

Regulatory dendritic cells protect against allergic airway inflammation in a murine asthmatic model

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Background: Dendritic cells (DCs) are crucial for the induction of immunity and tolerance. Despite an improved understanding of the DC-mediated control of T_H1-biased immunity, little is known about how DCs regulate T_H2-mediated immunity.

Objective: The effects of immunostimulatory mature DCs (maDCs) and regulatory DCs (DCregs) on T_H2-driven allergic immunity involving IgE production were examined.

Methods: A murine model of airway hyperresponsiveness; the adoptive transfer of maDCs, DCregs, and T cells; and T-cell function were studied.

Results: Antigen-pulsed maDCs inhibited antigen-specific IgE production but enhanced the production of antigen-specific IgG1 and IgG2a. Analysis of *Ifng*^{-/-} mice and *Il21r*^{-/-} mice revealed that the inhibitory effect of antigen-pulsed maDCs on antigen-specific IgE production involved IL-21-producing T follicular helper cells but not IFN- γ -producing T_H1 cells. In contrast, antigen-pulsed DCregs impaired the production of antigen-specific IgE, IgG1, and IgG2a. *In vivo* blockade experiments showed that antigen-specific CD4⁺CD25⁺Foxp3⁺ regulatory T cells mainly mediated the suppressive effect of antigen-pulsed DCregs on the production of antigen-specific IgE. Antigen-pulsed maDCs promoted airway inflammation, whereas antigen-pulsed DCregs markedly suppressed the pathogenesis.

Conclusion: DCregs abolish T_H2-mediated IgE production and allergic inflammation based on antigen-specific dominant tolerance, whereas maDCs exacerbate the pathogenesis despite inhibiting the IgE response through the activation of diverse types of T_H cell responses.

Clinical implications: Immunotherapy with DCregs is a promising treatment for allergic airway inflammation. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Dendritic cells, T_H2 cells, T regulatory cells, tolerance, IgE, airway hyperreactivity

Dendritic cells (DCs), the most potent antigen-presenting cells (APCs), are defined by their dendritic morphology and consist of heterogeneous subsets with a myeloid or lymphoid lineage, as well as differing maturity in both lymphoid and peripheral tissues.¹ Immature DCs (iDCs) sense the presence of invading pathogens through various pattern-recognition receptors and process the pathogens intracellularly in inflamed tissues, developing into mature DCs (maDCs) with the upregulated expression of MHC and costimulatory molecules in inflammatory microenvironments.¹ Subsequently, maDCs home into secondary lymphoid tissues, where they present the processed antigens to naive T cells to generate effector T cells.¹ Consequently, DCs play a crucial role in the link between innate and adaptive immunity.¹

Accumulating indirect evidence suggests that iDCs are involved in the induction of peripheral tolerance under steady-state conditions *in vivo* mediated through the generation of anergic T cells and T regulatory (Treg) cells, including thymic-derived naturally occurring CD4⁺CD25⁺Foxp3⁺ Treg (nTreg) cells and inducible IL-10-producing T_R1 cells.^{2,3} Moreover, the modification of iDCs with certain immunosuppressive molecules generates tolerogenic DCs, which not only show a reduced T-cell stimulatory capacity but also produce anergic T cells and Treg cells.^{2,3} Although several types of tolerogenic DCs exert protective effects on T_H1-mediated immunopathogenesis,^{2,3} how T_H2-mediated allergic responses are regulated is unclear.

We have previously established modified DCs with a greater capacity to produce anergic T cells and Treg cells than previously known tolerogenic DCs *in vivo* and *in vitro*, which we therefore designated as regulatory DCs (DCregs).^{4,5} In this study we examined the effect of immunostimulatory maDCs and DCregs on T_H2-driven allergic immunity involving IgE production.

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Abbreviations used

APC:	Antigen-presenting cell
BALF:	Bronchoalveolar lavage fluid
DC:	Dendritic cell
DCreg:	Regulatory dendritic cell
DNP-OVA:	2,4-Dinitrophenol-conjugated ovalbumin
ICOS:	Inducible costimulator
iDC:	Immature dendritic cell
maDC:	Mature dendritic cell
nTreg:	Naturally occurring CD4 ⁺ CD25 ⁺ Foxp3 ⁺ T regulatory
T _{FH} :	T follicular helper
Treg:	T regulatory
WT:	Wild-type

METHODS**Mice**

Wild-type (WT) BALB/C and C57BL/6 mice were purchased from Charles River Laboratories, (Raleigh, NC). *Ifng*^{-/-} C57BL/6 mice⁶ were purchased from the Jackson Laboratory (Bar Harbor, Me). *Il21r*^{-/-} C57BL/6 mice⁷ were provided by W. J. Leonard (Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md). All mice were used between 5 and 12 weeks of age and maintained in specific pathogen-free conditions and in accordance with the guidelines of the Institutional Animal Care Committee of the RIKEN Institute.

DC preparation

The preparation of DCs has been described previously.^{4,5} Briefly, maDCs were prepared by culturing bone marrow cells with murine GM-CSF (20 ng/mL; Wako Pure Chemical Industries, Osaka, Japan) for 8 days, followed by stimulation with LPS (1 µg/mL; Sigma-Aldrich, St Louis, Mo) for 24 hours. DCregs were generated from bone marrow cells cultured with murine GM-CSF (20 ng/mL), murine IL-10 (20 ng/mL, Wako Pure Chemical Industries), and human TGF-β1 (20 ng/mL, Wako Pure Chemical Industries) for 8 days, followed by stimulation with LPS (1 µg/mL) for 24 hours. These DCs were pulsed with or without 2,4-dinitrophenol-conjugated ovalbumin (DNP-OVA; 50 µg/mL; LSL, Tokyo, Japan) for 3 hours, washed with PBS, and used for subsequent experiments (DNP-OVA/maDCs or DNP-OVA/DCregs, respectively). Plasmacytoid DCs were purified from spleen tissue by using anti-mPDCA-1 microbeads and AutoMACS (both from Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were unstimulated or stimulated with CpG ODN (0.1 µmol/L; Hokkaido System Science, Sapporo, Japan) for 24 hours, and the culture supernatants were assayed for IFN-α with ELISA kits (>5 pg/mL; BioSource International, Camarillo, Calif).

Isolation of CD4⁺ T cells

CD4⁺ T cells were purified from spleen tissue in normal and immunized mice on day 7 with the mouse CD4 T lymphocyte Enrichment Set-DM (BD Bioscience, San Diego, Calif). CD4⁺ T cells were then sorted into CD4⁺CXCR5⁻, CD4⁺CXCR5⁺, CD4⁺CD25⁻, or CD4⁺CD25⁺ T cells with high purity (>98%) by using AutoMACS and FACS Vantage (Becton Dickinson Immunocytometry Systems, San Jose, Calif).

Immunization and treatment protocol

Mice (5 per group) were intraperitoneally immunized with DNP-OVA (10 µg per mouse) in alum (2 mg per mouse) on day 0 and bled

to obtain sera on day 14 to measure the primary immunoglobulin response. Mice were then bled to collect sera and immunized intraperitoneally with DNP-OVA in alum on day 42 and bled to obtain sera on day 49 to measure the secondary immunoglobulin response. In other experiments the immunized mice received OVA by means of inhalation (20 mg/mL for 10 minutes) 7 times after the second immunization, and acetylcholine-induced airway resistance, bronchoalveolar lavage fluid (BALF) cells, and lung histology with hematoxylin and eosin staining (magnification ×100) were assessed 24 hours after the last inhalation.⁸ For the treatment of mice, a single or repetitive intravenous injection or injections of unpulsed or DNP-OVA-pulsed DCs (10⁶ per mouse) was given 7 days before the first immunization or on days 21, 28, and 35 after the first immunization to determine the preventive or therapeutic effect, respectively. For the adoptive transfer experiments, CD4⁺CXCR5⁺, CD4⁺CXCR5⁻, or CD4⁺CD25⁺ T cells (2 × 10⁶ per mouse) were intravenously injected into BALB/C mice 24 hours before the primary immunization, secondary immunization, or both. For the *in vivo* blockade experiments, intraperitoneal injections of neutralizing anti-IL-10 mAb (BD Bioscience), anti-CD25 mAb (BD Bioscience), or preimmune rat IgG used as a control immunoglobulin (each 250 µg per mouse) were performed on days 5, 3, and 1 before immunization.

Measurement of serum total and DNP-specific immunoglobulin

Detection of total and DNP-specific IgE, IgG1, and IgG2a was performed according to a previous report.⁹

Flow cytometry

Cells were stained with fluorescein-conjugated mAbs to mouse CD11c, CD40, CD80, CD86, H-2Kd, I-A/I-E, CXCR5, Ly-6G, B220, OX40, CD200, isotype-matched control mAb (BD Bioscience), inducible costimulator (ICOS), ICOS-L, Foxp3 (eBioscience, San Diego, Calif), CD25, and mPDCA-1 (Miltenyi Biotec). Analysis of fluorescence staining was performed with a FACSCalibur flow cytometer and CELLQuest Software (both from Becton Dickinson Immunocytometry).

RT-PCR

cDNA was synthesized from RNA in CD4⁺ T cells (10⁶), and PCR analysis was performed with specific primers for IL-21,¹⁰ BCL-6 (5'-AGCAAGAACGCCTGCATCCTTC-3' and 5'-CATCTCTGTATGCTGTGGGGACTG-3'), and β-actin.¹⁰

***In vitro* priming of CD4⁺ T cells**

CD4⁺ T cells (5 × 10⁶) obtained from DNP-OVA-immunized mice were cultured with DNP-OVA-pulsed DCs (5 × 10⁵) in 6-well flat plates (Becton Dickinson) for 3 days. After incubation, CD4⁺ T cells were negatively selected with biotinylated anti-I-A/I-E mAb (BD Bioscience) plus IMag streptavidin Particles Plus-DM (BD Bioscience). These preparations (<0.1% of I-A/I-E⁺ DCs) were rested in medium containing IL-2 (5 U/mL, Wako Pure Chemical Industries) for 3 days and used for subsequent experiments.

