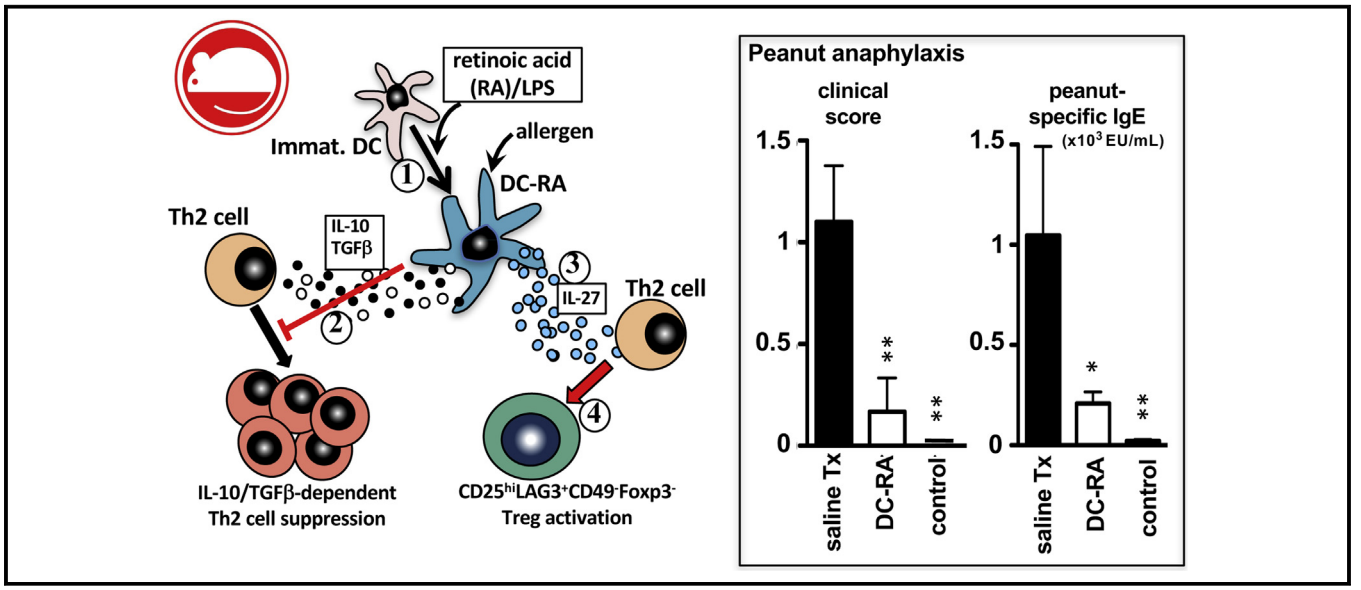
# Therapeutic reversal of food allergen sensitivity by mature retinoic acid–differentiated dendritic cell induction of LAG3$^{+}$CD49b$^{-}$Foxp3$^{-}$ regulatory T cells

**Wojciech Dawicki, PhD,[a] Chunyan Li, MSc,[b] Jennifer Town, PhD,[b] Xiaobei Zhang, BMed,[a] and John R. Gordon, PhD[a]**

*Saskatoon, Saskatchewan, Canada*

**GRAPHICAL ABSTRACT**




**Background: Anaphylaxis is a life-threatening condition for which we have limited therapeutic options. Although specific immunotherapy for food allergies is becoming more effective, it is still laborious and carries substantial risk of adverse events. On the other hand, regulatory dendritic cell (DC) therapy is effective in mouse models of allergic disease and has been shown to work with $T_H2$ cells from atopic asthmatic patients.**

**Objective: We assessed whether DC immunotherapy could reverse food allergen sensitivity in mouse models to provide proof of concept relating to their use in the clinic.**

**Methods: We generated and characterized mature retinoic acid–skewed dendritic cells (DC-RAs) and assessed their abilities to reverse ovalbumin or peanut allergies in mouse models, as well as their operative mechanisms.**

**Results: DC-RAs displayed a mature yet tolerogenic phenotype, expressing IL-10, TGF-β, IL-27, and aldehyde dehydrogenase 1A2 but not IL-12 or IL-35; IL-10 and TGF-β together drove their suppression of $T_H2$ cell proliferation. Delivery of specific allergen-presenting DC-RAs to half-maximally sensitized mice with ovalbumin or peanut allergy reduced anaphylactic responses to oral allergen challenge by 84% to 90%, as well as diarrhea, mast cell activation, and $T_H2$ cytokine responses and serum allergen-specific IgE/$IgG_1$ levels. DC-RA expression of IL-27 was important to their induction of CD25$^{+}$ lymphocyte activation gene 3 (LAG3)$^{+}$, CD49b$^{-}$, forkhead box P3 (Foxp3)$^{-}$ regulatory T cells *in vitro*, such that β subunit of IL-27 *(Ebi)*$^{-/-}$ (ie, IL-27–incompetent) DC-RAs were ineffective in inducing food allergen tolerance.**

**Conclusion: Our data indicate that regulatory DC immunotherapy can be effective for food allergies and suggest that induction of Foxp3$^{-}$ regulatory T cells might be a useful strategy for tolerance induction in this context. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)**

From the Departments of [a]Medicine and [b]Veterinary Microbiology, University of Saskatchewan.

Supported by grants from the Canadian Institutes of Health Research (MOP53167) and the AllerGen Networks of Centers of Excellence (CanFAST6).

Disclosure of potential conflict of interest: W. Dawicki receives grant support and travel support from the Canadian Institutes for Health Research and AllerGen Networks of Centers of Excellence. J. Town receives grant and travel support from the Canadian Institutes for Health Research and AllerGen Networks of Centers of Excellence. X. Zhang receives grant and travel support from the Canadian Institutes for Health Research and AllerGen Networks of Centers of Excellence. J. R. Gordon receives grant support and travel support from the Canadian Institutes for Health Research and the AllerGen Networks of Centers of Excellence. C. Li declares no relevant conflicts of interest.

Received for publication December 6, 2015; revised June 30, 2016; accepted for publication July 20, 2016.

Corresponding author: John R. Gordon, PhD, Division of Respirology, Critical Care and Sleep Medicine, Rm 3D30.8 Health Sciences Bldg, 107 Wiggins Rd, Saskatoon, Saskatchewan S7N 5E5, Canada. E-mail: john.gordon@usask.ca.

0091-6749/$36.00



http://dx.doi.org/10.1016/j.jaci.2016.07.042

***Key words:*** *Regulatory dendritic cells, immunotherapy, food allergy, anaphylaxis, tolerance*

Dendritic cells (DCs) are well known for their ability to activate T cells to which they present antigens, but they are also important to the induction of tolerance in some settings. For example, pulmonary DCs present innocuous aeroallergens while expressing low levels of MHC class II and costimulatory molecules and higher levels of IL-10 and thereby induce naive $CD4^+$ T cells to differentiate into $CD25^+$ forkhead box P3 $(Foxp3)^+$ regulatory T (Treg) cells that mediate aeroallergen tolerance.[1] In an analogous manner intestinal $CD103^+$ DCs that present innocuous food or gut commensal antigens to naive T cells in the gut-draining lymph nodes secrete TGF-β and retinoic acid (RA) while doing so and thereby induce the differentiation of gut-homing (ie, α4β7 integrin/CCR9-expressing) $CD25^+Foxp3^+$ Treg cells.[2] Such regulatory dendritic cells (DCregs) can also be induced *in vitro* by exposure to an array of tolerogenic mediators (eg, dexamethasone and IL-10), and indeed, it has been reported by multiple laboratories that such DCregs can suppress the responsiveness of effector T cells in multiple settings.[3] We and others have similarly reported that IL-10–skewed tolerogenic dendritic cells (DC10s) can reverse the asthma phenotype in mouse models,[4-6] in which they induce $T_H2$ cells to differentiate into $CD25^+Foxp3^+$ Treg cells in an IL-10–dependent fashion.[4,6] Although the tolerance so induced is progressive, long-lasting, and resistant to repeated challenge with physiologic levels of allergen,[5] others have reported that $Foxp3^+$ Treg cell immunotherapy in mice with colitis fails because the colitis-associated inflammation suppresses Foxp3 expression in these Treg cells, which subsequently convert into $T_H17$ cells that exacerbate rather than ameliorate the colitis phenotype.[7] This suggests that it could be important in the context of immunotherapy, for example, for inflammatory diseases to induce $Foxp3^-$ rather than $Foxp3^+$ Treg cells. Although induction of $Foxp3^+$ Treg cell–driven tolerance can be effective in experimental asthma therapy, regulatory dendritic cell (DCreg) treatment of food allergies could present challenges not found in the lung. For example, just as colitis inflammation can suppress Foxp3 expression by and therefore the regulatory activities of Treg cells,[7] allergen-induced inflammation in patients with food allergy could also affect the phenotype of $Foxp3^+$ Treg cells that might be present or induced in the gut.

Given the associations between intestinal RA and the induction of tolerogenic DCs,[2] we have examined conditions that would lead RA-induced tolerogenic DCs to foster differentiation of $Foxp3^-$ as opposed to $Foxp3^+$ Treg cell responses. Herein we report that specific allergen-presenting, RA-differentiated DCs that have been exposed to strong Toll-like receptor (TLR) 4 signals during differentiation express high levels of IL-27 and thereby induce food allergen tolerance in mouse models of ovalbumin (OVA) and peanut allergies, replacing allergen-specific $CD4^+$ $T_H2$ responses with $CD25^+$ lymphocyte activation gene 3 $(LAG3)^+$ $CD49b^-Foxp3^-$ Treg cell responses, which suppress allergic responses to oral allergen challenge.

*Abbreviations used*

| | |
|---|---|
| AhR: | Aryl hydrocarbon receptor |
| Aldh1A2: | Retinaldehyde 1A2 |
| CFSE: | Carboxyfluorescein succinimidyl ester |
| CPE: | Crude peanut extract |
| CTLA-4: | Cytotoxic lymphocyte antigen 4 |
| DC: | Dendritic cell |
| DC10: | IL-10–skewed tolerogenic dendritic cell |
| DC-LPS: | LPS-matured immunostimulatory dendritic cell |
| DC-RA: | Retinoic acid–skewed dendritic cells |
| DCreg: | Regulatory dendritic cell |
| *Ebi*: | β Subunit of IL-27 |
| FACS: | Fluorescence-activated cell sorting |
| Foxp3: | Forkhead box P3 |
| LAG3: | Lymphocyte activation gene 3 |
| mMCP-1: | Mouse mast cell protease 1 |
| OVA: | Ovalbumin |
| PDL: | Programmed death ligand |
| qRT-PCR: | Quantitative RT-PCR |
| RA: | Retinoic acid |
| TLR: | Toll-like receptor |
| Treg: | Regulatory T |
| WT: | Wild-type |

## METHODS

### Animals

BALB/c, C57Bl/6, OT II, and β subunit of IL-27 *(Ebi)* $3^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Me); 6 to 12-week-old female mice were used in all experiments. The animals were housed under specific pathogen-free conditions, with food and water provided *ad libitum*. All experiments were approved by our institutional animal ethics office in accordance with the guidelines of the Canadian Council on Animal Care.

