ORIGINAL ARTICLE

Immature Dendritic Cells Expressing Indoleamine 2,3-Dioxygenase Suppress Ovalbumin-Induced Allergic Airway Inflammation in Mice

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Abstract

Background: Proliferation of activated $CD4^+$ T lymphocytes is inhibited by indoleamine 2,3-dioxygenase (IDO).
Objective: We undertook the present study to test the hypothesis that IDO-expressing immature DCs (imDCs) can restore immune tolerance in mice suffering from allergic airway inflammation.
Methods: imDCs were generated from murine bone marrow cells using granulocyte-macrophage colony-stimulating factor. The imDCs were subsequently transfected with an IDO expression vector (pEGFP-N1-IDO). Surface marker expression, including CD11c, MHC II, CD80, and CD86, was analyzed using flow cytometry. IDO-expressing imDCs were injected into the trachea of ovalbumin (OVA)-sensitized mice, and lung histopathology and cytokine expression in bronchoalveolar lavage fluid were assessed. The splenic $CD4^+$ T cells of OVA-sensitized mice were isolated and co-cultured with pEGFP-N1-IDO–expressing imDCs, and apoptosis of $CD4^+$ T cells was detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay.
Results: Expression of IDO in imDCs did not alter cell surface molecule expression. We observed marked lung inflammation, elevated total cell and eosinophil count, and altered cytokine levels in OVA-sensitized mice. These parameters improved upon inoculation with IDO-expressing imDCs. Co-culture with IDO-expressing imDCs also induced apoptosis, inhibited IL-4 and IL-5 expression, and upregulated IFN-γ expression in $CD4^+$ T cells.
Conclusions: IDO-expressing imDCs induced T_H2 cell apoptosis and reduced T_H2 cell activation and allergic airway inflammation in OVA-sensitized mice. Thus, upregulation of IDO expression may provide a novel immunointervention strategy for asthma treatment.

Key words: Immune tolerance. Indoleamine 2,3-dioxygenase. Immature dendritic cell.

Resumen

Antecedentes: La indoleamina 2,3-dioxigenasa (IDO) inhibe la proliferación de linfocitos T $CD4^+$ activados.
Objetivo: Este estudio se realizó para probar la hipótesis de que las células dendríticas inmaduras (imDC) que expresan la IDO pueden restablecer la inmunotolerancia en ratones que presentan inflamación alérgica de las vías respiratorias.
Métodos: Las imDC se generaron a partir de células murinas de médula ósea utilizando factor estimulante de las colonias de macrófagos y granulocitos. A continuación, estas células se transfectaron con un vector de expresión de la IDO (pEGFP-N1-IDO). La expresión de marcadores de superficie, como CD11c, MHC II, CD80 y CD86, se analizó mediante citometría de flujo. Se inyectaron imDC que expresan la IDO en la tráquea de ratones sensibilizados a la ovoalbúmina (OVA), y se evaluaron la histopatología pulmonar y la expresión de citocinas en el líquido de lavado broncoalveolar. Los linfocitos T $CD4^+$ esplénicos de los ratones sensibilizados a la OVA se aislaron y cocultivaron con imDC que expresan pEGFP-N1-IDO, y se detectó la apoptosis de linfocitos T $CD4^+$ mediante la técnica TUNEL.
Resultados: La expresión de la IDO en las imDC no alteró la expresión molecular de la superficie celular. Se observó una inflamación pulmonar importante, un recuento total de células y un número de eosinófilos elevados y una alteración de los niveles de citocinas en los ratones sensibilizados a la OVA. Estos parámetros mejoraron con la inoculación de imDC que expresan la IDO. El cocultivo con imDC que expresan la IDO también produjo apoptosis, inhibió la expresión de IL-4 e IL-5, y aumentó la expresión de IFN-γ en los linfocitos T $CD4^+$.
Conclusiones: Las imDC que expresan la IDO produjeron la apoptosis de las células T_H2 y redujeron la activación de las células T_H2 y la inflamación alérgica de las vías respiratorias en ratones sensibilizados a la OVA. Por consiguiente, el aumento de la expresión de la IDO puede proporcionar una estrategia de inmunointervención nueva en el tratamiento del asma.

Palabras clave: inmunotolerancia; indoleamina 2,3-dioxigenasa; célula dendrítica inmadura.

Introduction

Asthma is a chronic inflammatory disorder of the airway caused by mast cells, lymphocytes, and eosinophils. Type 2 helper T cell (T_H) activation is central in allergen-induced airway inflammation; elevation of T_H2-specific cytokines (eg, interleukin [IL] 4, IL-5, and IL-13) increases activation of allergen-specific immunoglobulin (Ig) E–producing B cells and recruitment of mast cells and eosinophils [1]. Murine asthma models suggest that T_H2-driven allergen-induced airway disease results from failure of immune tolerance rather than from defective T_H1 immunity [2].