Antigen presentation assay

CD4⁺ T cells (2 × 10⁵) obtained from normal or DNP-OVA-immunized mice were cultured in 96-well flat-bottom plates (Becton Dickinson) with irradiated (15 Gy from a 137Cs source; Gammacell 40 Exactor; MDS Nordion, Ontario, Canada) syngeneic hemolyzed splenocytes used as APCs (2 × 10⁵) in the presence or absence of DNP-OVA (50 µg/mL). Irradiated unpulsed or DNP-OVA-pulsed DCs (2 × 10⁴) were also used as APCs. The culture was also performed in the presence or absence of IL-2 (10³ U/mL) or mAbs to CD3 and CD28 (10 µg/mL, both from BD Bioscience). Tritiated

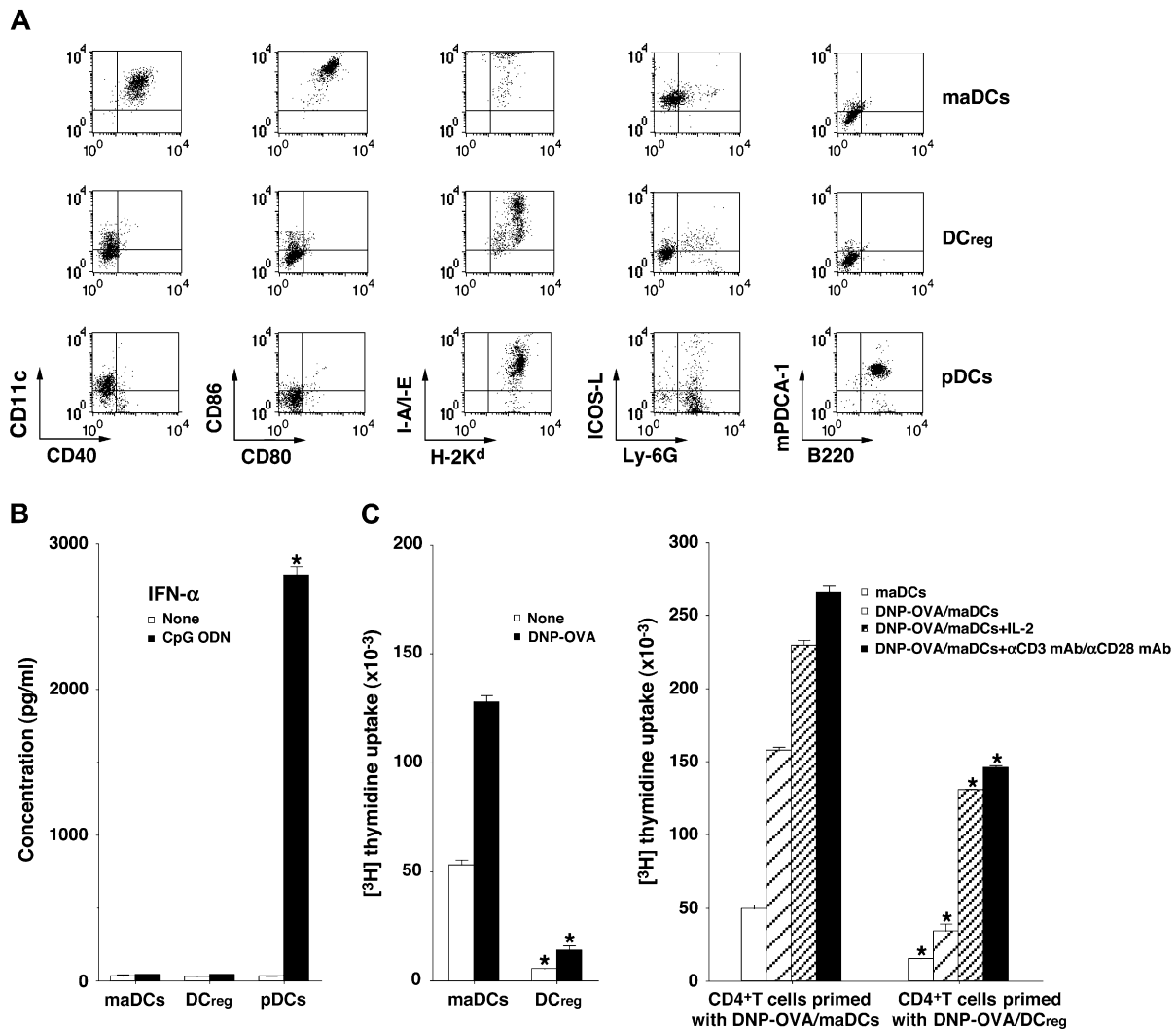


FIG 1. DCregs induce anergy in T_H2-polarized cells *in vitro*. **A** and **B**, Phenotype (Fig 1, A) and production of IFN-α (Fig 1, B) in DCs. **P* < .01 compared with maDCs. **C**, Proliferative responses of CD4⁺ T cells compared with the CD4⁺ T cells primed with maDCs (left) or DNP-OVA/maDCs (right). The results are representative of 4 experiments with similar results. pDCs, Plasmacytoid DCs.

thymidine (Amersham Biosciences Corp, Piscataway, NJ) incorporation was measured on day 3 for the last 18 hours. In another experiment the culture supernatants were assayed for IFN-γ (>1 pg/mL), IL-2 (>8 pg/mL), IL-4 (>5 pg/mL), and IL-10 (>13 pg/mL) with ELISA kits (BioSource International).

T/B-cell coculture

CD4⁺ T cells (10⁵) obtained from maDC-treated, DNP-OVA-immunized mice were cultured with B cells (10⁵; >98% B220⁺ cells), which were purified from spleen tissue in WT mice with the B lymphocyte Enrichment Set-DM (BD Bioscience) in 96-well flat-bottom plates for 11 days, and the culture supernatants were assayed for total IgG1 production.

Analysis of Treg cell function

CD4⁺ T cells obtained from DNP-OVA-immunized mice or CD4⁺CD25⁻ T cells obtained from normal mice (5 × 10⁴) were cultured with APCs (5 × 10⁴) in combination with DNP-OVA

(50 μg/mL) or anti-CD3 mAb (10 μg/mL) in the presence or absence of CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells (6.25 × 10³ to 5 × 10⁴). The proliferation was evaluated as described above.

Statistical analysis

For comparisons of multiple parameters, we used ANOVA.⁸ Statistical significance was set at a *P* value of less than .01. Data are expressed as means ± SD.

RESULTS

DCregs generate anergic T_H2-polarized cells

Mature DCs expressed high levels of CD11c, costimulatory, and MHC molecules, whereas DCregs had relatively high levels of MHC molecules and extremely low levels of CD11c and costimulatory molecules (Fig 1, A). In addition, both maDCs and DCregs neither expressed

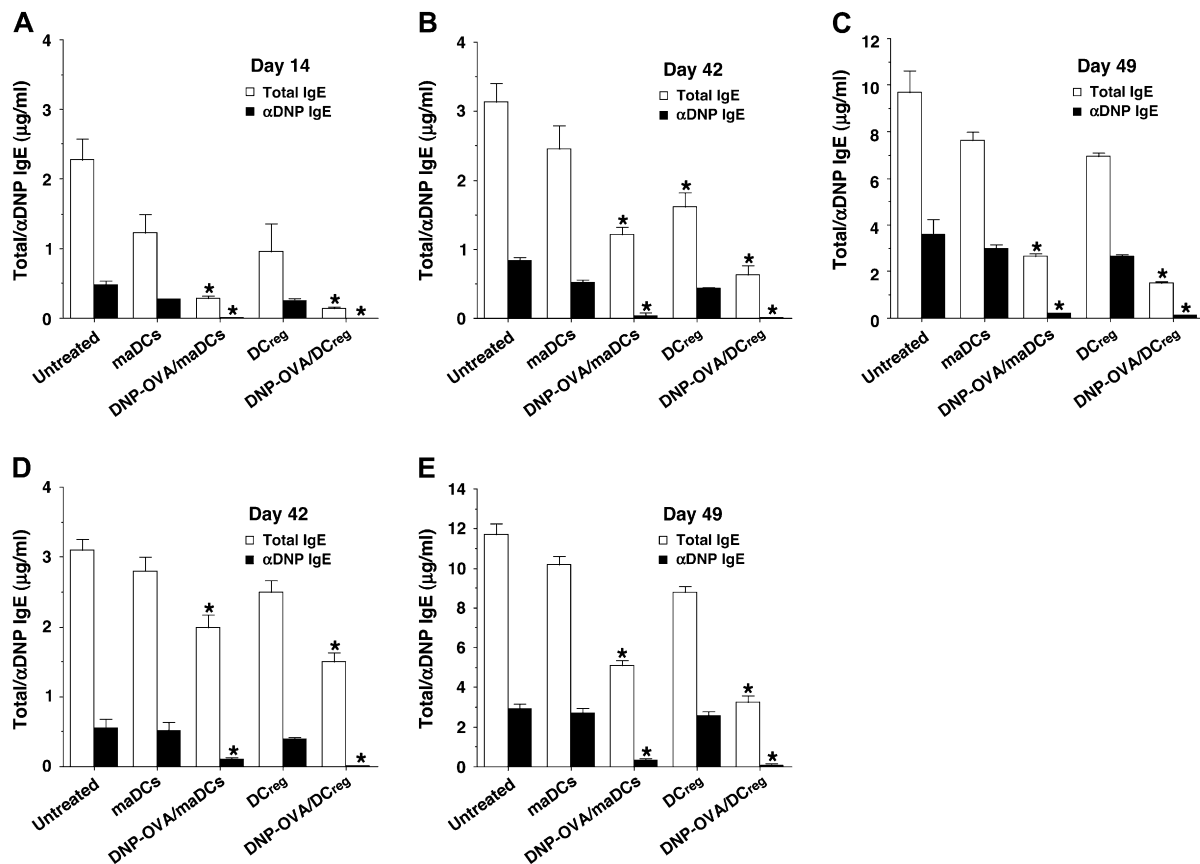


FIG 2. DCs regulate the IgE response. Preventive (A–C) or therapeutic (D and E) effect of DCs on the production of total and αDNP IgE in antigen-immunized mice (5 per group) is shown. * $P < .01$ compared with the untreated control group. The results are representative of 3 experiments with similar results.