### Reagents

Antibodies to mouse CD49b (clone DX5), CD80 (clone 16-10A1), CD11c (clone N418), CD4 (clone 11-0042-81), CD69 (clone H1-2F3), CD40 (clone HM40-3), CD25 (clone DC61.5), CD103 (clone 2E7), CD276 (clone M3.2D7), latency-activated protein (LAP; clone TW7-16B4), LAG3 (clone eBioC9B7W), CCR9 (clone eBioCW-1.2), OX40 ligand (clone RM134L), programmed death ligand 1 (PDL) 1 (clone MIH5), and PDL2 (clone 122) and isotype control antibodies were purchased from eBioscience (San Diego, Calif), whereas anti-mouse CD86 (clone GL1), CD54 (clone 3E2), $IgG_1$, and IgE were from BD Biosciences (San Jose, Calif). Anti-CD4–specific magnetic sorting beads and columns were from Miltenyi Biotec (Auburn, Calif). *E coli* O55:B5 LPS, OVA, and all-trans RA were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Ore), crude peanut extract (CPE) was purchased from Greer Laboratories (Lenoir, NC), and tritiated thymidine was purchased from American Radiolabeled Chemicals (St Louis, Mo). ELISA capture and detection antibodies and recombinant protein standards for IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-27p28, IFN-γ, and TGF-β were purchased from R&D Systems (Minneapolis, Minn).

### Flow cytometry

Cells were washed 3 times with fluorescence-activated cell sorting (FACS) buffer (0.01 mol/L azide, 2% FBS, and PBS), incubated with blocking antibody for 10 minutes at 4°C, and then labeled with marker-specific or isotype control fluorochrome-labeled antibodies for an additional 20 minutes at 4°C. After washing 2 times with FACS buffer, the cells were analyzed with an EPICS XL Flow Cytometer (Beckman Coulter, Mississauga, Ontario, Canada), and FACS data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

### Generation of DCs

All DCs were differentiated from bone marrow cells, as previously noted,[5] by plating cells at $5 \times 10^5$/mL in 6-well plates for 7 days in RPMI 1640

medium supplemented with 10% FBS, L-glutamine, and antibiotics; every 2 to 3 days, 75% of the medium in each culture was replaced. To generate tolerogenic retinoic acid–skewed dendritic cells (DC-RAs), we supplemented our standard DC differentiation cultures (20 ng/mL GM-CSF and 10 ng/mL IL-4) with 1 μmol/L RA; immature DC-RAs were used as is, whereas our standard mature DC-RAs were also exposed to LPS (1 μg/mL) for the final 18 hours of culture. It had been reported previously that immature RA-induced DCs can induce differentiation of $Foxp3^+$ Treg cells[8] but also that TLR4 signaling in dexamethasone-differentiated DCs upregulates their expression of IL-10 and IL-27.[9,10] Stimulatory LPS-matured immunostimulatory dendritic cells (DC-LPSs) were differentiated in 20 ng/mL GM-CSF (without IL-4 or RA) and pulsed with 1 μg/mL LPS for the final 18 hours of culture. Differentiated DCs were either pulsed with allergen (50 μg/mL) for the last 24 hours before harvest or not treated (control DC-RAs).

## Quantitative real-time PCR assays

RNA was isolated from cells by using RNeasy kits (Qiagen, Toronto, Ontario, Canada), whereas cDNAs were synthesized with qScript kits (Quanta Biosciences, Gaithersburg, Md), according to the manufacturer's specifications. Quantitative RT-PCR (qRT-PCR) reactions were performed with a PerfeCTa SYBR Green FastMix (Quanta Biosciences) with appropriate primers in a C1000 Touch (Bio-Rad Laboratories, Mississauga, Ontario, Canada) thermocycler. Primers sequences are listed in Table E1 in this article's Online Repository at www.jacionline.org. Data were analyzed with the Bio-Rad CFX Manager (Bio-Rad Laboratories) software, with β-actin as a normalizer.

## ELISA

Concentrations of IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-27p28, IFN-γ, and TGF-β in biological samples were determined by means of ELISA with matched capture and detection antibodies and cytokine standards, as noted previously.[5,11] The sensitivity of these assays was routinely 5 to 10 pg/mL. The mouse mast cell protease 1 (mMCP-1) ELISA reagents were purchased from eBioscience, and the assay was carried out according to the manufacturer's protocol. Concentrations of allergen-specific $IgG_1$ were determined by using a sandwich ELISA with plate-bound allergen to capture the antibodies from serum and biotinylated anti-IgG for detection, whereas concentrations of allergen-specific IgE were determined by using a sandwich ELISA with plate-bound anti-IgE to capture the antibodies from serum and biotinylated allergen for detection.[5,12] Streptavidin–alkaline phosphatase was used to visualize the captured biotinylated detection reagents. Serial dilutions of pooled reference plasma from mice immunized twice with 2 μg (OVA) or 20 μg (peanut) of allergen per milligram of alum was used as a standard, with arbitrarily assigned reference units set at 1000 for the undiluted reference plasma.

## Magnetic sorting of $CD4^+$ cells and suppression assays

Spleen and lymph node cells were dispersed mechanically and then passed through a 70-μm cell strainer, and the contaminating red blood cells were lysed with hypotonic Tris-ammonium chloride. $CD4^+$ cells were purified with CD4-specific paramagnetic beads, according to the supplier's protocol, and used immediately in subsequent assays. $CD4^+$ cell purity and viability was in each case 95% or greater.

$CD4^+$ $T_H2$ cells were purified from the spleens and lymph nodes of OVA- or CPE-alum–immunized B6 mice or naive OT II mice and cocultured with irradiated antigen-pulsed stimulatory DCs (DC-LPSs) for 48 hours in U-bottom 96-well plates ($10^5$ T cells and $3.7 \times 10^3$ DC-LPSs/well), as noted[4]; we determined in preliminary experiments that these conditions induced half-maximal T-cell proliferation. At 48 hours, 0.5 μCi of tritiated thymidine was added to the cultures, and the cells were harvested 24 hours later. The suppressive activities of putatively tolerogenic DC-RAs or DC-RA–induced Treg cells were determined by titrating cells that had been irradiated into T-cell/DC-LPS cocultures. In some experiments we also added neutralizing anti–IL-10 and/or anti–TGF-β antibodies into the cultures (10 μg/mL) to determine the roles of these cytokines in the regulatory activities of DC-RAs or DC-RA-induced Treg cells.

## *In vitro* production of DC-RA–induced Treg cells

Magnetically sorted $CD4^+$ $T_H2$ cells from OVA-alum–immunized mice were cocultured for 5 days with DC-RAs or, as a negative control, DC-LPSs ($10^5$ T cells and $3 \times 10^4$ DCs/well) in 96-well U-bottom plates, after which the induced putative $CD4^+$ Treg cells from these cultures were purified by means of magnetic sorting. In some assays the $T_H2$ cells were prelabeled with CFSE to assess their proliferation responses to allergen-presenting DC-RAs or DC-LPSs in these cultures, whereas in other assays the putatively induced Treg cells from these cultures were analyzed by means of FACS for the indicated markers or were assessed for their abilities to suppress DC-LPS–induced $T_H2$ cell proliferative responses, as above. In some experiments we added exogenous rIL-2 (0.2 ng/mL) to the cultures.

## Anaphylaxis sensitization

BALB/c mice were injected intraperitoneally twice 2 weeks apart with 200 μL of either OVA-alum (2 μg of OVA/2 mg of alum) or CPE-alum (20 μg of CPE/1 mg of alum). Beginning 2 weeks later, the mice were gavaged every second day with 2 mg of OVA or 50 mg of peanut butter, respectively (with 3 hours of fasting before each gavage), until they displayed anaphylactic responses to the oral challenge (usually 6-9 such serial gavages). We found that once signs of anaphylaxis were first observed during the sensitization phase, more than 3 additional challenges would most often lead to lethal outcomes. We used a standard 5-point scoring system for anaphylactic responses as follows: 0, no clinical symptoms; 1, repetitive vigorous nose/ear scratching; 2, lethargy and puffy eyes/mouth; 3, periods of motionless for more than 1 minute and lying prone; 4, no response to whisker stimuli and reduced or no response to prodding; and 5, tremor, convulsion, and death. For humane reasons, animals with a clinical score of 4 were euthanized immediately.

Because our purpose was to evaluate the effect (positive or negative) of DC treatments on the anaphylaxis phenotype, we titrated our OVA model by adjusting the numbers of pre-experiment challenges to yield half-maximal mean scores (ie, ±2). Most animals that displayed allergen sensitivity also experienced explosive diarrhea approximately 30 to 40 minutes after allergen challenge.

## DCreg treatment of animals with food allergy

To ensure that our treatment DCs were not exposed to a potentially confounding inflammatory environment *in situ* after delivery, we allowed 2 weeks after the animals had achieved full allergen sensitivity before beginning DCreg immunotherapy and then assessed the effect of the therapy on anaphylactic responses to challenge 4 weeks later. For treatment, each animal was injected intraperitoneally with $1 \times 10^6$ treatment or control DCs or an equal volume of saline, and 4 weeks later, they received 1 more oral challenge with OVA or peanut butter, as appropriate. The mice were observed over the ensuing 40 minutes for clinical demeanor and incidence of diarrhea, whereas at 50 minutes, a blood sample was taken from each mouse for mMCP-1 analysis. The next day, the mice were killed, and blood, peritoneal lavage fluid, and various tissues were collected.

## Statistical analysis

In experiments with only 2 study groups, Student *t* tests were performed, whereas in all other experiments, 1-way ANOVA was used with Tukey *post hoc* testing to compare pairs of experimental groups. All analyses were performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). Because the occurrence of diarrhea in our study was scored as its incidence, we could not undertake meaningful statistical analyses with this parameter.

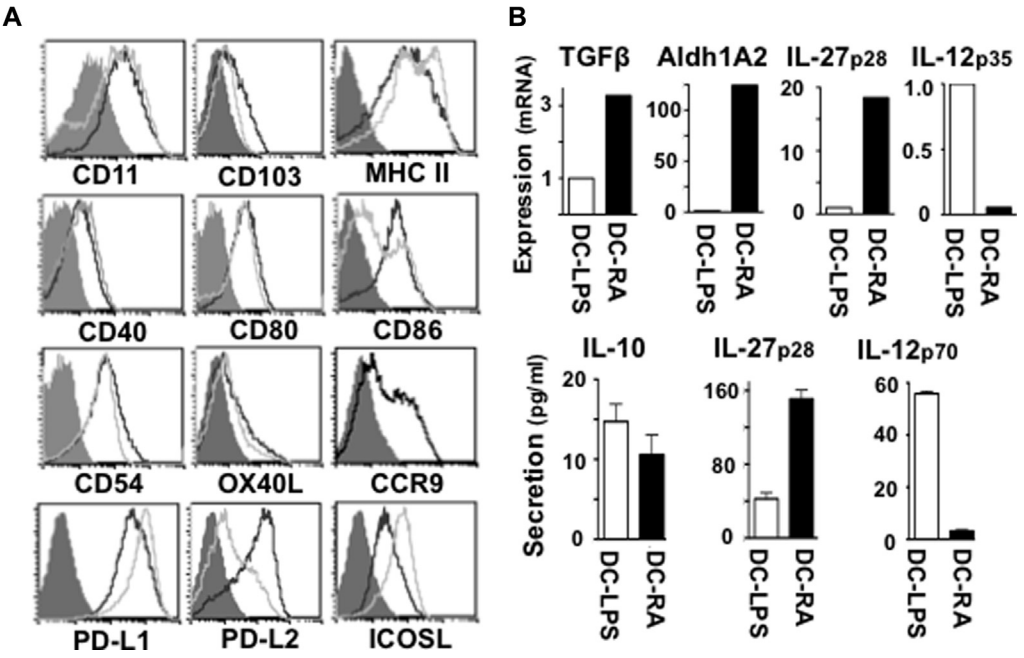



**FIG 1.** Characterization of mouse bone marrow–derived DCs that were differentiated in the presence of RA/LPS or LPS alone. **A,** DCs differentiated in the presence or absence of RA and then exposed to LPS for 18 hours (DC-RAs or DC-LPSs, respectively) were stained for FACS analysis by using marker-specific (*black,* DC-RAs; *gray* line, DC-LPSs) or isotype control *(gray-shaded histogram)* antibodies. **B,** They were also analyzed by using qRT-PCR or ELISA for expression of the indicated markers, as noted in the Methods section. DC-RAs expressed a mature yet tolerogenic phenotype.