Tryptophan is an amino acid that is essential for protein synthesis during T-cell activation and proliferation, and its metabolism is regulated by indoleamine 2,3-dioxygenase (IDO). Furthermore, dendritic cells (DCs) expressing IDO inhibit T-cell proliferation and may contribute to immunotolerance [3]. An IDO-expressing human monocyte–derived DC subpopulation, nonadherent $CD123^+$/chemokine receptor 6^+ ($CCR6^+$) cells, have been shown to suppress the allogeneic T-cell response [4]. Although these cells did not constitutively express IDO, upregulation of IDO in response to interferon (IFN) γ was observed [4]. In addition, IDO contributes to DC maturation induced by lipopolysaccharide, tumor necrosis factor-alpha (TNF-α), and poly(I:C). Mature DCs subsequently expand $CD4^+CD25^{high}$ regulatory T cells in an IDO-dependent manner [5]. Thus, IDO may induce immunotolerance.

The present study sought to determine whether IDO-expressing immature DCs (imDCs) could relieve airway inflammation and restore immunotolerance in ovalbumin (OVA)-sensitized mice. The role of exogenous IDO in proliferation, apoptosis, and cytokine secretion of airway $CD4^+$ T cells was also investigated. We hypothesized that injection of IDO-expressing imDCs could restore immune tolerance in mice suffering from allergic airway inflammation.

Materials and Methods

Animals

Twenty-four healthy BALB/c mice (4-6 weeks of age, male or female, weighing 18-22 g) were divided equally into 3 groups (n=6): the control group (including the OVA sensitized-group), the group exposed to IDO-expressing imDCs, and the group exposed to imDCs expressing the empty expression plasmid.

OVA-Sensitized Mouse Model

On days 0, 7, and 14, 0.1 mL of an OVA (Sigma, St. Louis, Missouri, USA)/$Al(OH)_3$ (Pierce, Rockford, Illinois, USA) mixture containing 50 μg OVA and 1 mg $Al(OH)_3$ was injected intraperitoneally. From days 21 to 25, the mice inhaled nebulized OVA in normal saline (10 g/L) for 30 minutes each day. Mice in the control group received injections and inhaled the same volume of normal saline at each time point.

On day 28, mice in the pEGFP-N1-IDO and the pEGFP-N1 groups were anesthetized by intraperitoneal injection of 0.3 mL 0.3% pentobarbital and IDO-expressing imDCs or DCs expressing the empty plasmid were injected into the trachea (1×10^7 imDCs/mouse). The control group and the OVA-sensitized mice did not receive cell injections. The mice were exposed to nebulized OVA in normal saline (10 g/L) 2 hours after intratracheal injection and for an additional 2 days. The mice were sacrificed after 48 hours.

Construction of the pEGFP-N1-IDO Expression Plasmid

High mouse IDO expression levels were selectively detected in certain cell types, including placenta cells [6]. Briefly, total RNA was isolated from the placenta of 15-day pregnant BALB/c mice and cDNA was synthesized using

the First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Polymerase chain reaction (PCR) was subsequently performed using a PCR kit following the manufacturer's instructions (Toyobo, Tokyo, Japan). Primers specific for mouse IDO were designed using Primer 5.0 and the mIDO gene sequence in GenBank (NM_008324). Additional nucleotides were included in the primer sequences to create the Bgl II and Kpn I restriction sites necessary for ligation into the pMD19T plasmid (Takara, Tokyo, Japan). After PCR, the 1243-bp fragment constituting the IDO cDNA was isolated and inserted into pMD19T, which were amplified after transformation in DH5α-competent *Escherichia coli* cells. The sequence of mIDO was also confirmed (Takara) and the mIDO cDNA was subsequently isolated and inserted into a mammalian expression plasmid, pEGFP-N1 (Clontech, Mountain View, California, USA), which contains the coding region for green fluorescent protein (GFP) under the control of the CMV promoter. The mIDO sequence was again confirmed (Takara).

Isolation and Liposome Transfection of Mouse Bone Marrow-Derived Immature DCs

imDCs were isolated from the bone marrow of healthy BALB/c mice aged 4-6 weeks and cultured as previously described [7,8]. Briefly, the bone marrow from femurs and tibias was flushed with phosphate buffered saline (PBS) and cultured in growth medium consisting of RPMI 1640 medium (Gibco, Grand Island, New York, USA) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2 mM of L-glutamin, 50 μM of 2-mercaptoethanol, and 10% heat-inactivated and filtered fetal calf serum (HyClone, Logan, Utah, USA). Bone marrow cells (2×10^6 cells) were cultured in 100-mm petri dishes in 10 mL of growth medium containing 200 U/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, New Jersey). After 3 days, another 10 mL of culture medium plus GM-CSF was added to the bone marrow cells. At days 6 and 8, the cell culture supernatant was removed and centrifuged, and the resulting pellet was resuspended in 10 mL of growth medium plus GM-CSF. At days 8-10, the cells were used in the subsequent experiments.

The pEGFP-N1-IDO expression plasmid (5 μg) was mixed with HBS buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) to a final volume of 50 μL. DOTAP liposome (30 μL; Roche, Mannheim, Germany) was added into 70 μL of HBS and left at room temperature for 15 minutes. The plasmid/HBS mixture was added to the liposome/HBS mixture and mixed thoroughly, and 2 mL of serum-free PRMI 1640 medium was added. The resulting medium was incubated in a 25-mL flask with imDCs. The cells were incubated for 8 hours at 37°C in 5% CO_2, after which the medium was replaced with medium containing 10% calf serum for 24 hours. To select for the growth of imDCs expressing the IDO gene, the cells were cultured for another 48 hours in medium containing 200 μg/mL G418 (Amresco, Solon, Ohio, USA). The control group (no transfection) and the group transfected with empty expression plasmid were used as controls. Intratracheal injection of the imDCs was carried out 36 hours after transfection.