Ly-6G, B220, or mPDCA-1 (Fig 1, A) nor produced IFN- α after stimulation with CpG ODN (Fig 1, B), indicating that DCregs are myeloid DC subsets distinct from conventional and plasmacytoid DC subsets. Furthermore, DNP-OVA/maDCs induced vigorous antigen-specific proliferation of T_H2 -polarized cells obtained from DNP-OVA-immunized mice (Fig 1, C), whereas DNP-OVA/DCregs not only were defective in the antigen-specific activation of T_H2 -polarized cells but also induced anergy to recall antigenic stimulation, which was partly overcome by additional IL-2 or anti-CD3 mAb plus anti-CD28 mAb (Fig 1, C).

DCregs suppress T_H2 -driven IgE response and asthma-like phenotype

Despite a good understanding of the role of DCs in the regulation of the T_H1 -mediated immune response, little is known about how a T_H2 -biased immunopathogenesis is regulated. Immunized mice showed a high serum level of IgE (Fig 2), and an asthma-like phenotype with histologic inflammation, increased numbers of BALF cells, and airway hyperresponsiveness was induced by sequential inhalation of antigen (Fig 3). Treatments with DNP-OVA/maDCs, but not maDCs, before and after immunization markedly inhibited the primary and secondary responses of total and DNP-specific IgE (Fig 2), whereas

the production of total and DNP-specific IgG1 (see Fig E1 in this article's Online Repository at www.jacionline.org) and IgG2a (see Fig E2 in this article's Online Repository at www.jacionline.org) was enhanced compared with that seen in the untreated group. Furthermore, DNP-OVA/maDCs not only promoted airway inflammation but also further increased the number of BALF cells compared with that seen in the untreated control group (Fig 3, A, B, D, and E), whereas they had little or no effect on airway hyperresponsiveness compared with the control group (Fig 3, C and F). In contrast, treatments with DNP-OVA/DCregs, but not DCregs, before and after immunization significantly inhibited the primary and secondary responses of total and DNP-specific IgE (Fig 2), IgG1 (see Fig E1 in the Online Repository at www.jacionline.org), and IgG2a (Fig E2), as well as asthma-like phenotype (Fig 3), compared with the untreated group.

Analysis of the function of $CD4^+$ T cells in antigen-primed mice

To clarify the mechanism responsible for the DC-mediated regulation of allergic responses, we analyzed the antigen-specific response of $CD4^+$ T cells obtained from antigen-primed mice that received DNP-OVA/maDCs or DNP-OVA/DCregs (DNP-OVA/maDC-treated group

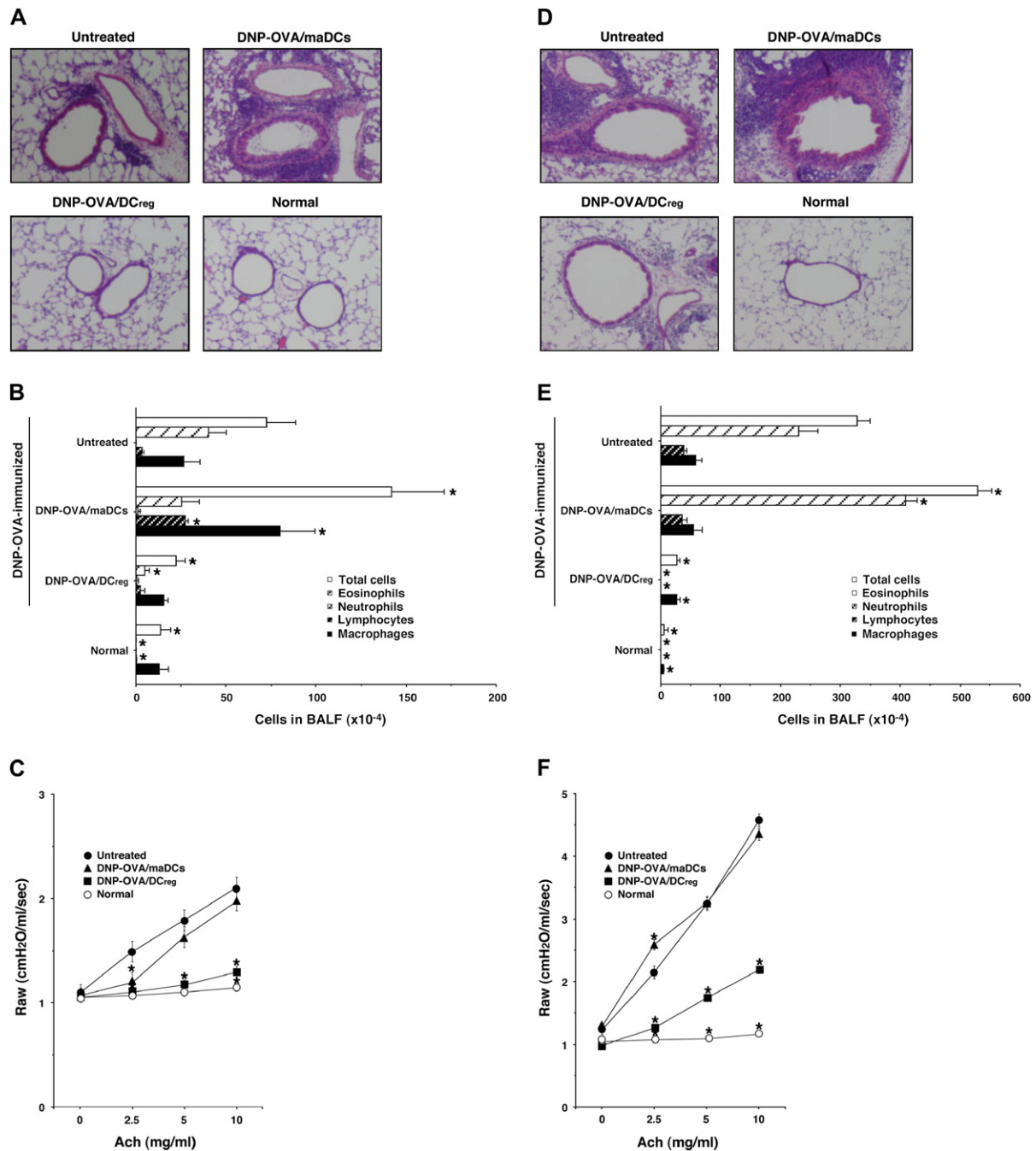
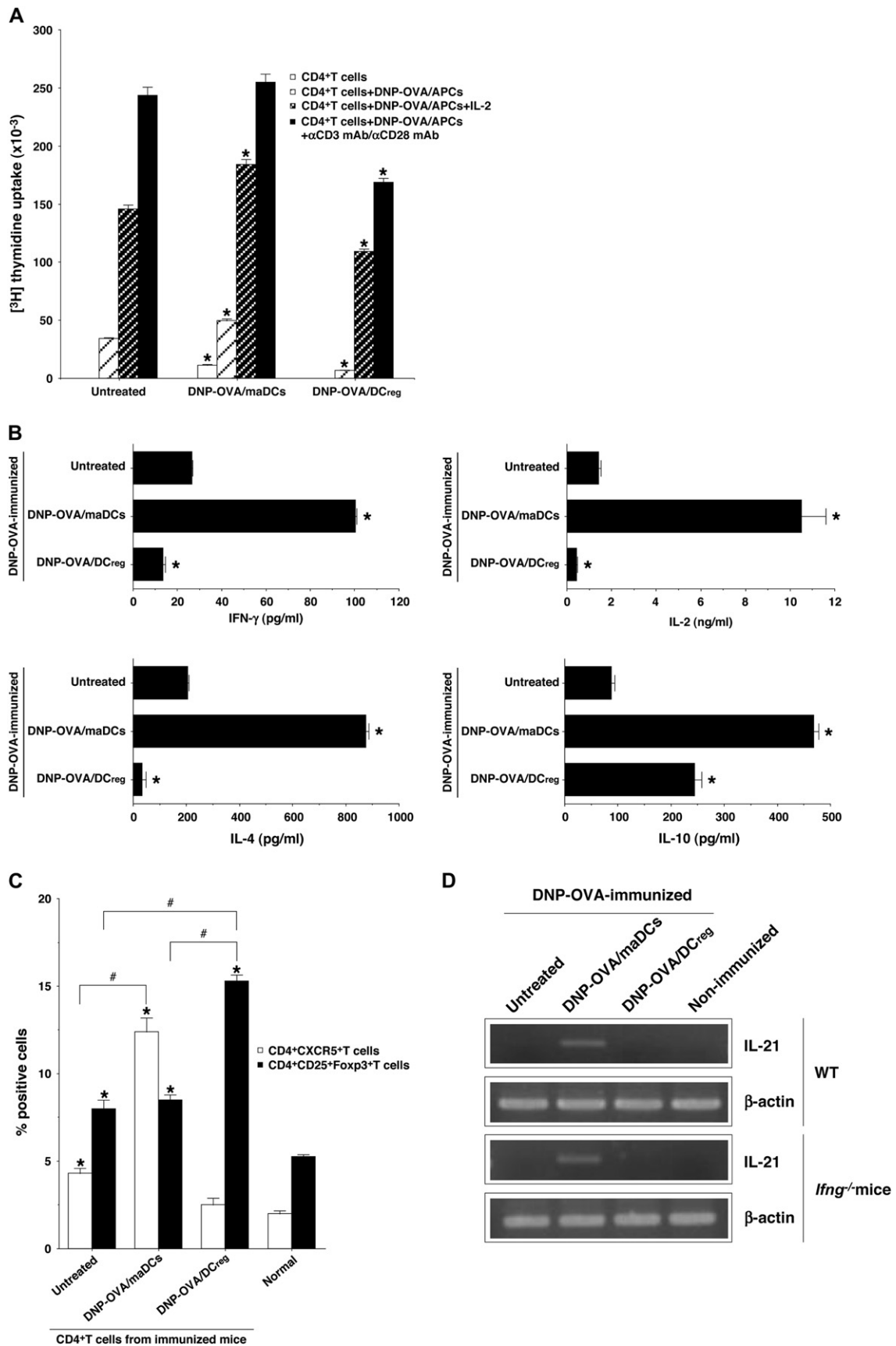