## RESULTS

### Characterization of mature RA-differentiated DCs

DCs differentiated in the presence of RA and subsequently pulsed with specific allergen while being exposed to *E coli* LPS expressed CD11c, the αE integrin CD103, and CCR9. They also strongly expressed MHC class II, CD54, CD80, and CD86 but also PDL1, inducible costimulator ligand, and uniquely higher levels of PDL2 than DC-LPSs (Fig 1, *A*). They expressed modest levels of TGF-β, high levels of IL-27 (IL-27p28/*Ebi3*) and the RA-metabolizing enzyme retinaldehyde 1A2 (Aldh1A2), little IL-10, and only background levels of IL-12, as determined by using IL-12p35 qRT-PCR and IL-12p70 ELISA. This latter outcome indicates also that DC-RAs do not express significant levels of the other IL-12p35–containing heterodimer, IL-35 (IL-12p35/*Ebi3*). Fully mature DC-LPSs also strongly expressed MHC class II, CD54, CD80, PDL1, and IL-12 but essentially only background or modest levels of TGF-β, Aldh1A2, or IL-27 (Fig 1, *B*).

When titrated into cocultures of allergen-presenting immunostimulatory DC-LPSs and either OVA TCR-transgenic (ie, OT II) T cells or $T_H2$ cells from C57/BL6 mice with OVA allergy, OVA-loaded DC-RAs suppressed 3-day T-cell proliferative responses in a dose-dependent fashion (Fig 2, *A*). This suppression occurred whether the DC-RAs and T cells were in direct contact with one another or were separated by a permeable membrane, indicating that cell-cell contact was not required (Fig 2, *B*).

We assessed whether expression of *Ebi3*-containing heterodimeric cytokines (ie, IL-27 [p28/*Ebi3*] or IL-35 [p35/*Ebi3*]) by DC-RAs was important for their suppression of effector T-cell proliferation in response to DC-LPS activation. As noted, our data suggest that DC-RAs do not express discernible levels of IL-35, although they do secrete ample IL-27 (Fig 1, *B*). We also confirmed that loss of *Ebi3* in DC-RAs did not affect other parameters relevant to their tolerogenic activities, including expression of Aldh1A2, IL-10, IL-12, and TGF-β, as well as MHC class I and II and an array of costimulatory markers, chemokine receptors, and inhibitory receptors (see Fig E1 in this article's Online Repository at www.jacionline.org). Both wild-type (WT) and $Ebi3^{-/-}$ DC-RAs were equally able to inhibit T-cell proliferation, suggesting that IL-27 was dispensable for DC-RA suppression of $T_H2$ cell responses in these 3-day cultures (Fig 2, *C*, left panel). In further assessing what drives the abilities of DC-RAs to inhibit $T_H2$ proliferative responses, we found that neutralizing IL-10 significantly reduced the cells' inhibitory activities ($P < .05$), whereas simultaneous inhibition of IL-10 and TGF-β fully reversed the DC-RA–dependent suppression of $T_H2$ cell activation by DC-LPSs ($P < .001$; Fig 2, *C*, right panel).

We also asked whether LPS-dependent maturation of RA-induced DCregs was a critical parameter in induction of their regulatory activities. Thus we titrated immature DC-RAs, LPS-matured DC-RA (DC-RAs), or, as a control, DC-LPSs into cultures of stimulatory DC-activated $CD4^+$ T cells magnetically sorted from OVA-sensitized (effector T cells) or unsensitized (naive T cells) OT II mice and assessed OT II T-cell proliferation (Fig 3, *A*). The LPS-matured DC-RAs were effective in attenuating the responses of both naive and sensitized $T_H2$ cells to allergen-presenting stimulatory DCs, but the immature DC-RAs were significantly less able to suppress the $T_H2$ responses, although they did have significant regulatory activities on both accounts (Fig 3, *A*). DC-LPSs had no such regulatory activities. Although the mature and immature DC-RAs expressed equivalent levels of Aldh1A2 and TGF-β, the mature cells

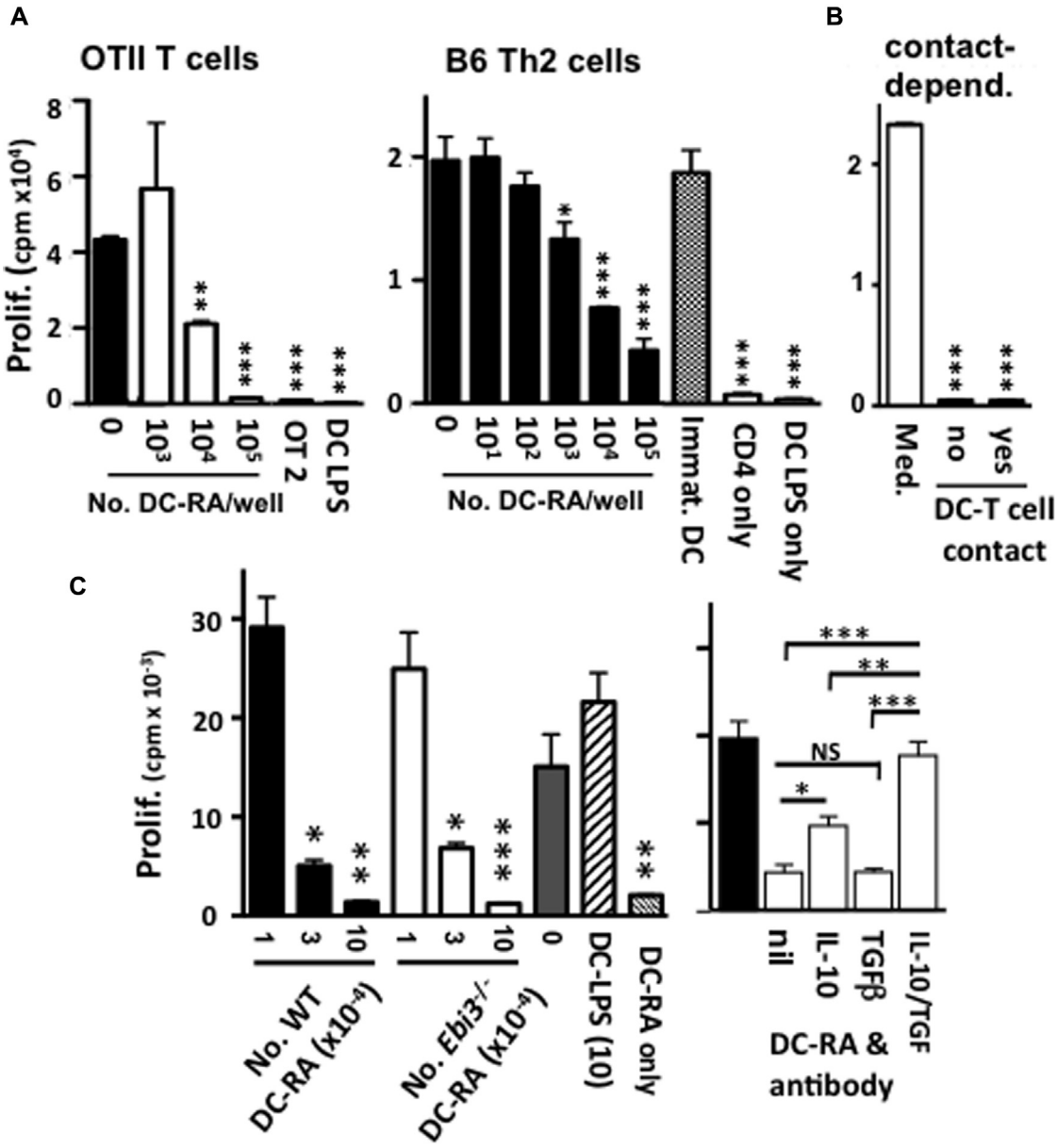



**FIG 2.** DC-RAs inhibit immunostimulatory DC-induced T-cell proliferation in an IL-10– and TGF-β–dependent but IL-27– and contact-independent manner. **A,** OVA-presenting DC-RAs were titrated into cultures of OVA-presenting DC-LPSs ($3.7 \times 10^3$ cells/well) and T cells ($1 \times 10^5$ cells/well) from OVA TCR-transgenic OT II or asthmatic mice to assess their suppression of T-cell proliferation. Control wells contained T cells, DC-LPSs, or immature DCs alone. Note that the scales for the TCR-transgenic OT II cell (100% OVA-specific T cells) and the B6 mouse $T_H2$ cells are different. **B,** Assessment of whether direct DC-RA–T-cell contact was required for this suppression. **C,** *Left panel,* Role of IL-27 expression by DC-RAs in suppression of $T_H2$ responses, as determined by using DC-RAs generated from WT or IL-$27^{-/-}$ mice. *Right panel,* Effect of neutralizing anti–IL-10 and/or TGF-β antibodies on DC-RA suppression of $T_H2$ cell proliferation. *NS,* $P \geq .05$. $^*P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$ versus DC-LPSs and T cells alone.

expressed substantially more IL-10 and IL-27p28, as determined by using qRT-PCR (Fig 3, *B*).