Flow Cytometry Analysis of CD11c, MHC II, CD80, and CD86 Expression

At 72 hours post-transfection, the cells were harvested and collected by centrifugation. After removal of the supernatant, the cells were diluted with PBS to a concentration of 1×10^6 cells/mL. The cell suspension (100 μL) was added to each tube along with 2 μg of PE-CD11c and 2 μg of FITC-CD80, FITC-CD86, or FITC-MHC II. A blank tube was used as a control. After incubation for 30 minutes at 4°C, the cells were washed 3 times with 0.1 mol/L PBS followed by centrifugation at 2000 rpm. After the cells were diluted in 0.4 mL of 0.1 mol/L PBS, expression of CD11c, MHC II, CD80, and CD86 on imDCs was analyzed using flow cytometry.

Isolation of mRNA

imDCs were harvested at 36 hours post-transfection with 1 mL of RNAiso (Takara Bio Inc., Japan) at room temperature for 15 minutes. After centrifugation (12000*g* at 4°C for 5 min), the supernatant was transferred to a new tube and incubated with chloroform at a 5:1 ratio. After vigorous mixing, the solution was incubated on ice for 15 minutes followed by centrifugation at 12000*g* and 4°C for 15 minutes. The aqueous supernatant containing the RNA was carefully removed and transferred to a new tube. An equal volume of ice-cold ethanol was added and mixed by shaking. After 60-minute incubation at –20°C, the RNA was isolated by centrifugation at 12000*g* and 4°C for 10 minutes. The pellet was washed with 1 mL of ice-cold 75% ethanol followed by centrifugation at 12000*g* and 4°C for 5 minutes. After the pellet was air-dried, the RNA was resuspended in 20-30 μL DEPC until it was dissolved completely. Isolated RNA was stored at –70°C and quantified by measuring its OD. The RNA concentration (μg/μL) was calculated as OD260×0.04×dilution times.

Reverse Transcription of cDNA

Reverse transcription was performed using a commercially available kit (Fermentas Life Sciences, Vilnius, Lithuania). Briefly, a total of 10μL of RNA (0.4 μg/μL) was added to a mixture containing OligodT18 (1 μg/μL; Promega, Madison, Wisconsin, USA), RNase-Free water, 5X reaction buffer, RNAase inhibitor (20 U/μL), dNTPs, and M-MuLV reverse transcriptase (200 U/μL). After an incubation period of 10 minutes at 25°C, a reverse transcription period of 90 minutes at 42°C was followed by inactivation at 95°C for 5 minutes.

Polymerase Chain Reaction

The IDO and ß-actin gene primers were designed using the mouse genome sequence published in GenBank and Primer 5.0 software. The primers were synthesized by the Beijing Parkson Corporation (Beijing, China). The primer sequences were as follows:

IDO forward, 5′-AGATCTCGCCACCATGGCACT CAGTAAAATATCT-3′, and IDO reverse, 5′-GGTACC GGCCAACTCAGAAGAGCTTTC-3′; ß-actin forward, 5′-TGAACCCTAAGGCCAACCG-3′, and ß-actin reverse, 5′-ATGCCACAGGATTCCATACCC-3′. The product

produced after IDO primer amplification was 1243 bp, whereas that produced after ß-actin amplification was 480 bp.

The primers were diluted with distilled water to obtain a 10-μM working solution and stored separately at –20°C. The PCR reaction mixture included 5 μL of 10×reaction buffer, 3 μL of $MgCl_2$ (25 mM), 1 μL of dNTPs (2.5 mM), 1 μL of cDNA, 1 μL of KOD plus Taq (2.5 U/μL), 1 μL each of forward and reverse primers specific for mIDO or ß-actin (10 μM each), and 37 μL of H_2O. The PCR reaction was run at 94°C for 5 minutes followed by 35 cycles of 98°C for 10 seconds, 54°C for 30 seconds, and 68°C for 40 seconds. A final extension step at 72°C for 10 minutes was then carried out followed by termination of the reaction.

PCR products were visualized by electrophoresis in 1% agarose gel. Specifically, 10 μL of PCR product mixed with 2 μL of loading buffer was separated by electrophoresis at 80 mV for 1-1.5 hours; the gel was observed using a UVP machine (Bio-Rad Company, USA).

