysis of DCs incubated with $DiIC^{18}$-labeled phagosomes. Incorporation of phagosomes did not induce DC maturation. **(E)** Cytokine secretion of DCs loaded with phagosomes. Incorporation of phagosomes by vitamin D_3-treated DCs did not change the cytokine secretion pattern of these cells. N/A = not activated.

T lymphocytes generated under these conditions maintain their naïve state, as seen by their high expression of CD62L and low expression of the early activation marker CD69. However, a small population of T cells expresses high levels of CD25 and CD62L (11%), which correspond to a T-regulatory phenotype. Thus, immature DCs generated in the presence of vitamin D_3 and loaded with OVA phagosomes are unable to induce T-cell activation but rather generate a small number of T cells with a regulatory phenotype.

Regulatory T lymphocytes are characterized by their expression of the transcription factor Foxp3. To confirm that the T cells generated in the above experiments represent a Treg population, we performed RT-PCR to detect Foxp3 mRNA in these cells. As seen in Fig 3B, OT-II T cells cocultured with vitamin D_3-treated DCs loaded with OVA phagosomes do express detectable levels of Foxp3 mRNA. These results indicate that immature DCs loaded with OVA phagosomes have the ability to induce naïve T cell to differentiate into a population of $CD4^+$, $CD25^+$, $CD62L^+$, $Foxp3^+$ Treg population of cells.

DISCUSSION

In this study, we developed a protocol for the generation of immature DCs able to incorporate alloantigens such as proteins from the MHC II or OVA without maturing. The generation of immature DCs with tolerogenic properties, as determined by their low expression of MHC II and costimulatory CD86 molecule, may be of great importance in the area of transplant tolerance and in the treatment of autoimmune diseases. Here we showed that differentiation of DCs from bone marrow precursors in the presence of vitamin D_3 generated a population of DCs with characteristics of immature cells. These cells have a pattern of inhibitory cytokines, as seen by their high production of IL-10 and low secretion of IL-2 and IL-12. This is characteristic of T-cell differentiation to T helper 2 (Th2) and tolerogenic responses.[10,11]

We further demonstrated that administration of alloantigen or an OVA peptide in the form of phagosomes to DCs generated in the presence of vitamin D_3 preserved the tolerogenic properties of the DCs, as seen by their low expression of MHC II and CD86 and by the preservation of their inhibitory cytokine secretion pattern. It has been described that antigen presentation by DCs in a noninflammatory context can generate a population of regulatory T lymphocytes (Treg) capable of suppressing an immune response instead of producing effector T lymphocytes.[12]

In this study, we demonstrated the generation of regulatory T cells from naïve T lymphocytes after coculture with phagosome-loaded vitamin D_3-treated immature DCs. These data suggested that these immature DCs are able to phagocytose peptides delivered from phagosomes and to process and present them to naïve T lymphocytes.

Most of the T cells exposed to DCs generated in the presence of vitamin D_3 and loaded with phagosomes re-

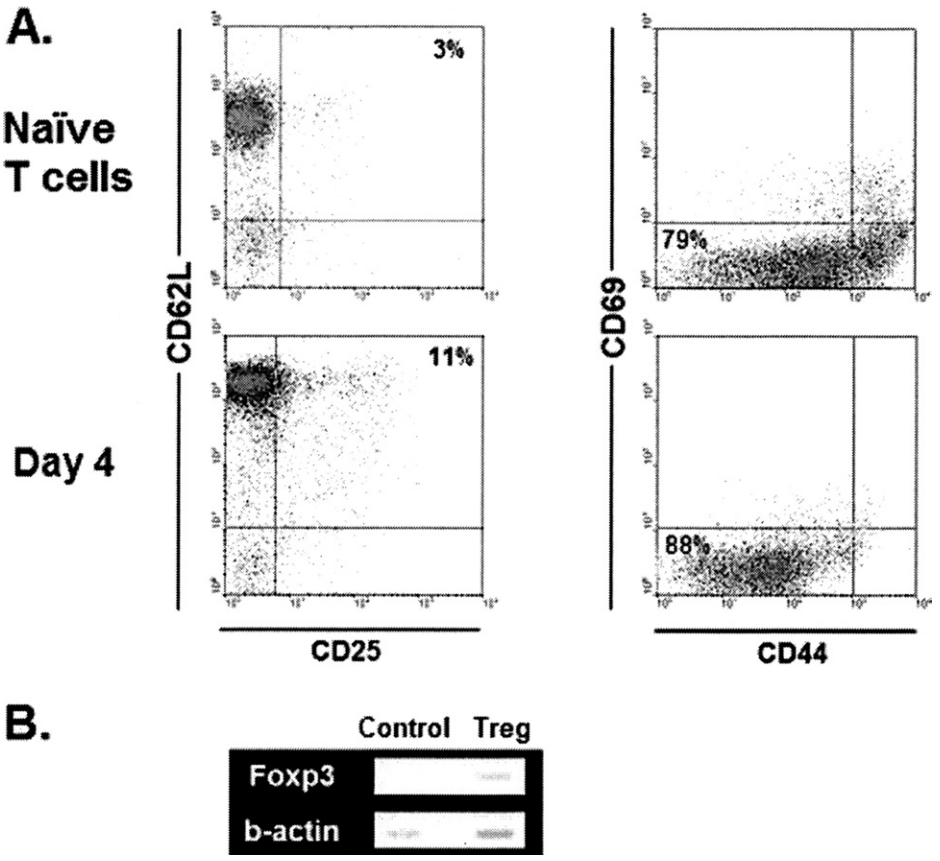

Fig 3. Vitamin D_3-treated DCs generate a regulatory $CD25^+$ $CD62L^+Foxp3^+$ T-lymphocyte population. **(A)** FACs analysis of T cells (gated on CD4) before and after 4 days of coculture with vitamin D_3-treated DCs and loaded with OVA phagosomes. **(B)** RT-PCR of Foxp3 mRNA in T cells generated in A. The β-actin gene was used as a control of RNA loading.

main in a naïve, probably anergic state, but most interestingly, we found a small population of T cells that express the Treg-specific markers $CD4^+CD25^+CD62L^+$ and the transcription factor Foxp3. Regulatory T lymphocytes have an important role in transplants because they potentiate the suppressive response against donor antigens.[13]

In conclusion, the use of immature DCs loaded with phagosomes carrying specific donor-derived peptides may have important clinical implications as it would permit selective expansion of specific regulatory T cells, thereby generating tolerance.[11]

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