## Specific allergen-presenting DC-RAs suppress allergen-induced anaphylaxis in OVA and peanut models of food allergies

We established OVA-induced anaphylaxis in mice, as noted in the Methods section, and then rested the mice for 2 weeks, treated them intraperitoneally with either saline or $1 \times 10^6$ control or allergen-pulsed DC-RAs, challenged them again 4 weeks later by means of gavage with allergen, and assessed their responses over the next 24 hours (Fig 4, *A*). As noted, we titrated our model to achieve half-maximal anaphylaxis responses to this final allergen challenge (ie, a score of ±2). In our OVA model the saline-treated mice had substantial nasal and ear urticaria, facial edema, and lethargy after allergen challenge but also displayed an explosive diarrhea response. They had high serum levels of

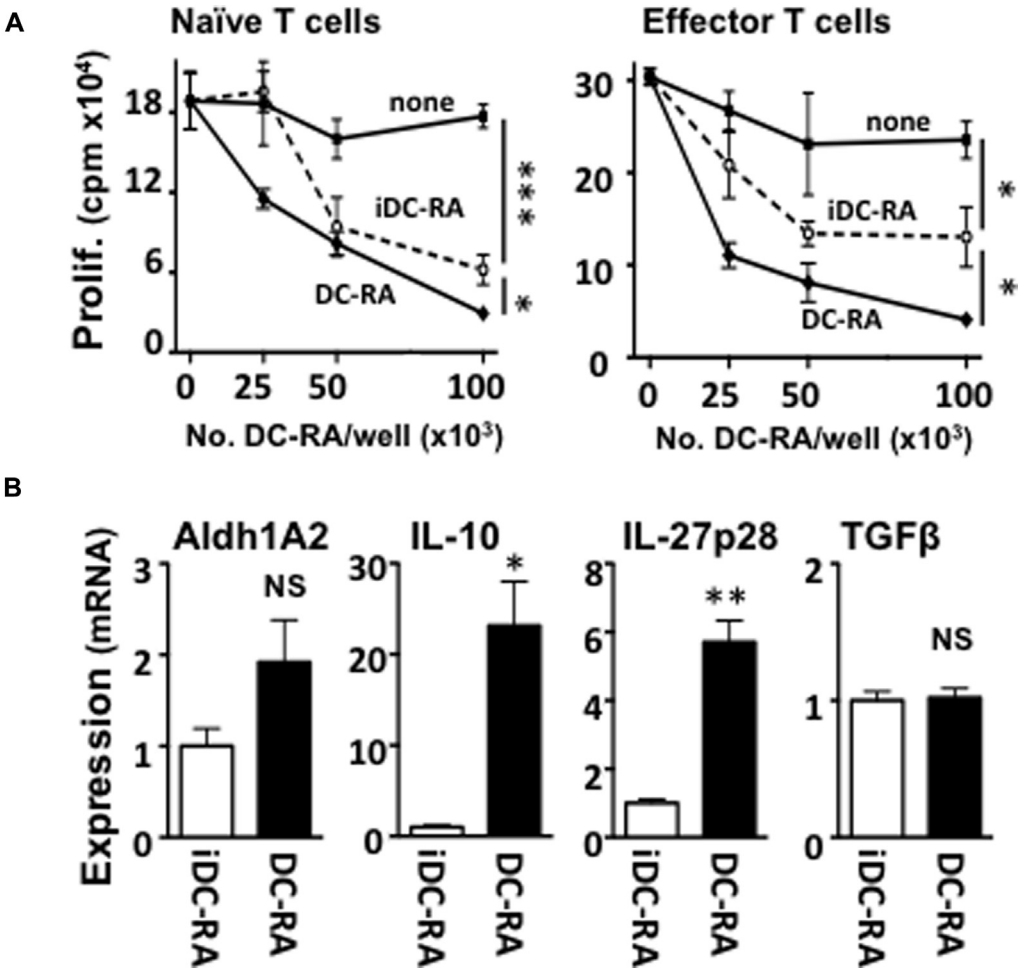



**FIG 3.** LPS maturation of DC-RAs enhances their suppressive ability. **A,** DC-LPSs or either immature (no LPS exposure) or LPS-matured DC-RAs were titrated into cocultures of OVA-presenting DC-LPSs and T cells obtained from either naive or OVA-immunized (effector T cells) OT II mice to assess their abilities to suppress T-cell proliferative responses. **B,** Expression of mRNA encoding the indicated tolerance-associated markers, as determined by using quantitative PCR. *Bars* represent means ± SEMs of 4 independent experiments. *$P \leq .05$, **$P \leq .01$, or ***$P \leq .001$. *NS*, $P > .05$.

OVA-specific IgE and $IgG_1$, such that they had a robust mast cell activation response to allergen challenge, as determined by circulating levels of mMCP-1 (Fig 4, *B* and *C*). As a surrogate measure of gut $T_H2$ sensitivity, we assessed levels of IL-4, IL-5, IL-9, and IL-13 but also IFN-γ in peritoneal wash fluids 24 hours after allergen challenge. Each of the $T_H2$ markers was upregulated in the anaphylactic mice, whereas very modest levels of IFN-γ were detected. Normal unsensitized control mice displayed no discernible indications of food allergies or allergen-specific IgE or $IgG_1$ antibodies in response to oral administration of allergen 24 hours beforehand (Fig 4, *D*).

Anaphylaxis phenotype mice that had been treated with $1 \times 10^6$ OVA-presenting DC-RAs ($^{OVA}$DC-RAs) had significant decreases in clinical scores, mast cell activation, IgE and $IgG_1$ levels, and levels of peritoneal wash fluid IL-4, IL-5, IL-9, and IL-13, but not IFN-γ, relative to levels seen in saline-treated mice ($P \leq .05$ to .001; Fig 4, *B-D*). To confirm the allergen specificity of these effects, we also assessed the effect of treating the mice with cells that had not be exposed to allergen (DC-RAs) and found that these cells had no effect on any gut-related anaphylactic events (clinical scores, diarrhea, and levels of mMCP-1, $T_H2$ cytokines, or IFN-γ) or serum $IgG_1$ levels (each $P > .05$ vs saline-treated mice), although for reasons that are not clear, the OVA-specific IgE levels were statistically significantly lower in these animals relative to those in saline-treated mice ($P \leq .05$). However, taken together, our data indicate that, like previous reports of the allergen specificity of murine and human DC10s,[5,6,13] the tolerogenic effects of DC-RAs in this model were largely antigen specific.

We also assessed whether DC-RA immunotherapy would protect against peanut allergen–driven anaphylaxis in our mice, which is arguably a more clinically relevant model (Fig 5), although the saline-treated peanut-sensitive mice displayed significantly lower anaphylaxis scores than the saline-treated OVA-allergic mice, which was potentially related to the higher doses of allergen we used for sensitization of these mice.[14] Nevertheless, here too we found that 4 weeks after treatment, CPE-presenting DC-RAs had substantially reduced clinical scores ($P \leq .01$), mast cell degranulation (serum mMCP-1 levels, $P \leq .05$; Fig 5, *A*), and circulating peanut allergen–specific $IgG_1$

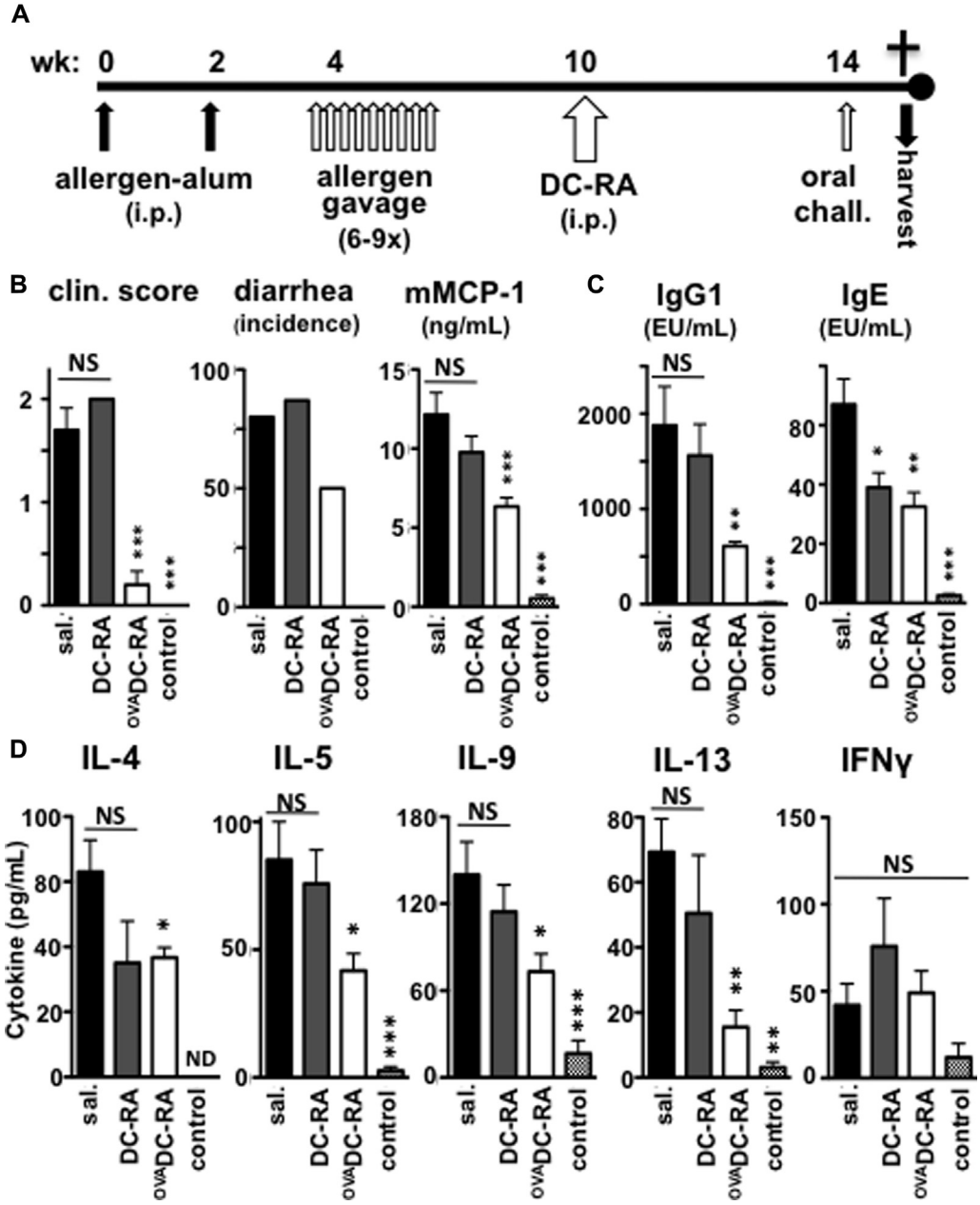



**FIG 4.** Specific allergen-presenting DC-RAs suppress anaphylactic responses to oral allergen challenge in a mouse model of OVA-induced anaphylaxis. **A,** Schematic diagram of the experimental protocol. Briefly, mice were either left unsensitized (control) or were sensitized with OVA-alum *(i.p.)*, followed by repeated gavage with OVA until they displayed overt anaphylactic responses to the oral challenge. They were then rested for 2 weeks before being given $1 \times 10^6$ otherwise untreated DC-RAs or OVA-pulsed DC-RAs or an equal volume of saline. All mice were challenged 4 weeks later by means of OVA gavage and assessed over the next 24 hours. **B,** At 30 minutes after challenge, the clinical scores and incidence of diarrhea were assessed, whereas at 50 minutes, serum was collected for analysis of mMCP-1 as a surrogate marker of intestinal mast cell activation. **C** and **D,** At 24 hours, serum was collected for analysis of OVA-specific $IgG_1$ and IgE (Fig 4, *C*), whereas peritoneal lavage fluid was assayed for the indicated cytokines (Fig 4, *D*). *NS*, $P \geq .05$. $^*P \leq .05$, $^{**}P \leq .01$, or $^{***}P \leq .001$. Results are the mean of 2 independent experiments (n = 9-10 mice per group).

($P \leq .01$) and IgE ($P \leq .05$) levels (Fig 5, *B*). Interestingly, while the mean peritoneal $T_H2$ cytokine values were lower and IFN-γ values were higher in CPE-pulsed DC-RA– versus saline-treated mice at 24 hours after challenge, these did not achieve statistical significance, although IL-10 levels were significantly increased ($P \leq .05$; Fig 5, *C*).