Western Blot Analysis

Total protein was extracted from imDC cells using PRO-PREP protein extraction solution (Intron, SungNam, Korea) and incubated at –20°C for 20 minutes. The cell lysates were centrifuged at 13000 RPM for 30 minutes and the supernatant was transferred to a new tube. The protein concentration was measured using the Bradford assay, and the remaining protein solution was stored at –70°C. Total cellular proteins (20 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were electro-transferred onto a polyvinylidene fluoride membrane followed by 3 washes with Tris-buffered saline and Tween 20 (TBST) for 5 minutes each. The membrane was incubated in 3 mL of blocking solution at room temperature for 60 minutes. A solution containing the primary antibody (rabbit anti-mouse IDO or ß-actin antibody) was diluted at 1:250 in blocking solution and incubated with the membrane at 4°C overnight. The membrane was washed 3 times with TBST for 5 minutes and incubated with horseradish peroxidase–labeled goat anti-rabbit secondary antibody diluted 1:1000 in blocking solution for 1 hour at 37°C. After 3 washes with TBST for 5 minutes, the membrane was developed with DAB until clear bands appeared.

Total Cell and Eosinophil Counts in Bronchoalveolar Lavage Fluid

The mice were anesthetized by intraperitoneal injection of 1% ketamine, after which tracheostomy, intubation, and lavage with 0.9% saline (0.5 mL, 5 times each) were performed. Bronchoalveolar lavage fluid (BALF) was centrifuged for 10 minutes at 20000 RPM, and the supernatants were collected and stored at –70°C. The cell pellets were treated with 0.83% Tris-NH_4Cl to disrupt the red blood cells; white blood cell levels were determined by hematoxylin-eosin staining of cell smears. White blood cells were identified as macrophages, lymphocytes, and eosinophils (EOS), and total cellular scores and EOS counts were determined in a blinded manner. At least 500 cells were counted for each slide.

Detection of Cytokines in BALF

IL-4, IL-5, and IFN-γ levels were determined in BALF by ELISA following the manufacturer's instructions (R&D Inc., Minneapolis, Minnesota, USA). The detectable range for IL-4 and IL-5 was 25-1000 pg/mL. The detection sensitivity of IFN-γ was 12.5-500 pg/mL.

Pulmonary Histopathology

Twenty-four hours after the final OVA provocation, the mice were anesthetized by intraperitoneal injection of 1% ketamine. Thoracotomy was performed to observe gross pulmonary changes. A 5-gauge needle was penetrated into the right ventricle and a tiny incision was made in the right atrial wall. Heart perfusion was performed using normal saline and 10% formaldehyde through the right atrial incision. Lung tissue was obtained, fixed overnight in 10% formaldehyde, dehydrated using an ethanol gradient, treated with xylenes, and embedded in paraffin. The tissues mounted in paraffin were sectioned, stained with hematoxylin-eosin, and observed using light microscopy for inflammatory cell infiltration, edema, and airway epithelial damage.

Apoptosis Analysis of Lymphocyte Cultures

$CD4^+$ T cells were isolated from the spleen of OVA-sensitized mice. $CD4^+$ T cells (1×10^4 cells/well) were mixed in a 96-well plate with imDCs (1×10^3, 1×10^4, or 5×10^4 cells/well) or imDCs expressing the empty vector or IDO. OVA (10 μg/mL) was added, and the mixed cells were cultured at 37°C in 5% CO_2 for 24 hours. TUNEL assays were performed to detect the rate of apoptosis. The cells were fixed with 10% formaldehyde, and the color was developed using DeadEnd Colorimetric Apoptosis Detection System (Promega). The apoptotic rate was calculated as a percentage using the following formula: (number of positive-staining nuclei/total nuclei) × 100%.

Statistical Analysis

Normally distributed continuous variables were compared by one-way analysis of variance. When a significant difference between groups was apparent, multiple comparisons of means were performed using a Bonferroni procedure with a type-I error adjustment. Data are presented as mean (SD). All statistical assessments were 2-sided and significance was set at .05. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, Illinois, USA).

Results

Effects of IDO on imDC Surface Marker Expression

Surface marker expression on imDCs derived from mouse bone marrow was assessed using flow cytometry. Of the cells analyzed, 8.81% were positive for both CD80 and CD11c, 9.92% expressed both CD86 and CD11c, and both MHCII and CD11c were expressed on 8.19%. Whereas 65%-70% of the cells expressed CD11c, expression of MHC-II, CD86,

Table 1. Surface Marker Expression on Immature DCs and Those Expressing IDO

Surface Marker Expression	Control Immature DCs	pEGFP-N1-IDO--Transfected
$CD11c^+$ 80^+	8.81%	5.83%
$CD11c^+$ 86^+	9.92%	9.42%
$CD11c^+$ MHC Class II^+	8.19%	6.19%

Abbreviation: DC, dendritic cells; IDO, indoleamine 2,3-dioxygenase; MHC, major histocompatibility complex.

and CD80 was low, which is similar to imDC surface marker expression (Table 1). Upon transfection with pEGFP-N1-IDO, no effects on surface marker expression were observed in imDCs; the double-positive expression rate was 5.83%, 9.42%, and 6.19% for CD80 and CD11c, CD86 and CD11c, and MHCII and CD11c, respectively (Table 1). In addition, expression of CD11c was detected in 60%-70% of the cells. Thus, IDO expression did not affect surface marker expression or maturation of these cells (Table 1).

Expression of IDO mRNA and Protein in imDCs

IDO mRNA expression in imDCs following 36 hours of gene transfection was assessed by RT-PCR (Figure 1). Whereas IDO mRNA was observed in imDCs transfected with the pEGFP-N1-IDO plasmid, no expression was detected in those transfected with the pEGFP-N1 empty vector or those not undergoing transfection. However, ß-actin expression was uniform in the cells.