FIG 3. DCs regulate an asthma-like phenotype. Preventive (A–C) or therapeutic (D–F) effect of DCs on the inflammation of lung tissue (Fig 3, A and D), numbers of BALF cells (Fig 3, B and E), and airway hyperresponsiveness (Fig 3, C and F) in antigen-immunized mice (5 per group) is shown. * $P < .01$ compared with the untreated control group. The results are representative of 2 experiments with similar results. *Raw*, Airway resistance; *Ach*, acetylcholine.

or DNP-OVA/DCreg-treated group, respectively). $CD4^+$ T cells obtained from the DNP-OVA/maDC-treated group showed increased proliferation (Fig 4, A), as well as production of IFN- γ , IL-4, IL-2, and IL-10 (Fig 4, B) in response to antigenic stimulation compared with those from antigen-primed mice (untreated group). In contrast, $CD4^+$ T cells obtained from the DNP-

OVA/DCreg-treated group were hyporesponsive to antigenic stimulation and additional stimulations partly restored this response compared with responses seen in cells obtained from the untreated control group (Fig 4, A). In addition, they showed less production of IFN- γ , IL-4, and IL-2 and greater production of IL-10 compared with the untreated group (Fig 4, B).



Analysis of maDC-mediated regulation of allergic responses

We examined the role of IFN- γ -producing T_H1 cells in the maDC-mediated suppression of IgE production in *Ifng*^{-/-} mice. Similar to its effect in WT mice (Fig 5, A, and see Fig E3, A, in this article's Online Repository at www.jacionline.org), treatment with DNP-OVA/maDCs significantly suppressed the production of total and DNP-specific IgE (Fig 5, B) and enhanced the production of total and DNP-specific IgG1 (see Fig E3, B, in this article's Online Repository at www.jacionline.org) compared with that seen in the untreated control group in *Ifng*^{-/-} mice, whereas *Ifng*^{-/-} mice did not produce total and DNP-specific IgG2a (see Fig E4, B, in this article's Online Repository at www.jacionline.org).

Il21r^{-/-} mice have higher levels of IgE but lower levels of IgG1 than WT mice after antigen immunization,⁷ and CXCR5⁺ICOS⁺CD200⁺BCL-6⁺OX40⁻ T follicular helper (T_{FH}) cells not only produced IL-21 but also enhanced the production of IgG1 from B cells (see Fig E5 in this article's Online Repository at www.jacionline.org).^{11,12} DNP-OVA/maDCs not only increased the proportion of CD4⁺CXCR5⁺ T cells compared with those obtained from normal and immunized mice (Fig 4, C) but also induced transcription of IL-21 in CD4⁺ T cells in WT and *Ifng*^{-/-} mice (Fig 4, D). In addition, there was no significant difference in the production of DNP-specific IgE between the untreated control group and the DNP-OVA/maDC-treated group (Fig 5, C), whereas DNP-OVA/maDCs enhanced the production of total and DNP-specific IgG1 (see Fig E3, C, in the Online Repository at www.jacionline.org) and IgG2a (see Fig E4, C, in this article's Online Repository at www.jacionline.org) in *Il21r*^{-/-} mice. The adoptive transfer of CD4⁺CXCR5⁺ T cells obtained from the DNP-OVA/maDC-treated group into WT mice before immunization significantly inhibited the production of total and DNP-specific IgE (Fig 5, D) and enhanced the production of total and DNP-specific IgG1 (see Fig E3, D, in this article's Online Repository at www.jacionline.org), whereas CD4⁺CXCR5⁻ T cells increased total and DNP-specific IgG1 (see Fig E3, D) and IgG2a (see Fig E4, D in this article's Online Repository at www.jacionline.org) compared with levels seen in the untreated control group. Furthermore, the adoptive transfer of CD4⁺CXCR5⁺ T cells had no effect on an asthma-like phenotype (see Fig E6 in this article's Online Repository at www.jacionline.org).

Analysis of DCreg-mediated regulation of allergic responses

We examined antigen-specific cytokine production by CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells (Fig 6, A). CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells obtained from the DNP-OVA/maDC-treated group exhibited more

potent production of IL-2 and IL-10, whereas CD4⁺CD25⁻ T cells obtained from the DNP-OVA/DCreg-treated group produced a greater amount of IL-10 and less IL-2 compared with levels seen in the untreated control group.

We further evaluated the regulatory function of CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells. Although CD4⁺CD25⁻ T cells did not show any regulatory function (Fig 6, B), CD4⁺CD25⁺ T cells obtained from the DNP-OVA/DCreg-treated group showed more potent suppression of the antigenic response of *in vivo* antigen-primed CD4⁺ T cells (Fig 6, C, left panel) and polyclonal response of CD4⁺CD25⁻ T cells obtained from normal mice to anti-CD3 mAb (Fig 6, C, right panel), whereas CD4⁺CD25⁺ T cells obtained from the DNP-OVA/maDC-treated group showed less suppression than those obtained from the untreated group (Fig 6, C). We also observed that CD4⁺CD25⁺ nTreg cells only suppressed the polyclonal activation of CD4⁺CD25⁻ T cells (Fig 6, C, right panel), possibly because of the small numbers of OVA-specific CD4⁺CD25⁺ nTreg cells in naive mice.¹³

In vivo blockade experiments with anti-IL-10 mAb and anti-CD25 mAb were performed to determine the role of IL-10 and CD4⁺CD25⁺ Treg cells. Treatment with anti-CD25 mAb impaired the suppressive effect of DNP-OVA/DCregs on the production of total and DNP-specific IgE (Fig 6, D), as well as IgG1 and IgG2a (see Fig E7, A and B, in this article's Online Repository at www.jacionline.org), more effectively than did treatment with anti-IL-10 mAb. On the other hand, DNP-OVA/DCregs significantly increased the proportion of CD4⁺CD25⁺Foxp3⁺ Treg cells in immunized mice compared with other groups (Fig 4, C), and the adoptive transfer of these CD4⁺CD25⁺ T cells suppressed the production of total and DNP-specific IgE (Fig 6, E), as well as IgG1 and IgG2a (see Fig E7, C and D, in this article's Online Repository at www.jacionline.org), more than that of CD4⁺CD25⁺ nTreg cells. In addition, the adoptive transfer of these CD4⁺CD25⁺ Treg cells significantly suppressed an asthma-like phenotype (see Fig E6 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Here we have reported the effect of immunostimulatory maDCs or DCregs on T_H2-driven allergic immunity involving IgE production. Antigen-pulsed maDCs inhibited the production of antigen-specific IgE, and this event involved the generation of IL-21-producing CXCR5⁺ T_{FH} cells, but not IFN- γ -producing T_H1 cells, whereas antigen-pulsed maDCs enhanced airways inflammation. In contrast, antigen-pulsed DCregs suppressed both antigen-specific IgE production and the allergic response

FIG 4. DCs regulate T_H2-polarized cells *in vivo*. Proliferation (A), cytokine production (B), phenotype (C), and transcriptional expression (D) in CD4⁺ T cells are shown. **P* < .01 compared with the untreated control group (Fig 4, A and B) or normal mice (Fig 4, C). #*P* < .01 compared among groups (Fig 4, C). The results are representative of 5 experiments with similar results.