## DC-RAs induce $T_H2$ cells to adopt an IL-27–dependent $CD25^+LAG3^+CD49b^-Foxp3^-$ Treg cell phenotype

We next investigated whether our tolerogenic DC-RAs were inducing the development of Treg cell responses among the $T_H2$ cells of allergic animals. We cocultured OVA-presenting DC-RAs

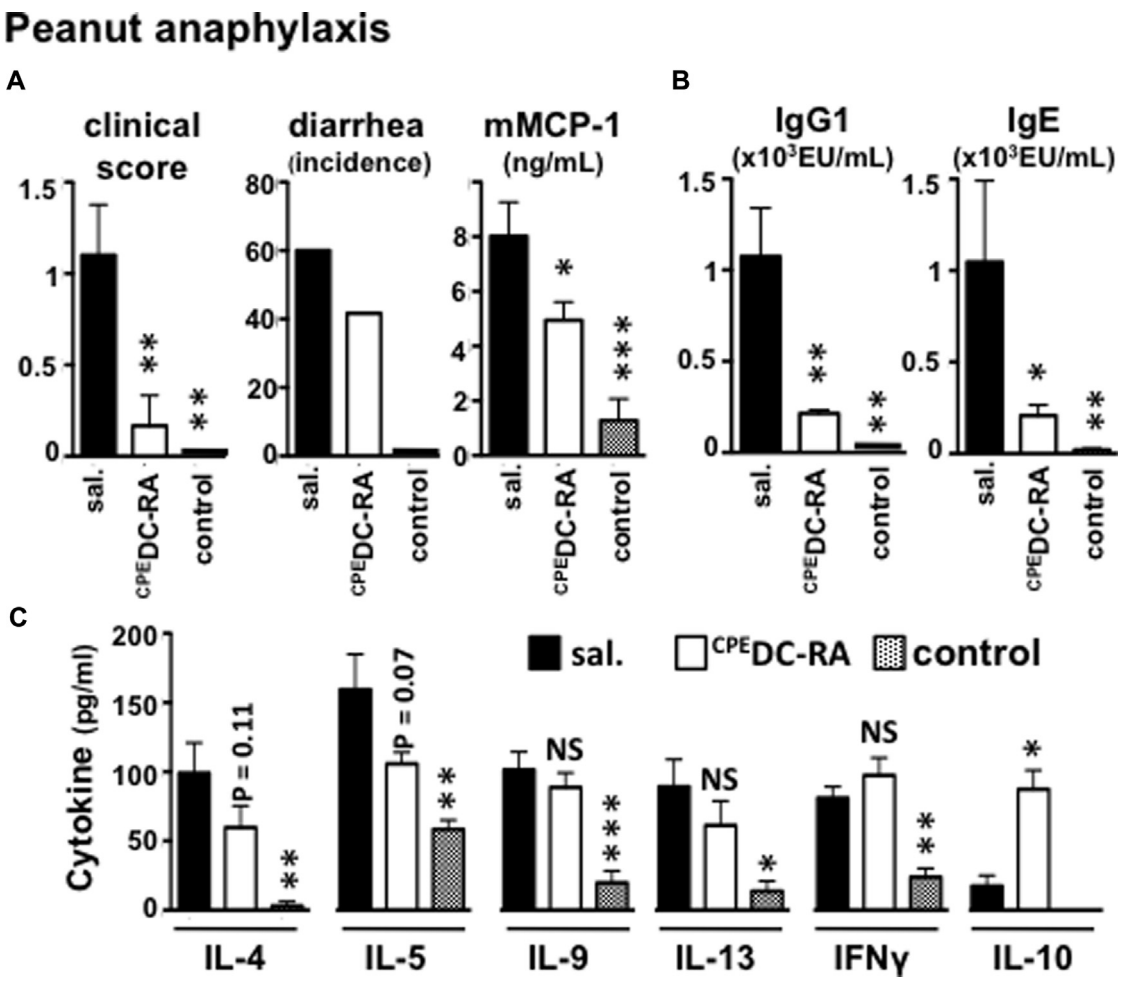



**FIG 5.** DC-RAs also suppress anaphylactic responses to allergen challenge in a mouse model of peanut-induced anaphylaxis. Mice were left unsensitized (control) or sensitized with CPE for initial exposure, followed by repeated gavage with peanut butter until they displayed overt anaphylactic responses. They were then rested for 2 weeks before being given $1 \times 10^6$ CPE-presenting DC-RAs or an equal volume of saline, as in Fig 4. Four weeks later, all mice were given an oral peanut butter challenge. **A** and **B,** Clinical scores, diarrhea, and mMCP-1 levels were assessed as in Fig 3 (Fig 5, *A*), as were serum peanut allergen–specific $IgG_1$ and IgE (Fig 5, *B*). **C,** At 24 hours, peritoneal lavage fluids were collected and assayed for the indicated cytokines by means of ELISA. *NS*, $P \geq .05$. *$P \leq .05$, **$P \leq .01$, or ***$P \leq .001$, respectively. Results are the mean of 2 independent experiments (n = 10 mice per group).

or DC-LPSs and CFSE-labeled splenic $CD4^+$ T cells from OVA-sensitized mice for 5 days and then assessed the proliferative responses that had occurred in these cultures (CFSE dilution assay; Fig 6, *A*) and expression of Treg cells markers, as determined by means of FACS of gated $CD4^+$ T cells. As expected, immunostimulatory DC-LPSs strongly induced T-cell proliferation, whereas the DC-RA–treated $CD4^+$ T cells did not proliferate at all, although their high-level expression of CD25 suggested that they had been activated (Fig 6, *B*). The numbers of $CD4^+$ T cells recovered from the DC-RA/$T_H2$ cocultures were equivalent to the numbers of $CD4^+$ cells placed in the cultures, and we observed no changes in the FACS forward-scatter or side-scatter parameters in these cultures, indicating that the T cells were not undergoing apoptosis or dying. Addition of IL-2 to these DC-RA/$T_H2$ cell cocultures had no effect on this T-cell response (data not shown), further indicating that these cells were not simply anergic.[15] When we magnetically sorted the $CD4^+$ T cells from these DC-RA cocultures and assessed their suppressive activity in our standard DC-LPS/OT II $T_H2$ cell assay, we found that the DC-RA–induced T cells displayed potent Treg cell activity, suppressing the OT II T-cell proliferation in a dose-dependent fashion (Fig 6, *C*, WT DC-RA bars). These induced Treg cells strongly expressed LAG3 and CCR9, but not CD49b or Foxp3 (Fig 6, *B*), which distinguishes them from classical $LAG3^+CD49b^+Foxp3^-$ $T_R1$ cells or $CD25^+Foxp3^+$ Treg cells.[4,11,16]

Given the role that IL-27 can play in Treg cell induction[17] and the strong expression of this cytokine by DC-RAs, we queried whether IL-27 was important to the regulatory phenotype of DC-RAs. Thus we repeated our 5-day $T_H2$ cell/DC-RA coculture experiment but used DC-RAs generated from the bone marrow of WT or *Ebi3*$^{-/-}$ mice. Again, we magnetically sorted the $CD4^+$ T cells from these cocultures and confirmed that, unlike T cells from WT DC-RA cocultures, $CD4^+$ T cells from *Ebi3*$^{-/-}$ DC-RA/$T_H2$ cocultures had no discernible regulatory activity in this assay (Fig 6, *C*); we noted above that

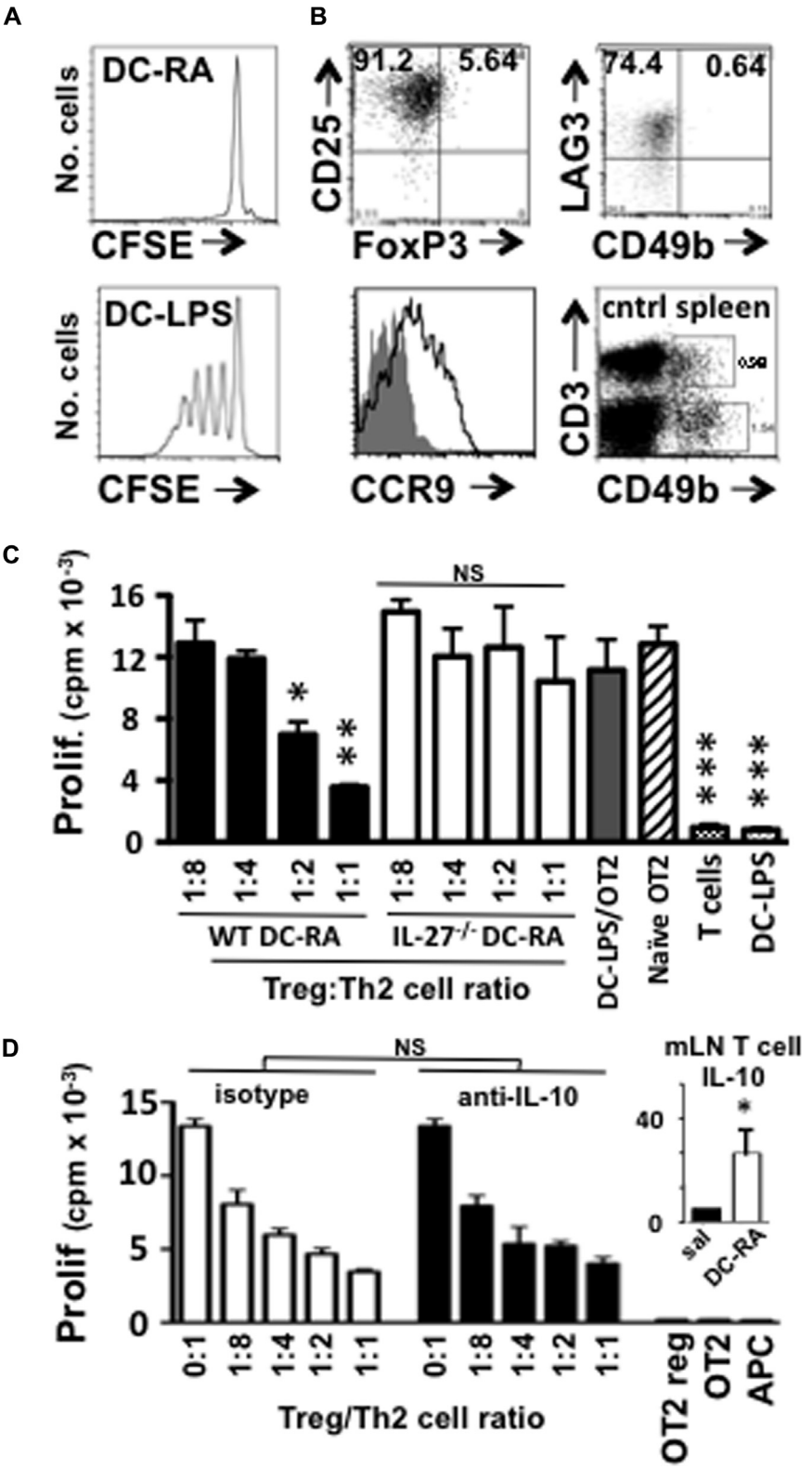



**FIG 6.** DC-RA expression of IL-27 is critical to induction of the Foxp3$^-$ Treg cells that suppress $T_H2$ responses. **A,** $CD4^+$ cells from OVA-immunized mice were labeled with CFSE and cocultured for 5 days with DC-RAs or DC-LPSs, and their proliferative responses were assessed by using a CFSE dilution assay. **B,** $CD4^+$ cells from 5-day DC-RA cultures (Fig 6, *A*) were assessed by means of FACS for expression of the indicated markers. **C,** $CD4^+$ cells sorted from 5-day WT or IL-$27^{-/-}$ DC-RA/$T_H2$ cell cocultures were titrated into secondary cocultures containing $10^5$ OT II cells and $3.7 \times 10^3$ OVA-pulsed DC-LPSs/well, and OT II cell proliferation was assessed. **D,** The impact of anti–IL-10 or isotype control antibodies (10 μg/mL) on the suppressive activities of WT DC-RA–induced Treg cells was assessed as in Fig 6, *C*. As a control, we also included wells with no DC-RA–induced Treg cells (0:1). *Inset,* IL-10 secretion over 48 hours by CD4 cells from the mesenteric lymph nodes *(mLN)* of mice taken 24 hours after allergen-induced anaphylaxis. *NS*, $P \geq .05$. *$P \leq .05$, **$P \leq .01$, or ***$P \leq .001$, respectively. Results are representative of 3 independent experiments (n = 10 mice per group).