As with the IDO mRNA, IDO protein was observed in only those imDCs transfected with the pEGFP-N1-IDO plasmid (Figure 2). Specifically, Western blot analysis revealed no IDO expression in untransfected imDCs or those transfected with the empty control vector. Analysis of glyceraldehyde-3-phosphate dehydrogenase expression revealed equal sample loading.

Effects of IDO-Expressing imDCs on BALF Cytology

Compared with the control group, total cell (Figure 3A)

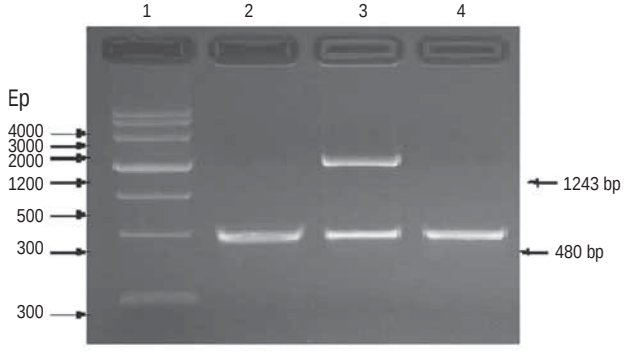

Figure 1. FIDO gene mRNA expression in imDCs from each group. Lane 1, DNA Marker λ-Hind III; Lane 2, Empty vector-transfected imDCs; Lane 3, IDO-expressing imDCs; Lane 4, Nontransfected immature DCs.

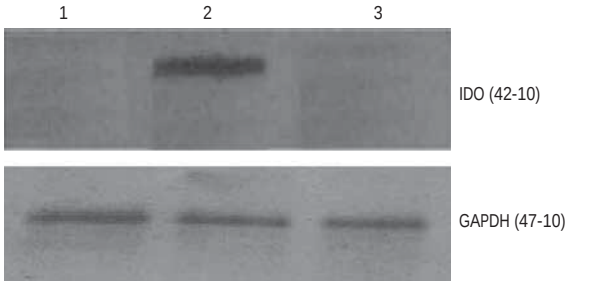

Figure 2. Western blot analysis of IDO expression in imDCs from each group. Lane 1, Empty vector-transfected imDCs; Lane 2, IDO-expressing imDCs; Lane 3, Nontransfected immature DCs.

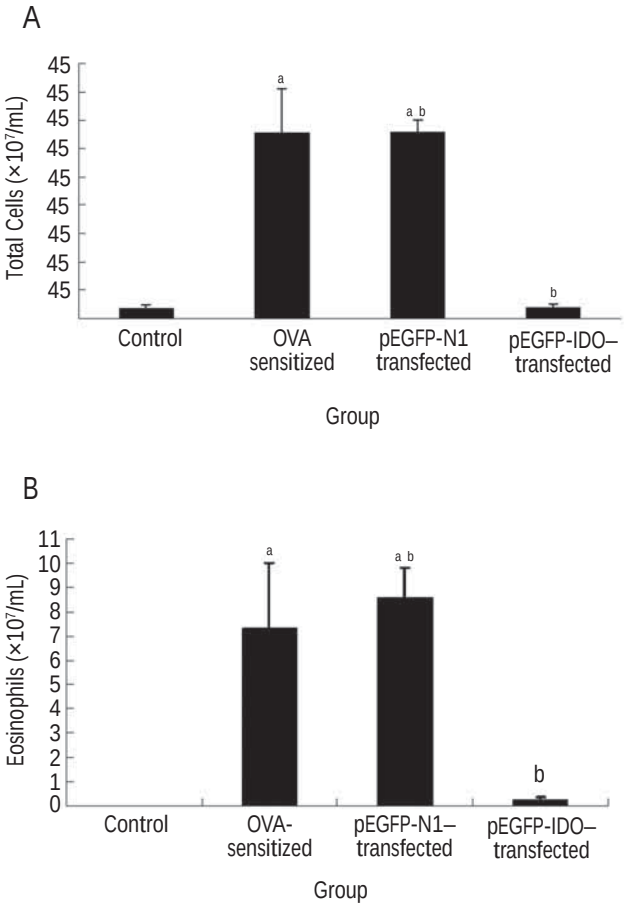

Figure 3. Effects of IDO-expressing imDCs on BALF cytology. Total cell counts (A) and (B) eosinophil counts were determined for each group. [a]Significant difference between the indicated group and the control group. [b]Significant difference between the indicated group and the OVA-sensitized group. Pair-wise multiple comparisons between groups were determined using a Bonferroni adjustment (α=0.001).

and EOS counts (Figure 3B) increased significantly in the OVA-sensitized group (P<.001). Mice exposed to empty vector-expressing imDCs also exhibited increased total cell and EOS counts in BALF (P<.001). Compared with the OVA-sensitized group, total cell and EOS counts decreased

significantly in the group exposed to IDO-expressing imDCs ($P<.001$). In addition, no significant differences in total cell and EOS counts were observed between the control group and the group exposed to IDO-expressing imDCs.