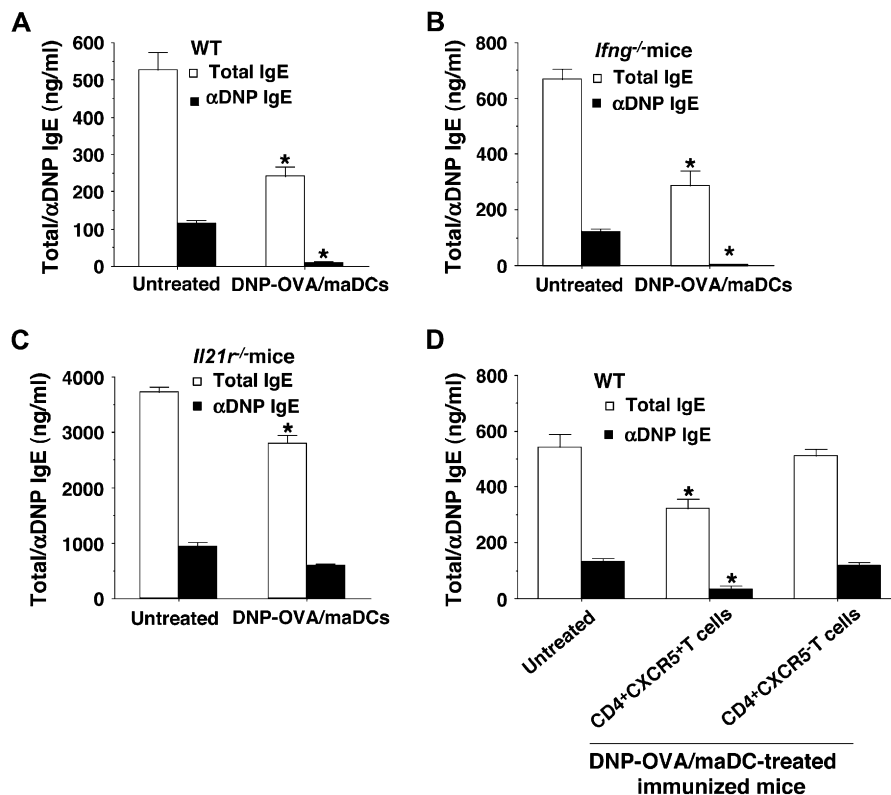


FIG 5. IgE response in the antigen-pulsed maDC-treated group. Preventive effect of antigen-pulsed maDCs (A–C) or the indicated CD4⁺ T subsets obtained from the antigen-pulsed maDC-treated group (D) on the production of total and αDNP IgE in antigen-immunized mice (5 per group) is shown. **P* < .01 compared with the untreated group. The results are representative of 3 experiments with similar results.

mediated through the antigen-specific expansion of CD4⁺CD25⁺Foxp3⁺ Treg cells.

Antigen-pulsed maDCs have been believed to be “powerful natural adjuvants” that suppress T_H2-mediated allergic diseases through IL-12-mediated induction of a T_H1-skewing immune response.¹ Indeed, antigen-pulsed maDCs significantly suppressed antigen-specific IgE production but enhanced antigen-specific IgG2a production. However, analysis of *Ifng*^{-/-} mice revealed that the inhibitory effect of antigen-pulsed maDCs on antigen-specific IgE production was inconsequential, whereas antigen-specific IgG2a production was suppressed. Furthermore, antigen-pulsed maDCs generated IL-21-producing T_{FH} cells in both WT and *Ifng*^{-/-} mice, and the inhibitory effect of antigen-pulsed maDCs on antigen-specific IgE production was markedly impaired in *Il21r*^{-/-} mice. We also showed that the adoptive transfer of CD4⁺CXCR5⁺ T cells induced by antigen-pulsed maDCs suppressed the antigen-specific IgE production. These results suggest that IL-21-producing CXCR5⁺ T_{FH} cells play a crucial role in the inhibitory effect of antigen-pulsed maDCs on antigen-specific IgE production, possibly mediated through the selective induction of apoptosis of B_e cells,¹⁴ whereas IFN-γ-producing T_H1 cells mediate antigen-specific IgG2a production.

A previous study reported that the injected myeloid DCs in the airways induced T_H2-dominated sensitization

to inhaled antigen, leading to eosinophilic airway inflammation.¹⁵ On the other hand, IL-21 inhibited the IgE response while it enhanced other T_H2 responses.^{7,16} We showed that treatment of antigen-primed mice with antigen-pulsed maDCs, as well as IL-21-producing CXCR5⁺ T_{FH} cells, failed to suppress the airway's allergic response, despite the inhibition of antigen-specific IgE production. We also observed adverse events: more than 60% of the immunized mice that received sequential injections of antigen-pulsed maDCs died on day 35 after primary immunization, possibly because of the excessive production of proinflammatory cytokines. Our results imply that T_H1 cells and T_H2 cells,¹⁷ rather than T_{FH} cells, are involved in the exacerbation of the inflammation of the airway in the DNP-OVA/maDC-treated group. Taken together, the use of antigen-pulsed maDCs might not be suitable for the treatment of T_H2-mediated allergic diseases.

We showed that antigen-pulsed DCregs not only induced an antigen-specific anergic state in antigen-primed T_H2-polarized cells but also generated CD4⁺CD25⁻ T_R1 cells and CD4⁺CD25⁺ Treg cells *in vivo*. Unlike CD4⁺CD25⁺ nTreg cells, these CD4⁺CD25⁺ Treg cells suppressed antigen-primed T_H2-polarized cells in an antigen-specific fashion. *In vivo* blockade experiments showed that CD4⁺CD25⁺ Treg cells mainly mediated the suppressive effect of

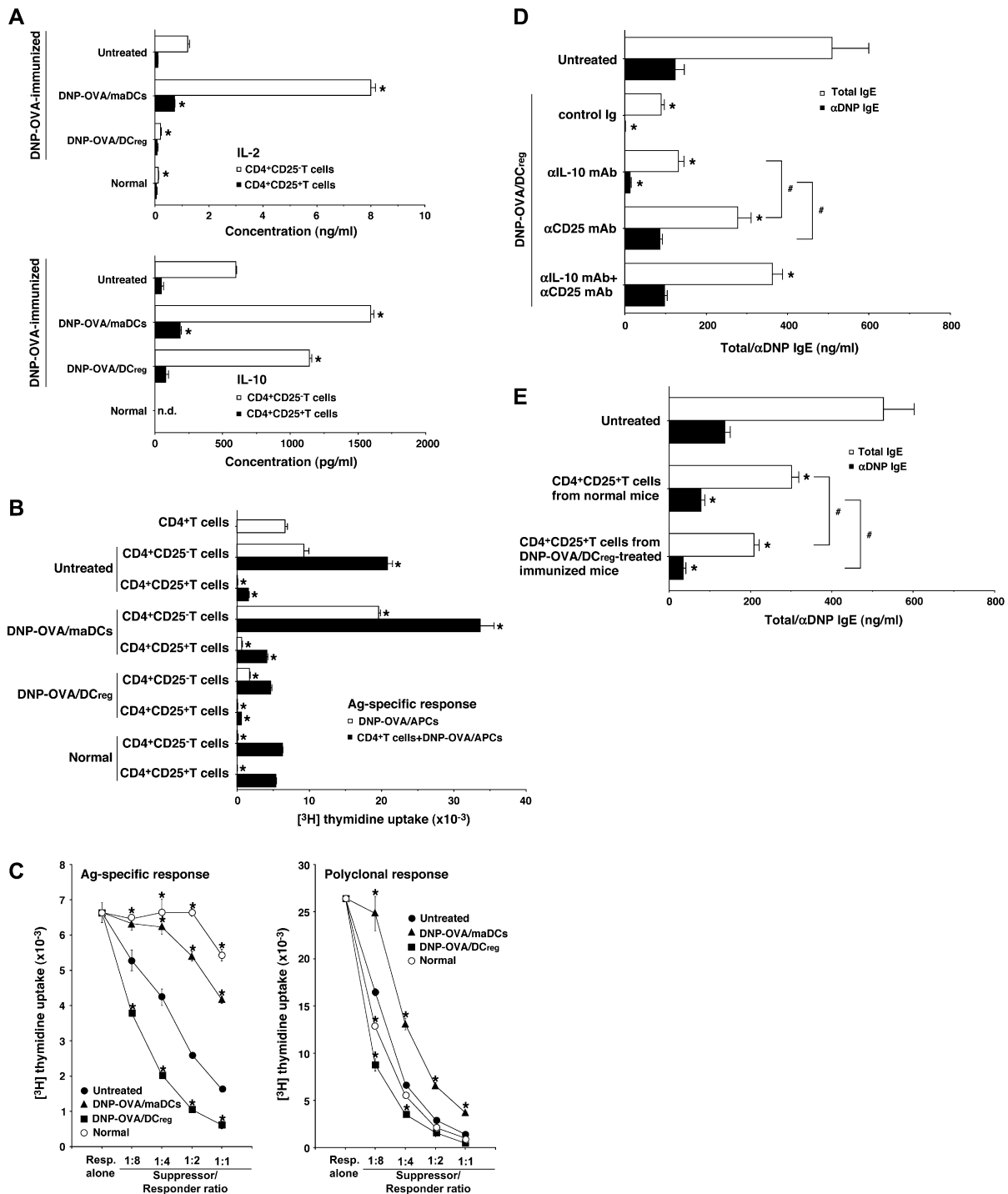


FIG 6. Analysis of the antigen-pulsed DCreg-treated group. Cytokine production (**A**) and regulatory effect of CD4⁺CD25⁺ T cells (**B** and **C**) are shown. Role of IL-10 (**D**), CD25⁺ cells (**D**), and CD4⁺CD25⁺ T cells (**E**) in IgE production in antigen-immunized mice (5 per group) is also shown. **P* < .01 compared with the untreated control group; #*P* < .01 among groups. The results are representative of 3 experiments with similar results.

antigen-pulsed DCregs on antigen-specific IgE production. In addition, the adoptive transfer of CD4⁺CD25⁺ Treg cells induced by antigen-pulsed DCregs not only suppressed antigen-specific IgE production more than CD4⁺CD25⁺ nTreg cells but also inhibited pathogenesis.