WT DC-RAs produce IL-27 (ie, IL-27p28/*Ebi3*) but not IL-35 (IL-12p35/*Ebi3*).

Because IL-27 induces IL-10 expression in T cells[18] and Foxp3$^-$ $T_R1$ cells use IL-10 as a primary regulatory mediator,[16] we next assessed whether IL-10 had a role in the regulatory activities of our DC-RA–induced Treg cells. We confirmed that mesenteric lymph node (Fig 6, *D*, inset) or splenic (data not shown) T cells from DC-RA–treated mice with OVA allergy did in fact secrete IL-10 (≤50 pg/3 × $10^6$ cells over 48 hours; $P \leq .05$ vs saline-treated anaphylactic mice).

We then assessed the effect of neutralizing anti–IL-10 antibodies on DC-RA–induced Treg cell suppression of $T_H2$ proliferative responses in DC-LPS cocultures. We titrated DC-RA–induced Treg cells into these cultures and added either isotype control or neutralizing anti–IL-10 antibodies but found that neither of these treatments had any effect on the regulatory activities of DC-RA–induced Treg cells (Fig 6, *D*), even though these levels of anti–IL-10 reduced the abilities of DC-RAs to suppress $T_H2$ cell proliferation (Fig 2, *D*).

We next examined the expression of a panel of markers by these Treg cells, comparing their expression with that of DC-LPS–activated effector $T_H2$ cells from asthmatic OT II mice and naive CD4$^+$ OT II T cells (Fig 7). The DC-RA–induced Treg cells expressed substantial levels of LAG3 and PDL1, although no more so than the comparator effector T cells, but negligible programmed death 1 receptor, cytotoxic lymphocyte antigen 4 (CTLA-4), PDL2, and neuropilin-1. We also found that our DC-RA–induced Treg cells expressed only low levels of IL-10, TGF-β, c-Maf, or the ligand-activated transcription factor aryl hydrocarbon receptor (AhR; see Fig E2 in this article's Online Repository at www.jacionline.org). Taken together, these data suggest that DC-RAs induce IL-10–independent CD25$^+$LAG3$^+$CD49b$^-$Foxp3$^-$ Treg cells in an IL-27–dependent manner.

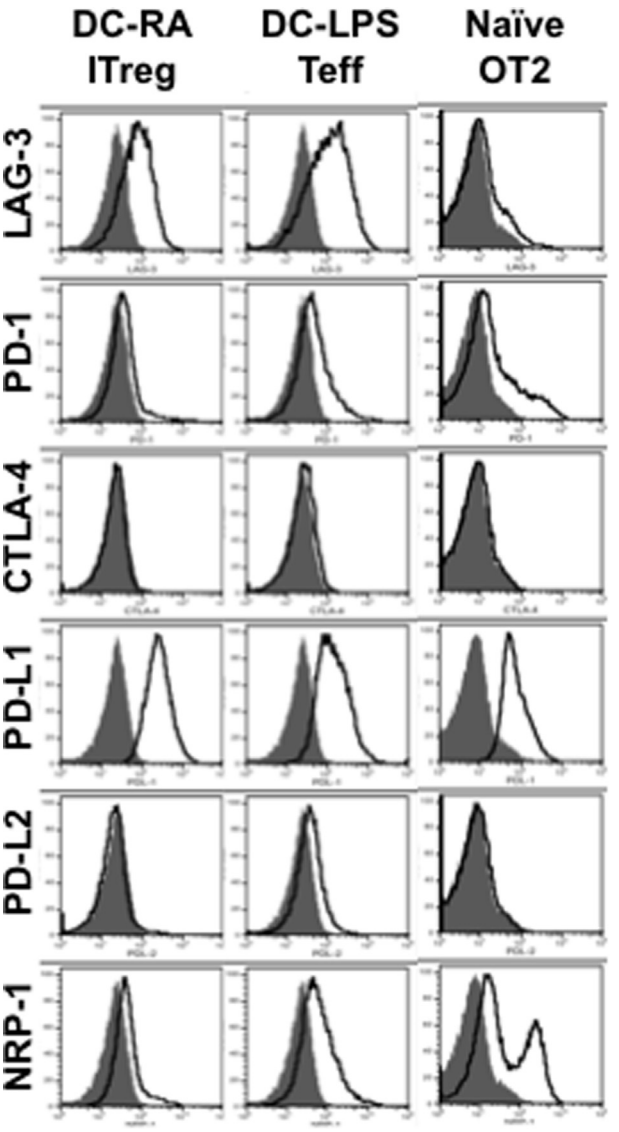



**FIG 7.** FACS characterization of DC-RA–induced Treg cells. CD4$^+$ T cells from OVA/alum-immunized OT II mice were cocultured with either DC-RAs or DC-LPSs. At the end of the 5-day cultures, naive OT II CD4$^+$ T cells or CD4$^+$ T cells from cocultures were assessed for expression of the indicated markers *(black line)*; isotype control antibody–stained cells *(solid gray)* were also examined. Results are representative of 3 independent experiments. *NRP-1*, Neuropilin-1.

## Induction of tolerance to allergen by DC-RAs is critically dependent on their expression of IL-27

To confirm that secretion of IL-27 by DC-RAs is important to their tolerogenic activities, we next assessed the abilities of OVA-presenting *Ebi3*$^{-/-}$ versus WT DC-RAs to suppress allergic responses in our OVA anaphylaxis model (Fig 8). As above, the WT DC-RAs reduced clinical scores, mast cell activation, serum OVA-specific $IgG_1$ and IgE levels, and peritoneal $T_H2$ cytokine responses in this model, whereas OVA-presenting *Ebi3*$^{-/-}$ DC-RAs had no discernible effect on clinical scores, mast cell activation, or peritoneal IL-4, IL-5, or IL-9 levels. Interestingly, the $IgG_1$, IgE, and IL-13 levels in mice treated with *Ebi3*$^{-/-}$ DC-RAs were not significantly different from those in WT DC-RA–treated animals. Taken together, however, these data suggest that IL-27 expression by DC-RAs is critical for their induction of a robust tolerance to anaphylaxis in animals with food allergy.

## DISCUSSION

In the gut, DCs that differentiate locally under the influence of RA and TGF-β expressed by intestinal epithelial cells foster tolerance to harmless commensal bacteria and food antigens.[2] We found that DCs differentiated in the presence of RA and exposed to LPS maturational signals (DC-RAs) express markers consistent with RA-induced gut DCs (CD103, CCR9, and Aldh1A2) and some found on fully mature DCs (MHC class II, CD40, CD54, CD80, and OX40 ligand) but, at the same time, numerous markers consistent with a tolerogenic phenotype. These specific allergen-presenting DC-RAs suppressed $T_H2$-type T-cell responses in an IL-10– and TGF-β–dependent fashion, but it was their secretion of IL-27 that was critical to their induction of the Foxp3$^-$ Treg cells that suppressed allergen-induced anaphylactic responses *in vivo*. These data provide proof of principle that DCregs can be used therapeutically for food allergies in mouse models, wherein they induce a novel phenotype of CD4$^+$Foxp3$^-$ Treg cells. As far as we are aware, this is the first such demonstration of successful implementation of DC immunotherapy in the context of intestinal allergic

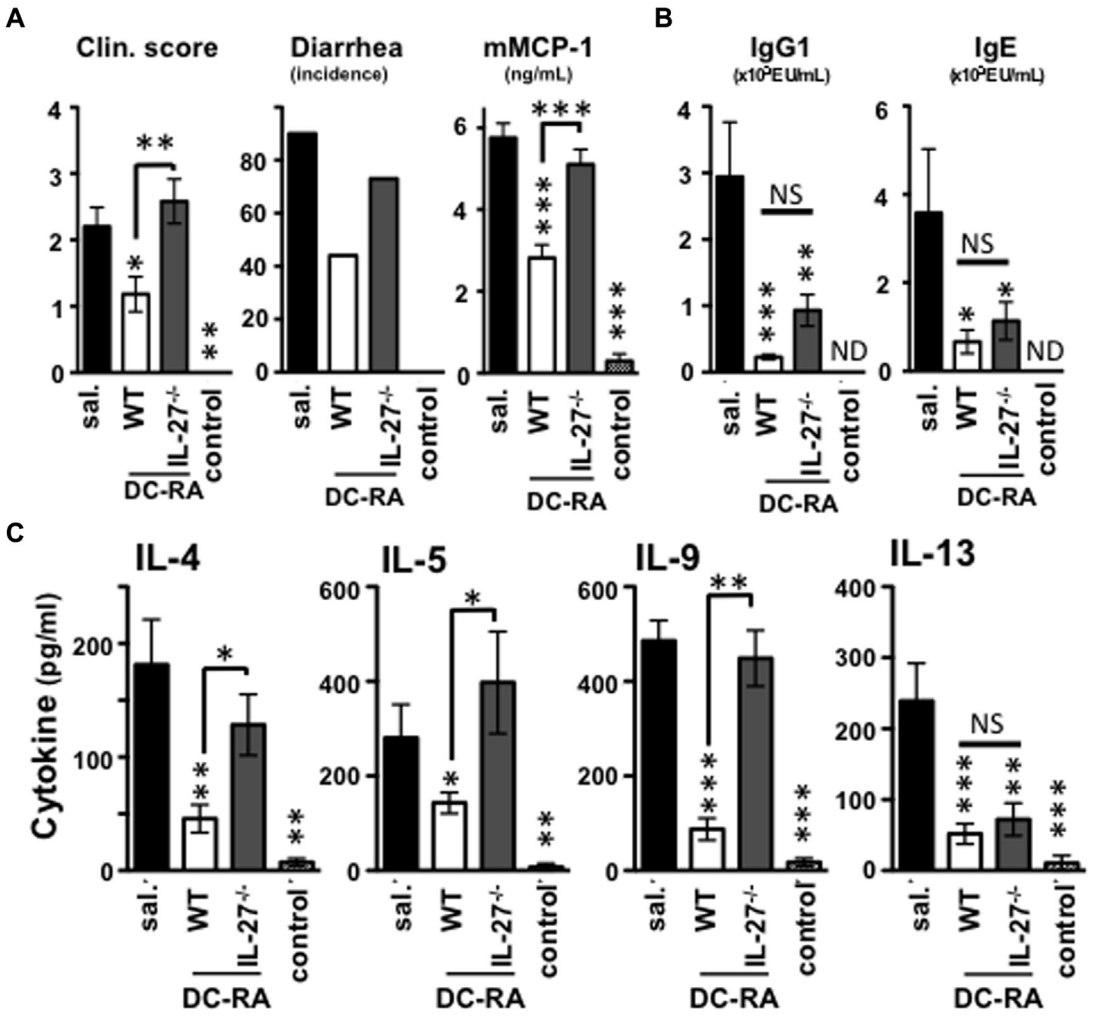



**FIG 8.** IL-27 is critical to DC-RA reversal of the anaphylactic phenotype after food allergen challenge. Mice were sensitized to OVA, treated with OVA-presenting DC-RAs, and challenged with OVA, as in Fig 4, but with either WT or IL-27$^{-/-}$ DC-RAs. Clinical scores, incidence of diarrhea, and serum mMCP-1 levels **(A)**; serum OVA-specific $IgG_1$ and IgE levels **(B)**, and peritoneal lavage fluid $T_H2$ cytokine levels **(C)** are shown. *NS*, $P \geq .05$. *$P \leq .05$, **$P \leq .01$, or ***$P \leq .001$. Results are the mean of 2 independent experiments (n = 10-12 mice per group).