Effects of IDO-Expressing imDCs on IL-4, IL-5, and IFN-γ Levels in BALF

As shown in Table 2, significant differences in BALF IL-4, IL-5, and IFN-γ cytokine expression were detected between the 4 groups. Increased IL-4 and IL-5 levels and decreased IFN-γ levels were observed in the OVA-sensitized group, as compared with the control group ($P<.001$). In addition, cytokine levels were unaltered in those treated with empty vector-expressing imDCs. However, mice treated with IDO-expressing imDCs exhibited reduced IL-4 and IL-5 levels and increased IFN-γ levels ($P<.001$).

Table 2. IL-4, IL-5, and IFN-γ Expression in Bronchoalveolar Lavage Fluid[a]

Cytokines (pg/mL)	Control (n=6)	OVA-Sensitized (n=6)	pEGFP-N1-Transfected (n=6)	pEGFP-IDO-Transfected (n=6)
IL-4	100.19 (25.67)	410.00 (43.24)[b]	392.24 (48.78)[b,c]	91.19 (23.25)[c]
IL-5	98.65 (12.43)	396.01 (37.68)[b]	402.46 (42.21)[b,c]	101.65 (23.16)[c]
IFN-γ	266.78 (28.52)	156.78 (21.33)[b]	121.85 (23.48)[b,c]	232.56 (36.96)[c]

Abbreviation: IFN, interferon; IL, interleukin.
[a]*P* values are based on analysis of variance. Pair-wise multiple comparisons between groups were determined using a Bonferroni adjustment (α=0.001).
[b]Significantly different from the control group.
[c]Significantly different from the OVA-sensitized group.

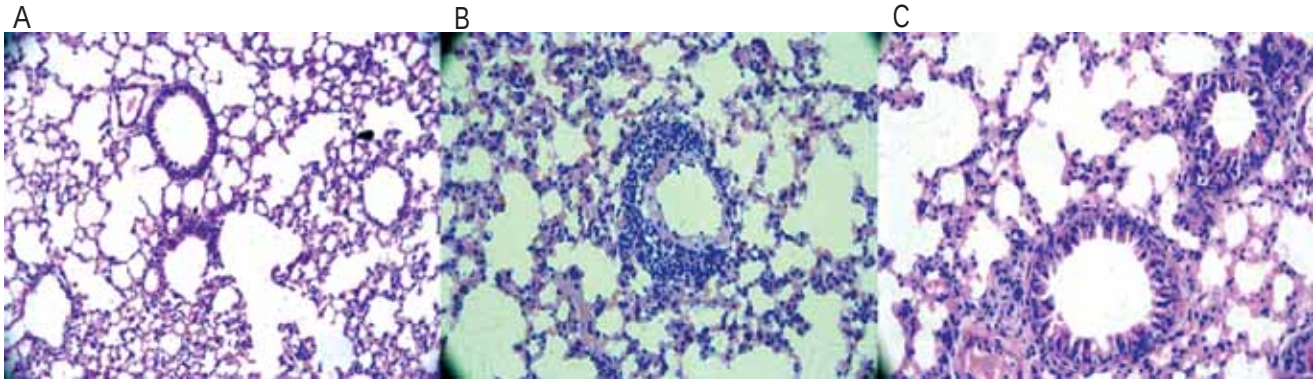

Figure 4. Effects of IDO-expressing imDCs on pulmonary histopathology. A, Control group (magnification ×200). B, Mouse lung tissue exposed to empty vector-transfected imDCs (magnification ×400). C, Mouse lung tissue exposed to IDO-expressing imDCs (magnification ×400).

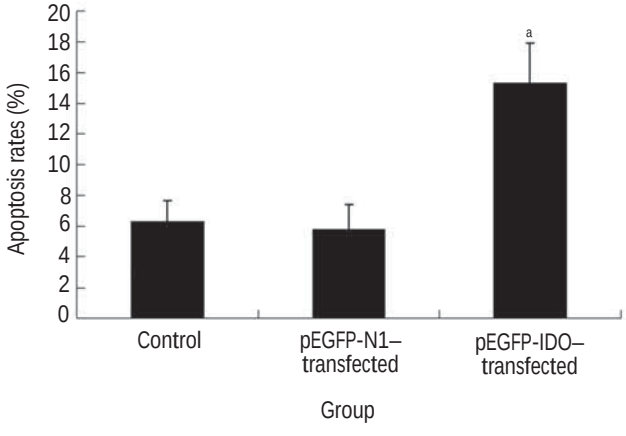

Figure 5. Effects of IDO-expressing imDC co-culture on CD4+T lymphocyte apoptosis. [a]Significant difference between the indicated and control groups. Pair-wise multiple comparisons between groups were determined using a Bonferroni adjustment (α=0.017).

Effects of IDO-Expressing imDCs on Pulmonary Histopathology

No apparent inflammatory changes in the pulmonary airways were observed in the control group (Figure 4A). Compared with the control group, significant inflammatory cell infiltration was observed in the bronchi, submucosal tissue of small vessels, and surrounding lung tissues, predominantly by EOS, lymphocytes, and neutrophils. In addition, exfoliation of epithelial cells, apparent vessel wall edema, and mucus plugs were detected in the OVA-sensitized and empty vector imDC groups (Figure 4B). Analysis of airways exposed to IDO-expressing imDCs revealed mild inflammation, reduced infiltration of small airways and small vessels by inflammatory cells, and lack of pulmonary airway edema. Epithelial damage and exfoliation were reduced (Figure 4C).