Therefore antigen-specific CD4⁺CD25⁺ Treg cells generated by DCregs are responsible for the impaired production of antigen-specific IgE, as well as IgG1 and IgG2a, through the direct suppression of T_H2 cells and B cells¹⁸⁻²⁰ and the protection against an asthma-like

phenotype. Collectively, the immunotherapeutic strategy using antigen-pulsed DCregs allows a benefit for treatment of T_H2 -driven allergic diseases without adverse events.

In summary, the findings reported here highlight a novel immunotherapeutic approach using DCregs for the treatment of experimental allergic asthma. Recent studies found that $CD4^+CD25^+$ nTreg cells protected against murine experimentally induced asthma.^{21,22} Moreover, human $CD4^+CD25^+$ nTreg cells occur at lower frequency than their murine counterparts, and a practical obstacle to prevent the clinical application of these experimental approaches is the difficulty in isolating sufficient numbers of $CD4^+CD25^+$ nTreg cells from human blood.^{23,24} We have previously reported that human DCregs exhibited a similar phenotype and T-cell regulatory function, including the generation of antigen-specific $CD4^+CD25^+$ Treg cells from naive $CD4^+$ T cells *in vitro* to murine counterparts.²⁵ Therefore the availability of a large number of human DCregs might provide for the antigen-specific control of allergic immune responses. To test this hypothesis, the development of immunotherapy with human DCregs is being conducted in our laboratories. In addition, further insight into the molecular mechanism underlying the DCreg-mediated regulation of T-cell function might lead to a better understanding of the induction of peripheral tolerance.

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REFERENCES

1. Sato K, Fujita S. Dendritic cells—nature and classification. *Allergol Int* 2007;56:183-91.
2. Morelli AE, Thomson AW. Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction. *Immunol Rev* 2003;196:125-46.
3. Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 2006;108:1435-40.
4. Sato K, Yamashita N, Yamashita N, Baba M, Matsuyama T. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity* 2003;18:367-79.
5. Fujita S, Seino K, Sato K, Sato Y, Eizumi K, Yamashita N, et al. Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response. *Blood* 2006;107:3656-64.
6. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* 1993;259:1739-42.
7. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science* 2002;298:1630-4.
8. Yamashita N, Tashimo H, Matsuo Y, Ishida H, Yoshiura K, Sato K, et al. Role of CCL21 and CCL19 in allergic inflammation in the ovalbumin-specific murine asthmatic model. *J Allergy Clin Immunol* 2006;117:1040-6.
9. Tomura T, Watarai H, Honma N, Sato M, Iwamatsu A, Kato Y, et al. Immunosuppressive activities of recombinant glycosylation-inhibiting factor mutants. *J Immunol* 1999;162:195-202.
10. Brandt K, Bulfone-Paus S, Foster DC, Ruckert R. Interleukin-21 inhibits dendritic cell activation and maturation. *Blood* 2003;102:4090-8.
11. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 2005;5:853-65.
12. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5⁺ follicular B helper T cell maintenance in vivo. *J Immunol* 2005;175:2340-8.
13. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-61.
14. Harada M, Magara-Koyanagi K, Watarai H, Nagata Y, Ishii Y, Kojo S, et al. IL-21-induced B_e cell apoptosis mediated by natural killer T cells suppresses IgE responses. *J Exp Med* 2006;203:2929-37.
15. Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* 2000;106:551-9.
16. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF Jr, Cheever AW, et al. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest* 2006;116:2044-55.
17. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175-83.
18. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, et al. Relation of $CD4^+CD25^+$ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;365:608-15.
19. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567-75.
20. Lim HW, Hillsamer P, Banham AH, Kim CH. Direct suppression of B cells by $CD4^+CD25^+$ regulatory T cells. *J Immunol* 2005;175:4180-3.
21. Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, et al. $CD4^+CD25^+$ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 2005;202:1549-61.
22. Joetham A, Takada K, Taube C, Miyahara N, Matsubara S, Koya T, et al. Naturally occurring lung $CD4^+CD25^+$ T cell regulation of airway allergic responses depends on IL-10 induction of TGF- β . *J Immunol* 2007;178:1433-42.
23. Godfrey WR, Spoden DJ, Ge YG, Baker SR, Liu B, Levine BL, et al. Cord blood $CD4^+CD25^+$ -derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 2005;105:750-8.
24. Li L, Godfrey WR, Porter SB, Ge Y, June CH, Blazar BR, et al. $CD4^+CD25^+$ regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. *Blood* 2005;106:3068-73.
25. Sato K, Yamashita N, Yamashita N, Baba M, Matsuyama T. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* 2003;101:3581-9.

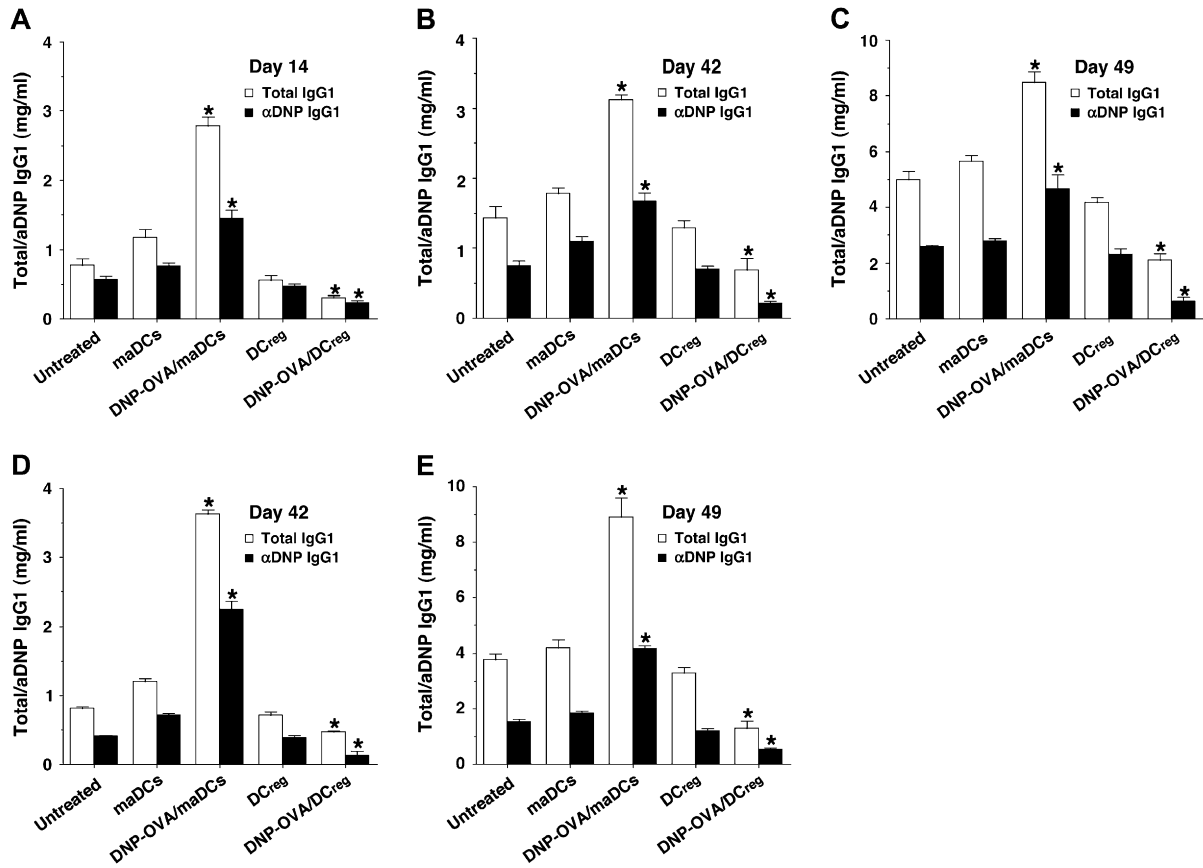


FIG E1. DCs regulate the IgG1 response. Preventive (A-C) or therapeutic (D and E) effect of DCs on the production of total and αDNP IgG1 in antigen-immunized mice (5 per group) is shown. * $P < .01$ compared with the untreated control group. The results are representative of 3 experiments with similar results.