inflammation. These Treg cells were neither classical $CD25^+Foxp3^+$ Treg cells nor $T_R1$ cells (ie, IL-10–dependent $CD25^-Maf^+AhR^+LAG3^+CD49b^+Foxp3^-$)[19]; they were IL-10–independent $LAG3^+CD25^+CD49b^-$ cells that only weakly express IL-10, c-Maf, and AhR. This suggests that we have another therapeutic option for DCreg immunotherapy that could potentially be useful in the face of intestinal inflammatory diseases, wherein inflammation can suppress Foxp3 expression in classical $Foxp3^+$ Treg cells and thereby also their tolerogenic influence.[7]

Within 4 weeks of administering a single dose of specific allergen-presenting DC-RAs, the treatment had reduced anaphylaxis clinical scores by 84% to 90% and IgE/$IgG_1$ levels by 65% to 90%. We expected that reductions in mast cell activation in this model would lag behind those for serum IgE or $IgG_1$ because the half-life of mast cell FcεRI-bound IgE is in the order of 4 to 6 weeks in mice, even after serum levels of IgE have become indiscernible.[20] We know that in mouse models of asthma, the therapeutic effects of DC10 immunotherapy are progressive in nature, with the IgE and $IgG_1$ responses lagging somewhat behind the DC10-induced corrections of the $T_H2$ response. Nevertheless, within 12 weeks of a single DC10 treatment, IgE levels are at background in this model and remain there for at least another 4 months, whereas 4 biweekly doses bring the asthma phenotype to near background within 2 months.[5] We did not assess the effects of delivering DC-RAs multiple times in our anaphylaxis models but would anticipate similarly accelerated outcomes with repeated DC-RA immunotherapy.

DC-RA suppression of *in vitro* $T_H2$ proliferative responses did not require direct DC-RA/$T_H2$ cell-cell contact, although the DC-RAs strongly expressed the PDL2 inhibitory ligand for programmed death 1 receptor, which was reciprocally expressed by the $T_H2$ cells in this study. PDL2 has been shown to be important to the induction of oral tolerance in mouse models.[21] Other clues that our DC-RAs might be tolerogenic could be found in their expression of Aldh1A2 and TGF-β. As noted above, intestinal $CD103^+$ DCs that develop under the influence of TGF-β and RA secreted by the intestinal epithelium (which in

turn secrete TGF-β and RA themselves)[2,22] induce the differentiation of gut-homing (ie, α4β7-and CCR9-expressing) Foxp3$^+$ Treg cells.[23] Although our DC-RA–induced Treg cells were CCR9$^+$, we did not assess their expression of α4β7 integrin but did find them to be Foxp3$^-$ cells; these Treg cells effectively induced food allergen tolerance, suggesting that they would have had some affinity for the gut mucosal compartment. TGF-β and RA are both important to the development of the gut-homing Treg cell phenotype, inasmuch as neutralization of TGF-β in cocultures of human monocyte-derived (immature) RA-derived DCs and T cells inhibits α4β7 but not CCR9 expression by the T cells, whereas addition of an RA receptor antagonist by itself inhibits expression of both α4β7 and CCR9.[24] However, CD103$^+$ DCs are also found in the lungs, where expression of RA and TGF-β by these cells is also important to their local induction of Foxp3$^+$ Treg cells[25] and tolerance. Although our DC-RAs expressed high levels of Aldh1A2 and modest amounts of TGF-β, we did not assess their production of RA. However, our data indicate that both IL-10 and TGF-β were jointly responsible for the DC-RA–dependent suppression of $T_H2$ cell proliferation we observed in our 3-day cultures.

Others have reported that IL-10 and TGF-β cooperate in suppressing alloantigen-driven mixed lymphocyte reactions, with neither IL-10 nor TGF-β alone being sufficient to realize full hyporesponsiveness,[26] and this is consistent with our findings herein. It has been reported previously that one mechanism by which IL-27 suppresses T-cell responses is through induction of IL-10 expression by its target T cells,[18,27] but secretion of IL-10 and TGF-β by DC-RAs would likely mask an IL-10–associated influence of IL-27 in such DC-RA/$T_H2$ cell cultures, such that any effect of removing IL-27–induced $T_H2$ cell IL-10 secretion (ie, by use of *Ebi*$^{-/-}$ DC-RAs) would not be apparent in $T_H2$ cell inhibition assays.

Although IL-27 did not discernibly affect $T_H2$ proliferative responses in our 3-day cultures, it was expressed at high levels by our DC-RAs and was important in their inducing $T_H2$ cells to differentiate into Foxp3$^-$ Treg cells across 5 days in our model. In turn, immature RA-induced DCs reportedly induce the differentiation of Foxp3$^+$ Treg cells,[8] but TLR ligands, such as we used with our DC-RAs, can regulate IL-27 expression. For example, TLR2 or TLR4 signaling in DCs that have been differentiated in the presence of dexamethasone, either alone or with vitamin D3, upregulates their expression of IL-10 and IL-27.[9,10] Interestingly, LPS stimulation of hepatic DCs induces expression of IL-10 and IL-27 and fosters the differentiation of Foxp3$^+$ Treg cells,[28] but $T_H3$ cells induce DCs to secrete IL-27, IL-10, and TGF-β, which in turn induce the development of Foxp3$^-$ $T_R1$ cells.[29] However, $T_H3$ cell–induced DCregs express more TGF-β and substantially less IL-27 than our DC-RAs,[29] suggesting perhaps that the relative levels of IL-10, TGF-β, and IL-27 expression by DCregs might be a factor in their induction of Foxp3$^-$ versus Foxp3$^+$ Treg cells.

It has been reported that IL-27 and TGF-β synergize in induction of c-Maf,[30] a critical component in IL-10 induction within $T_R1$ cells, although others reported that IL-27 induced c-Maf, IL-21, and inducible costimulator expression are all critical to the induction of $T_R1$ cells.[31] IL-27 also induces T-cell expression of AhR, which binds to c-Maf to coordinately activate the IL-10 and IL-21 promotors.[32] Our DC-RA–induced Foxp3$^-$ Treg cells expressed only very low levels of c-Maf or AhR, suggesting that DC-RAs potentially provide additional signals that alter T-cell responses to IL-27.

Another factor affected by IL-27 signaling in T cells, and more specifically Treg cells, is LAG3, which was strongly expressed on our DC-RA–induced Treg cells and has been reported to play a significant role in inflammatory bowel disease tolerance.[33] Thus although DC-RA–induced Foxp3$^-$ Treg cells share some features in common with $T_R1$ cells, they also differ in multiple respects, including lack of or significantly reduced expression of CD49b and IL-10 but augmented expression of CD25.

Specific allergen-presenting DC10s can also be effective in preventing the induction of or reversing the allergic phenotype, as seen in mouse models of OVA and house dust mite–related asthma,[4-6,34,35] albeit through mechanisms distinct from the DC-RAs in the present study. DC10s abrogate airway hyperresponsiveness within 3 weeks of treating asthmatic mice and progressively diminish allergen-specific $T_H2$ responses for up to 8 months.[5] Moreover, DC10-induced tolerance is resistant to repeated challenge with physiologic levels of allergen (ie, levels that induce early and late responses in asthmatic patients). Mouse DC10s induce allergen-specific $T_H2$ cells to transdifferentiate into CD25$^+$Foxp3$^+$ Treg cells that can passively transfer asthma tolerance,[4,11] whereas semimature human DC10s generated from atopic asthmatic patients can induce autologous $T_H2$ cell tolerance and $T_H2$ cell conversion into LAG3$^+$CTLA-4$^+$ILT2$^+$CD25$^+$Foxp3$^+$ Treg cells.[13] As noted, in contrast to our LPS-exposed DC-RAs, DCs-RA that have not been exposed to LPS induce the differentiation of CD25$^+$Foxp3$^+$ Treg.[23] Our rationale herein for choosing differentiation conditions that lead to induction of CD25$^+$Foxp3$^-$ Treg cells relates to the report that Foxp3$^+$ Treg cells lose expression of Foxp3 when introduced into mice with colitis and subsequently convert into $T_H17$ cells that exacerbate rather than ameliorate colitis pathology.[7]

We query whether gut inflammation *per se*, such as might occur in the setting of food allergen–induced anaphylaxis or in patients with food allergy with colitis, could similarly suppress Foxp3 expression within nascently induced Foxp3$^+$ Treg cells and thereby reduce their therapeutic effectiveness. We hypothesize that Foxp3$^-$ Treg cells could be more resistant to these types of inflammation and therefore might better retain their therapeutic effectiveness. Certainly, in principle, our findings herein provide for an alternate therapeutic option above and beyond the use or induction of Foxp3$^+$ Treg or $T_R1$ cells, expanding our options as we diversify the array of indications for DCreg immunotherapy.

We thank Mark Boyd for his assistance with FACS analysis.

**Key messages**

- **DCreg therapy can be used successfully to reverse food allergen sensitivity.**
- **Mature DCs-RAs induce non–$T_R1$-type Foxp3$^-$ Treg cells through IL-27 secretion.**