Effects of imDC and CD4+T Cell Co-culture on Apoptosis and Cytokine Expression

The CD4+ T-cell apoptotic response varied according to

Table 3. IL-4, IL-5, and IFN-γ Levels in Cell Culture Supernatant Were Detected by ELISA[a]

Cytokines (pg/mL)	Control (n=6)	pEGFP-N1-Transfected (n=6)	pEGFP-IDO-Transfected (n=6)
IL-4	296.39 (55.12)	253.45 (28.57)	87.91 (13.20)[b]
IL-5	356.27 (61.42)	287.67 (27.56)	104. 65 (21.34)[b]
IFN-γ	99.15 (32.78)	122.54 (37.48)	266.78 (15.52)[b]

Abbreviations: ELISA, enzyme-linked immunoassay; IFN, interferon; IL, interleukin.
[a]P values are based on ANOVA test. Pair-wise multiple comparisons between groups were determined using a Bonferroni adjustment (α=0.017).
[b]Significantly different from the control group.

co-culture with imDCs, empty vector-expressing imDCs, and IDO-expressing imDCs (Figure 5). Compared with the control group, the percentage of apoptotic $CD4^+$ T cells co-cultured with IDO-expressing imDCs was significantly higher (15.3% [2.6%] vs 6.3% [1.4%], P<.017).

The effects of imDC co-culture on cytokine expression were also analyzed. As shown in Table 3, IL-4 and IL-5 levels were significantly reduced and IFN-γ levels were elevated when $CD4^+$ T cells were co-cultured with IDO-expressing imDCs (P<.001).

Discussion

We used an OVA-sensitized mouse model to analyze the effects of IDO-expressing imDCs on total cell and EOS counts, cytokine expression, pulmonary histopathology, and apoptosis of $CD4^+$ T cells. Specifically, IDO-expressing imDCs altered cytokine levels to those of controls, reduced total cell and EOS counts, and improved pulmonary histopathology in OVA-sensitized mice. Furthermore, the mechanism by which IDO-expressing imDCs improve lung pathobiology could be through stimulation of $CD4^+$ T cell apoptosis. These findings suggest that IDO-expressing imDCs relieved allergic airway inflammation in OVA-sensitized mice, thus inducing immunotolerance.

DC function depends on the development of immune reactions, including cellular immunity, humoral immunity, and immunotolerance [9]. In vitro alteration of the biological properties of DCs as well as functional regulation help prevent and treat immune-related diseases (eg, hypersensitivity diseases [10] and autoimmune diseases).

In peripheral organs and tissues, DCs are primarily immature and incapable of T-cell activation. In phenotypical and functional terms, imDCs expressed low levels of surface MHC II, and DCs arising from myeloid- or lymphoid-derived precursors exhibited an immature phenotype characterized by a high phagocytic capacity and low expression of costimulatory molecules such as CD40, CD80, and CD86 [11]. Splenic and thymic DCs expressed moderate levels of MHC II (4% and 19%, respectively) and very low levels of CD86 [11]. Surface marker expression in long-term DC cultures revealed that 6.9% were MHC II–positive and 5.9% were CD80-positive [12]. In the present study, 8%-6% of the imDCs were MHC II–positive and 5%-8% were CD80-positive, which was consistent with the results of previous studies [11,12]. Importantly, no changes in surface marker expression were observed upon IDO expression.

Peripheral T cell–mediated immunotolerance may be mediated by cytotoxic T lymphocyte–related antigen-4 (CTLA4). Munn et al [13] demonstrated that ligation of CTLA4 with the DC costimulator B7 induced IDO expression in DCs. In addition, the mechanism by which CTLA4-expressing Treg-induced immunotolerance involved IDO-mediated catabolism of tryptophan and suppression of T-cell differentiation. In mice, IDO-expressing DCs can induce naïve T cells to differentiate into regulatory T cells through CTLA4/B7 ligation [14]. In the present study, increased apoptosis was observed in $CD4^+$ T cells cocultured with IDO-expressing imDCs.

Unlike immunotolerance induced by allergen vaccines, the present study investigated a novel therapeutic intervention. Allergen-targeting DNA vaccines effectively relieve pulmonary allergic airway inflammation in mice and decrease airway hyperreactivity in asthmatic mice [15-17]. However, the precise mechanism, which is possibly related to induction of T_H1 responses and correction of T_H1/T_H2 imbalance, remains unclear [18]. In vitro analysis by Klostermann et al [19] indicated that DCs isolated from patients with allergic diseases and expressing allergen-encoding genes mainly induced T_H1 responses, thus, correcting the T_H1/T_H2 imbalance. The present study revealed that imDCs expressing allergen genes not only altered in vitro cytokine levels but also reduced pulmonary inflammation in vivo.