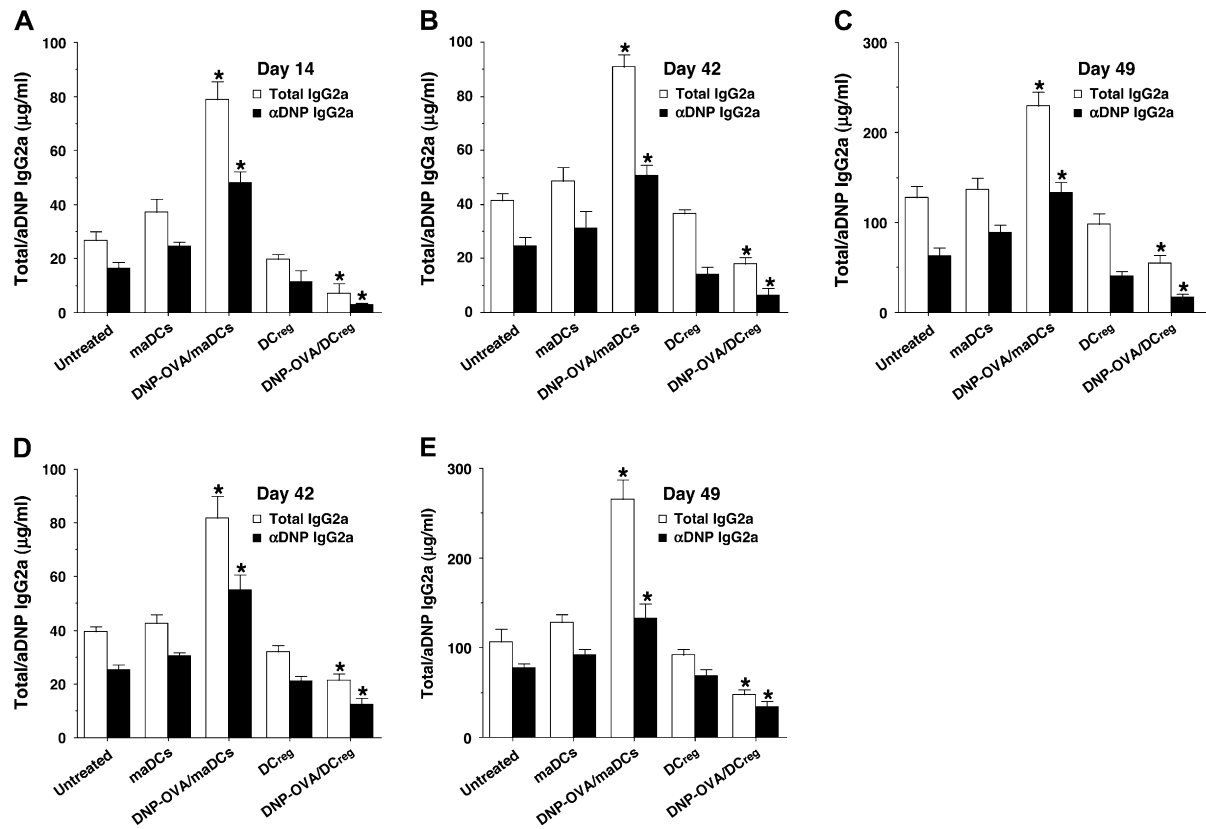


FIG E2. DCs regulate the IgG2a response. Preventive (A-C) or therapeutic (D and E) effect of DCs on the production of total and αDNP IgG2a in antigen-immunized mice (5 per group) is shown. * $P < .01$ compared with the untreated control group. The results are representative of 3 experiments with similar results.

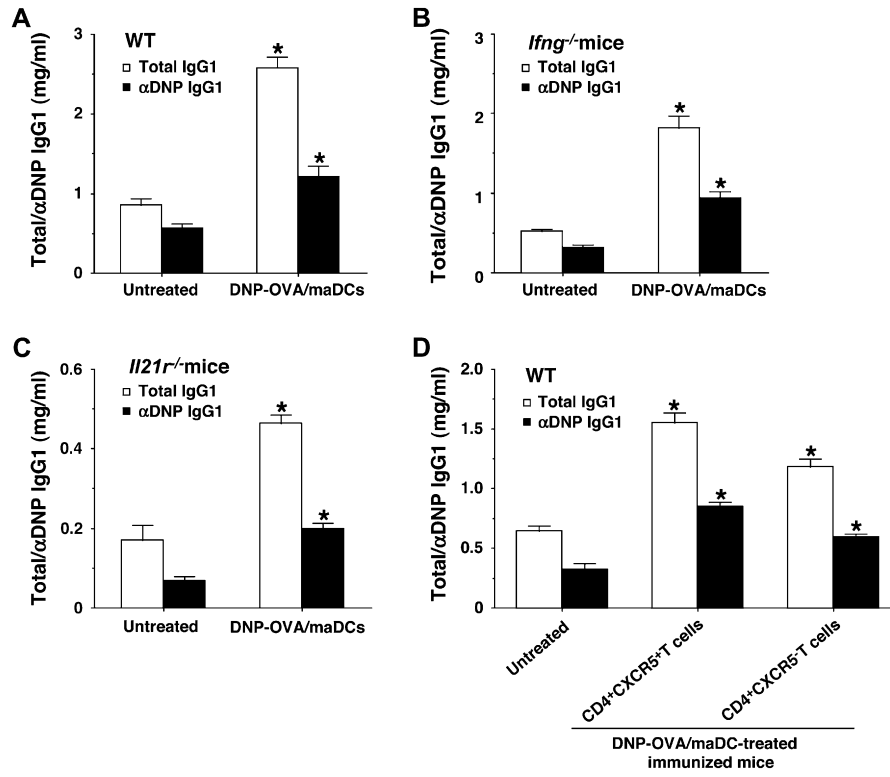


FIG E3. IgG1 response in the antigen-pulsed maDC-treated group. Preventive effect of antigen-pulsed maDCs (A-C) or the indicated CD4⁺ T subsets obtained from the antigen-pulsed maDC-treated group (D) on the production of total and αDNP IgG1 in antigen-immunized mice (5 per group) is shown. **P* < .01 compared with the untreated group. The results are representative of 3 experiments with similar results.

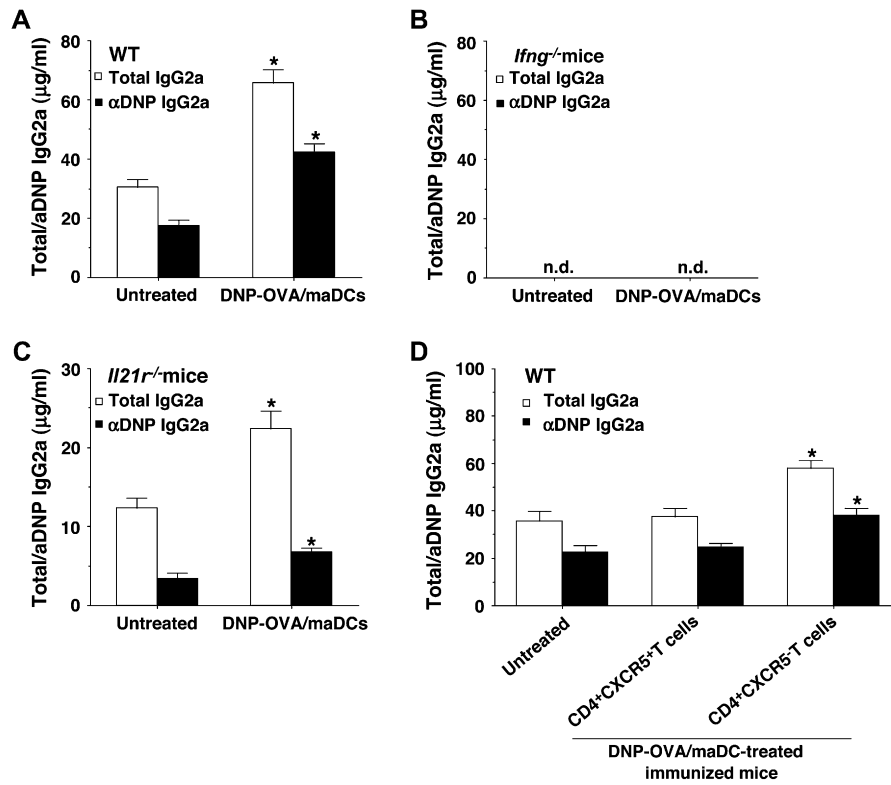


FIG E4. IgG2a response in the antigen-pulsed maDC-treated group. Preventive effect of antigen-pulsed maDCs (A-C) or the indicated CD4⁺ T subsets obtained from the antigen-pulsed maDC-treated group (D) on the production of total and αDNP IgG2a in antigen-immunized mice (5 per group) is shown. **P* < .01 compared with the untreated group. The results are representative of 3 experiments with similar results.

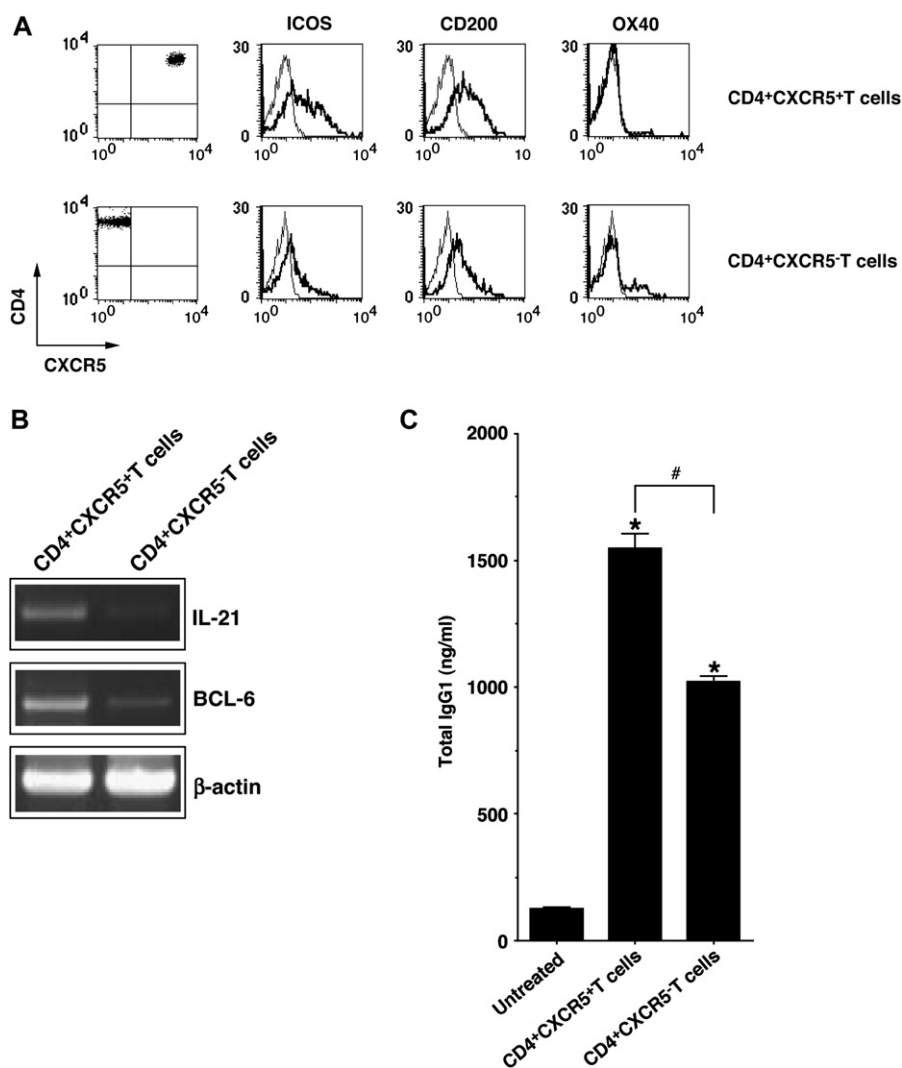


FIG E5. Analysis of CD4⁺CXCR5⁺ and CD4⁺CXCR5⁻ T cells generated by antigen-pulsed maDCs. Phenotype (**A**) and transcriptional expression (**B**) in CD4⁺CXCR5⁺ and CD4⁺CXCR5⁻ T cells are shown. Production of total IgG1 by B cells (**C**) is also shown. * $P < .01$ compared with the untreated group; # $P < .01$ among groups. The results are representative of 3 experiments with similar results.