## REFERENCES

1. Holt PG, Upham JW. The role of dendritic cells in asthma. Curr Opin Allergy Clin Immunol 2004;4:39-44.

2. Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. Immunol Rev 2011; 241:241-59.
3. Gordon JR, Ma Y, Churchman L, Gordon SA, Dawicki W. Regulatory dendritic cells for immunotherapy in immunologic diseases. Front Immunol 2014;5:7.
4. Huang H, Dawicki W, Zhang X, Town J, Gordon JR. Tolerogenic dendritic cells induce CD4(+)CD25(hi)Foxp3(+) regulatory T cell differentiation from CD4(+)CD25(-/lo)Foxp3(-) effector T cells. J Immunol 2010;185:5003-10.
5. Nayyar A, Dawicki W, Huang H, Lu MP, Zhang X, Gordon JR. Induction of prolonged asthma tolerance by IL-10-differentiated dendritic cells: differential impact on airway hyperresponsiveness and the Th2 immunoinflammatory response. J Immunol 2012;189:72-9.
6. Lu M, Dawicki W, Zhang X, Huang H, Nayyar A, Gordon JR. Therapeutic induction of tolerance by IL-10-differentiated dendritic cells in a mouse model of house dust mite-asthma. Allergy 2011;66:612-20.
7. Yurchenko E, Shio MT, Huang TC, Da Silva Martins M, Szyf M, Levings MK, et al. Inflammation-driven reprogramming of CD4+ Foxp3+ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition in vivo. PLoS One 2012;7:e35572.
8. Chang J, Thangamani S, Kim MH, Ulrich B, Morris SM Jr, Kim CH. Retinoic acid promotes the development of Arg1-expressing dendritic cells for the regulation of T-cell differentiation. Eur J Immunol 2013;43:967-78.
9. Naranjo-Gomez M, Raich-Regue D, Onate C, Grau-Lopez L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. J Transl Med 2011;9:89.
10. Brosbol-Ravnborg A, Bundgaard B, Hollsberg P. Synergy between vitamin D(3) and Toll-like receptor agonists regulates human dendritic cell response during maturation. Clin Dev Immunol 2013;2013:807971.
11. Huang H, Ma Y, Dawicki W, Zhang X, Gordon JR. Comparison of induced versus natural regulatory T cells of the same TCR specificity for induction of tolerance to an environmental antigen. J Immunol 2013;191:1136-43.
12. Schneider AM, Li F, Zhang X, Gordon JR. Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease following sensitization with limiting doses of ovalbumin-alum. Cell Immunol 2001;212:101-9.
13. Li XL, Yang A, Huang H, Zhang X, Town J, Davis B, et al. Induction of type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. Am J Respir Cell Mol Biol 2010;42:190-9.
14. Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. J Allergy Clin Immunol 2000;106:150-8.
15. Chappert P, Schwartz RH. Induction of T cell anergy: integration of environmental cues and infectious tolerance. Curr Opin Immunol 2010;22:552-9.
16. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med 2013;19:739-46.
17. Vasanthakumar A, Kallies A. IL-27 paves different roads to Tr1. Eur J Immunol 2013;43:882-5.
18. Batten M, Kljavin NM, Li J, Walter MJ, de Sauvage FJ, Ghilardi N. Cutting edge: IL-27 is a potent inducer of IL-10 but not FoxP3 in murine T cells. J Immunol 2008;180:2752-6.
19. Yao Y, Vent-Schmidt J, McGeough MD, Wong M, Hoffman HM, Steiner TS, et al. Tr1 cells, but not Foxp3(+) regulatory T cells, suppress NLRP3 inflammasome Activation via an IL-10-dependent mechanism. J Immunol 2015;195:488-97.
20. Kubo S, Nakayama T, Matsuoka K, Yonekawa H, Karasuyama H. Long term maintenance of IgE-mediated memory in mast cells in the absence of detectable serum IgE. J Immunol 2003;170:775-80.
21. Zhang Y, Chung Y, Bishop C, Daugherty B, Chute H, Holst P, et al. Regulation of T cell activation and tolerance by PDL2. Proc Natl Acad Sci U S A 2006;103: 11695-700.
22. Iliev ID, Spadoni I, Mileti E, Matteoli G, Sonzogni A, Sampietro GM, et al. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut 2009;58:1481-9.
23. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007;204:1757-64.
24. Saurer L, McCullough KC, Summerfield A. In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. J Immunol 2007;179:3504-14.
25. Khare A, Krishnamoorthy N, Oriss TB, Fei MJ, Ray P, Ray A. Cutting edge: inhaled antigen upregulates retinaldehyde dehydrogenase in lung CD103(+) but not plasmacytoid dendritic cells to induce Foxp3 de novo in CD4(+) T cells and promote airway tolerance. J Immunol 2013;191:25-9.
26. Zeller JC, Panoskaltsis-Mortari A, Murphy WJ, Ruscetti FW, Narula S, Roncarolo MG, et al. Induction of CD4(+) T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta(1). J Immunol 1999;163:3684-91.
27. Yoshida H, Nakaya M, Miyazaki Y. Interleukin 27: a double-edged sword for offense and defense. J Leukoc Biol 2009;86:1295-303.
28. Chen Y, Jiang G, Yang HR, Gu X, Wang L, Hsieh CC, et al. Distinct response of liver myeloid dendritic cells to endotoxin is mediated by IL-27. J Hepatol 2009;51: 510-9.
29. Awasthi A, Carrier Y, Peron JP, Bettelli E, Kamanaka M, Flavell RA, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol 2007;8:1380-9.
30. Xu J, Yang Y, Qiu G, Lal G, Wu Z, Levy D, et al. c-Maf regulates IL-10 expression during Th17 polarization. J Immunol 2009;182:6226-36.
31. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol 2009;183:797-801.
32. Apetoh L, Quintana FJ, Pot C, Joller N, Xiao S, Kumar D, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. Nat Immunol 2010;11:854-61.
33. Do JS, Visperas A, Sanogo YO, Bechtel JJ, Dvorina N, Kim S, et al. An IL-27/Lag3 axis enhances Foxp3+ regulatory T cell–suppressive function and therapeutic efficacy. Mucosal Immunol 2016;9:137-45.
34. Henry E, Desmet CJ, Garze V, Fievez L, Bedoret D, Heirman C, et al. Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma. J Immunol 2008;181:7230-42.
35. Koya T, Matsuda H, Takeda K, Matsubara S, Miyahara N, Balhorn A, et al. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. J Allergy Clin Immunol 2007;119:1241-50.

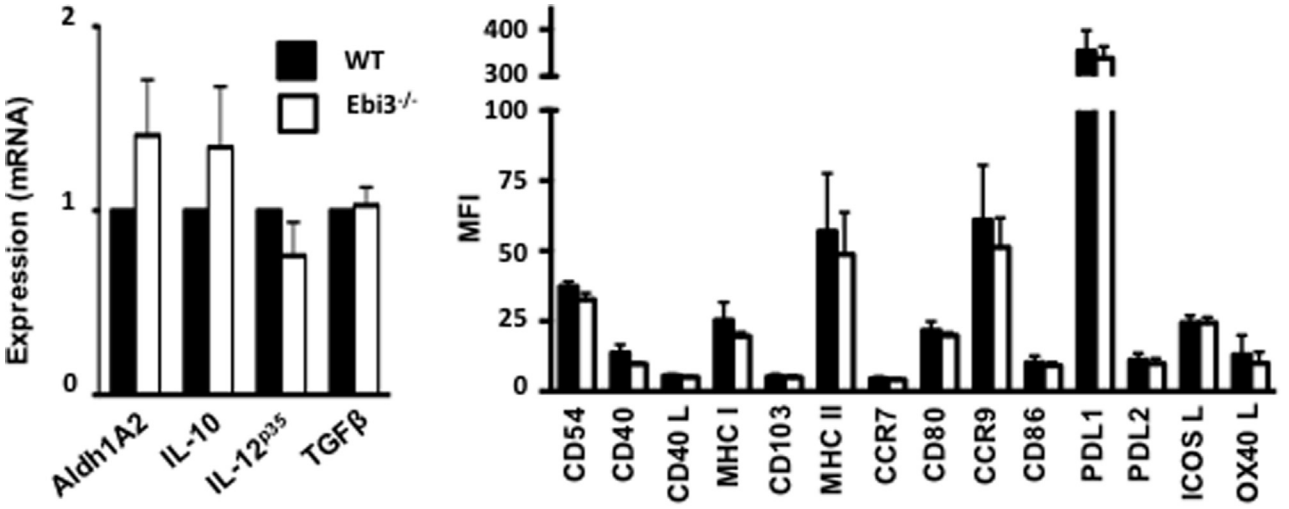



**FIG E1.** Characterization of DC-RAs generated from WT or *Ebi3*$^{-/-}$ mice. DC-RAs were generated from the bone marrow of C57BL/6 or *Ebi3*$^{-/-}$ mice. *Left panel*, Expression of mRNA for the indicated markers was assessed by using quantitative PCR. *Right panel*, Cell-surface marker expression was determined by means of FACS. *Bars* represent means ± SEMs of 3 independent experiments.

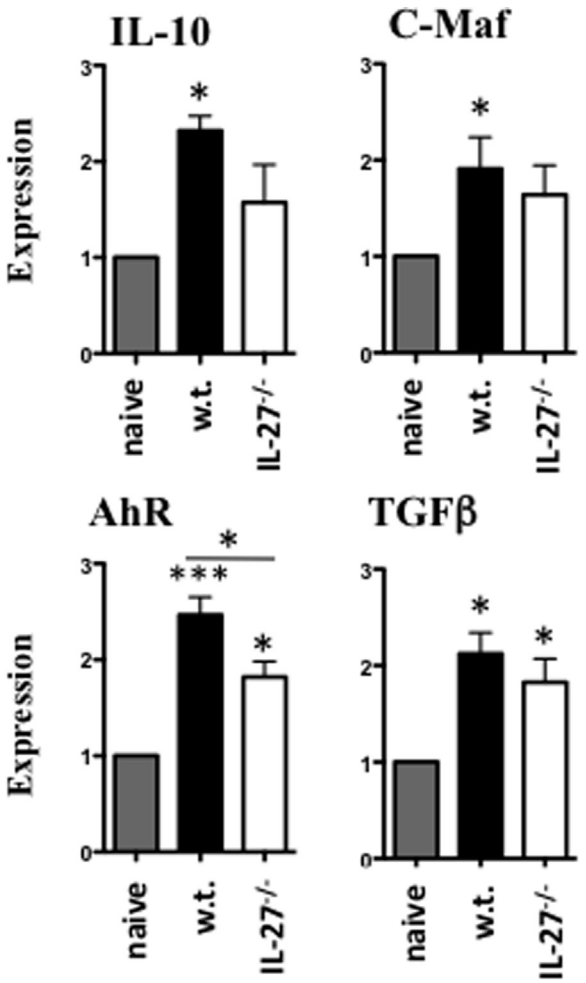



**FIG E2**. Characterization of WT and IL-27$^{-/-}$ DC-RA–induced Treg cells. Magnetically sorted WT or IL-27$^{-/-}$ DC-RA–induced Treg cells generated as in Fig 7 or naive CD4$^{+}$ T cells (as normalization controls) were assessed for expression of indicated genes by using quantitative PCR. Expression is normalized to actin. DC-RA–induced CD25$^{+}$LAG3$^{+}$CD49b$^{-}$Foxp3$^{-}$ Treg cells express modest but statistically significant levels of IL-10, c-Maf, the AhR, and TGF-β, as determined by using qRT-PCR. *$P \leq .05$ or ***$P \leq .001$. Results are the mean of 3 independent experiments.

**TABLE E1.** Sequences of primers used for qRT-PCR analysis of DCreg markers

| Target | Sequence |
|---|---|
| c-Maf F | AGCAGTTGGTGACCATGTCG |
| c-Maf R | TGGAGATCTCCTGCTTGAGG |
| IL-12p35 F | GCCCTCCTAAACCACCTCAGT |
| IL-12p35 R | CAGGCAACTCTCGTTCTTGTGTA |
| AhR F | CGCTGCTTCCTCCACAACTG |
| AhR R | TAAGCTGCCCTTTGGCATCAC |
| IL-27p28 F | CTCTGCTTCCTCGCTACCAC |
| IL-27p28 R | GGGGCAGCTTCTTTTCTTCT |
| TGF-β1 F | GACTCTCCACCTGCAAGACCAT |
| TGF-β1 R | GGGACTGGCGAGCCTTAGTT |
| Aldh1A2 F | AATGGGTGAGTTTGGCTTACGG |
| Aldh1A2 R | AGAAACGTGGCAGTCTTGGCAC |
| B-Actin F | AGAGGGAAATCGTGCGTGAC |
| B-Actin R | CAATAGTGATGACCTGGCCGT |
| IL-10 F | AAGCCTTATCGGAAATGATCCA |
| IL-10 R | GCTCCACTGCCTTGCTCTTATT |

*F*, Forward primer; *R*, reverse primer.