DCs are principal antigen-presenting cells (APCs) in DNA vaccine–induced cell immunity and boost the immunological effects induced by DNA vaccines [20,21]. Gene-transfected killer DCs induce immunotolerance by eliminating antigen-specific T-cell clones. In the present study, increased $CD4^+$ T cell apoptosis was observed in mice receiving IDO-expressing imDCs, as compared to those in the control groups. In contrast, allergen vaccines take effect by inducing hyporeactivity of T cells to allergens; therefore, long-term maintenance therapy is required.

In conclusion, imDCs expressing IDO favorably altered cytokine expression and inflammatory cell numbers in OVA-sensitized mice. In addition, in vivo pulmonary inflammation

was reduced in response to IDO-expressing imDCs. Finally, the mechanism by which IDO-expressing imDCs influence allergic airway inflammation may be through stimulation of $CD4^+$ T-cell apoptosis. Thus, allergen-expressing imDCs may provide a novel immunointervention strategy for asthma through amelioration of the T_H2 cell response.

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References

1. Mazzarella G, Bianco A, Catena E, De Palma R, Abbate GF. Th1/Th2 lymphocyte polarization in asthma. Allergy. 2000;55:6-9.
2. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged inhaled allergen exposure can induce persistent tolerance. Am J Respir Cell Mol Biol. 2007;36:573-84.
3. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, Marshall B, Chandler P, Antonia SJ, Burgess R, Slingluff CL Jr, Mellor AL. Potential regulatory function of human dendritic cells expressing indoleamine 2, 3-dioxygenase. Science. 2002;297:1867-70.
4. Terness P, Chuang JJ, Bauer T, Jiga L, Opelz G. Regulation of human auto- and alloreactive T cells by indoleamine 2, 3-dioxygenase (IDO)-producing dendritic cells: too much ado about IDO? Blood. 2005;105:2480-6.
5. Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, Lavainne F, Rémy S, Brion R, Hubert FX, Heslan M, Rimbert M, Berthelot L, Moffett JR, Josien R, Grégoire M, Anegon I. IDO expands human CD4+CD25 high regulatory T cells by promoting maturation of LPS-treated dendritic cells. Eur J Immunol. 2007;37:3054-62.
6. Dai X, Zhu BT. Indoleamine 2, 3-dioxygenase tissue distribution and cellular localization in mice: implications for its biological functions. J Histochem Cytochem. 2010;58:17-28.
7. Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223:77-92.
8. Lutz MB, Suri RM, Niimi M, Ogilvie AL, Kukutsch NA, Rössner S, Schuler G, Austyn JM. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. Eur J Immunol. 2000;30:1813-22.
9. Shortman K, Liu YJ. Mouse and human dendritic cells subtypes. Nat Rev Immunol. 2002;2:151-61.
10. Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol. 2004;19:225-52.
11. Wilson NS, El-Sukkari D, Belz GT, Smith CM, Steptoe RJ, Heath WR, Shortman K, Villadangos JA. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. Blood. 2003;102:2187-94.
12. Quah B, Ni K, O'Neill HC. In vitro hematopoiesis produces a distinct class of immature dendritic cells from spleen progenitors with limited T cell stimulation capacity. Int Immunol. 2004;16:567-77.
13. Munn DH, Sharma MD, Mellor AL. Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2, 3-dioxygenase activity in dendritic cells. J Immunol. 2004;172:4100-10.
14. Mellor AL, Chandler P, Baban B, Hansen AM, Marshall B, Pihkala J, Waldmann H, Cobbold S, Adams E, Munn DH. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2, 3 dioxygenase. Int Immunol. 2004;16:1391-401.
15. Pichler CE, Marquardsen A, Sparholt S, Løwenstein H, Bircher A, Bischof M, Pichler WJ. Specific immunotherapy with Dermatophagoides pteronyssinus and D. farinae results in decreased bronchial hyperreactivity. Allergy. 1997;52:274-83.
16. Kwon SS, Kim NS, Yoo TJ. Vaccination with DNA encoding human T-cell epitopes suppresses Der p induced allergic responses in mice. J Asthma. 2005;42:119-25.
17. Jarman ER, Lamb JR. Reversal of established CD4+ type 2 T helper-mediated allergic airway inflammation and eosinophilia by therapeutic treatment with DNA vaccines limits progression towards chronic inflammation and remodeling. Immunology. 2004;112:631-42.
18. Cui Z. DNA vaccine. Adv Genet. 2005;54:257-89.
19. Klostermann B, Bellinghausen I, Böttcher I, Petersen A, Becker WM, Knop J, Saloga J. Modification of the human allergic immune response by allergen-DNA-transfected dendritic cells in vitro. J Allergy Clin Immunol. 2004;113:327-33.
20. Coombes BK, Manhony JB. Dendritic cell discoveries provide new insight into the cellular immunobiology of DNA vaccines. Immunol Lett. 2001;78:103-11.
21. Sumida SM, McKay PF, Truitt DM, Kishko MG, Arthur JC, Seaman MS, Jackson SS, Gorgone DA, Lifton MA, Letvin NL, Barouch DH. Recruitment and expansion of dendritic cells in vivo potentiate the immunogenicity of plasmid DNA vaccines. J Clin Invest. 2004;114:1334-42.

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