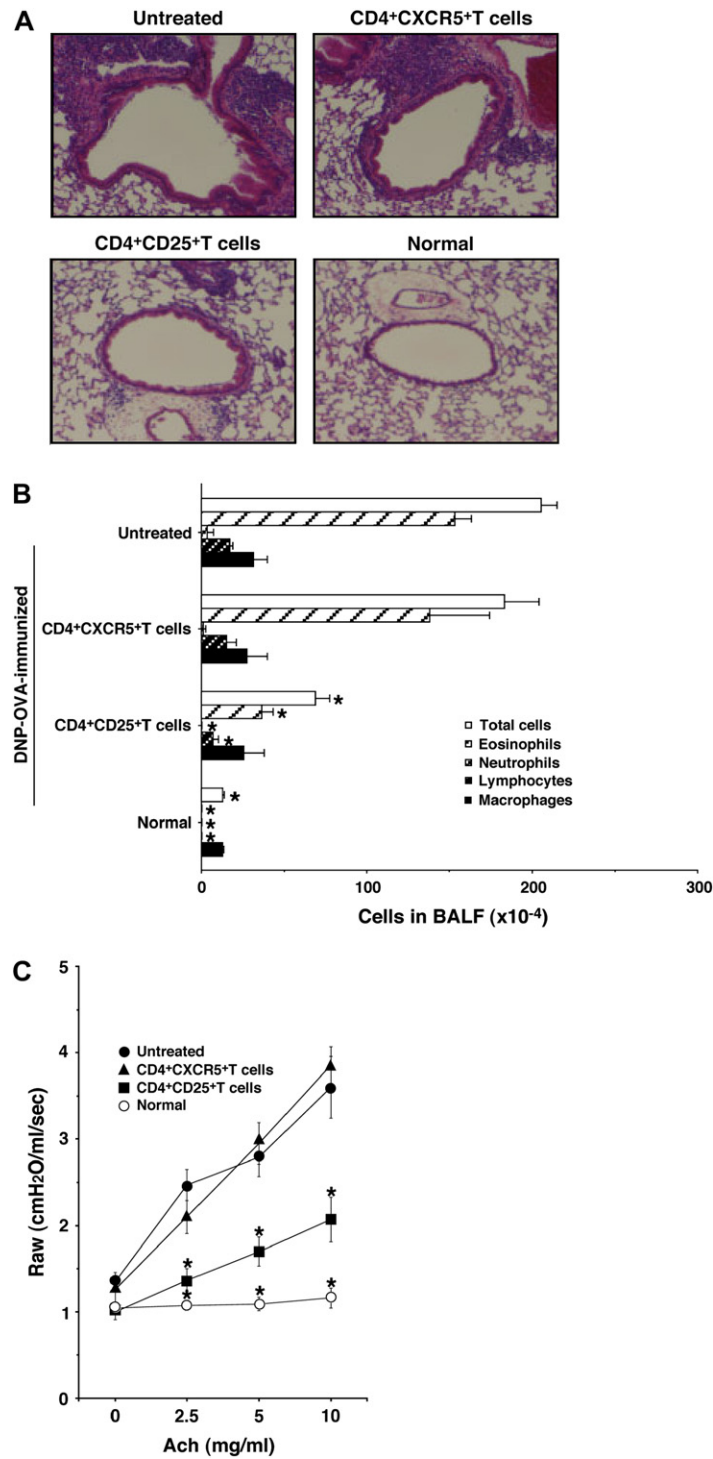


FIG E6. CD4⁺CD25⁺ T cells regulate an asthma-like phenotype. Effect of CD4⁺ T-cell subsets on the inflammation of lung tissue (**A**), numbers of BALF cells (**B**), and airway hyperresponsiveness (**C**) in antigen-immunized mice (5 per group) is shown. * $P < .01$ compared with the untreated control group. The results are representative of 2 experiments with similar results. Raw, Airway resistance; Ach, acetylcholine.

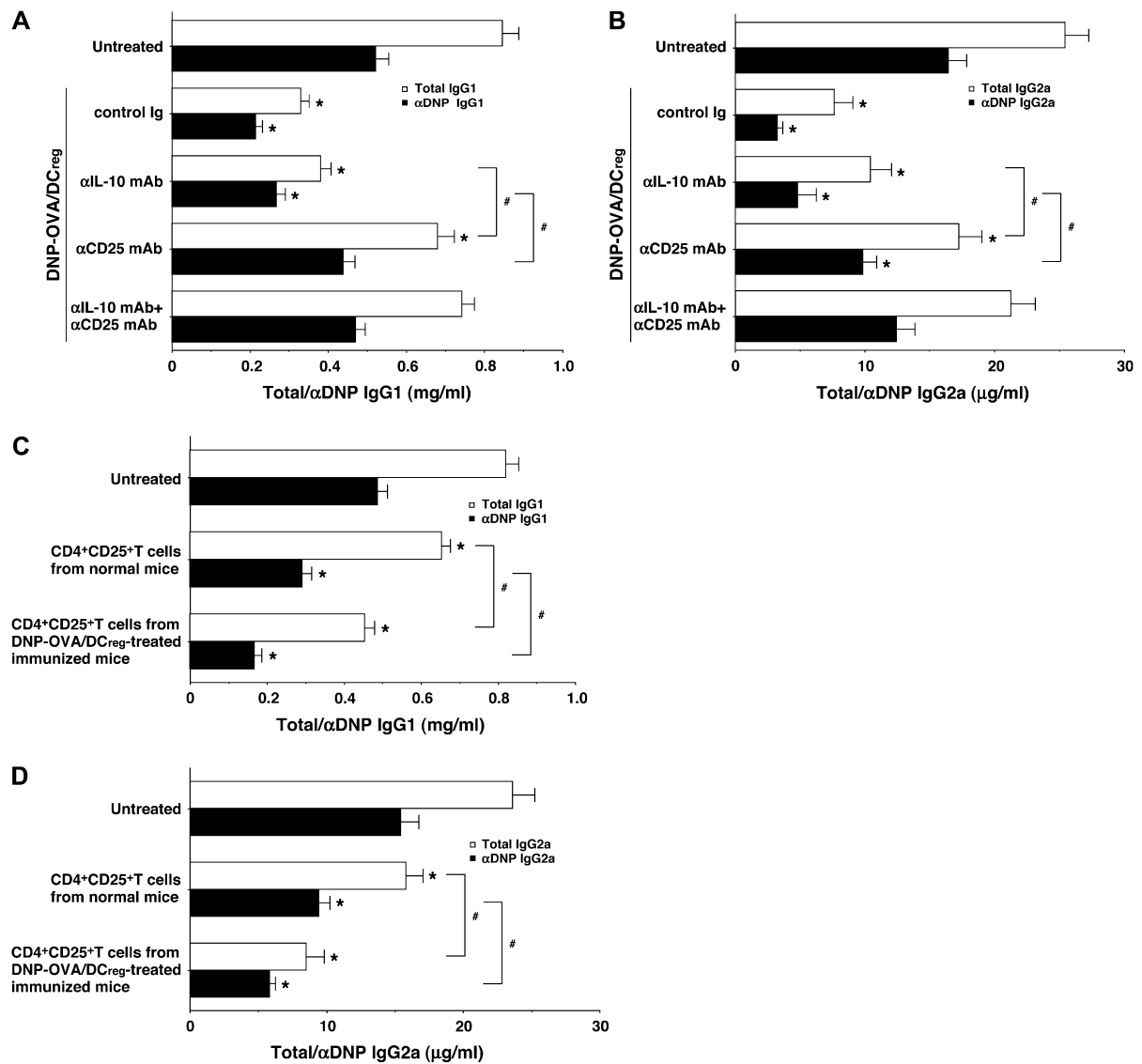


FIG E7. IgG1 and IgG2a responses in the antigen-pulsed DC_{reg}-treated group. Role of IL-10 (**A** and **B**), CD25⁺ cells (**Fig E6**, **A** and **B**), and CD4⁺CD25⁺ T cells (**C** and **D**) in IgG1 (**Fig E6**, **A** and **C**) and IgG2a (**Fig E6**, **B** and **D**) production in antigen-immunized mice (5 per group) is shown. **P* < .01 compared with the untreated control group; #*P* < .01 among groups. The results are representative of 3 experiments with similar results.