Therapeutic reversal of food allergen sensitivity by mature retinoic acid-differentiated dendritic cell induction of LAG3\(^+\)CD49b\(^-\)Foxp3\(^-\) Treg

Wojciech Dawicki, PhD\(^*\), Chunyan Li, MSc\(^†\), Jennifer Town, PhD\(^†\), Xiaobei Zhang, BMed\(^*\),
and John R. Gordon, PhD\(^*\)

\(^*\)Department of Medicine, and the \(^†\)Department of Veterinary Microbiology,
University of Saskatchewan, Canada

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Address for correspondence: Dr. John R. Gordon, Division of Respirology, Critical Care and Sleep Medicine, Rm 3D30.8 Health Sciences Bld, 107 Wiggins Road, Saskatoon, SK, Canada S7N 5E5. Phone: 306-966-1901; Email: john.gordon@usask.ca.

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Abbreviations used: AhR, the ligand-activated transcription factor aryl hydrocarbon receptor; Aldh1A2, retinaldehyde 1A2; CFSE, carboxyfluoryl succinylate ester; c-maf, the proto-oncogene c-maf; CCR9, CC subfamily chemokine receptor 9; CPE, crude peanut extract; CTLA4, cytotoxic lymphocyte antigen 4; DC10, IL-10-skewed tolerogenic dendritic cells; DC-LPS, LPS-matured immunostimulatory dendritic cell; DC-RA, retinoic acid-skewed dendritic cells; Ebi, the \(\beta\) sub-unit of interleukin-27; FBS, fetal bovine sérum; Foxp3, the transcription factor forkhead box P3; LAG3, lymphocyte activation gene 3; LPS, lipopolysaccharide; mMCP-1, mouse mast cell protease-1; OVA, ovalbumin; OT II mice, transgenic mice expressing an OVA-specific T cell receptor; PD1, programmed death 1 (receptor); PD-L1 & L2, programmed death ligand 1 and 2; TLR4, toll-like receptor 4; Treg, regulatory T cell
ABSTRACT

Background. Anaphylaxis is a life-threatening condition for which we have limited therapeutic options. While specific immunotherapy for food allergies is becoming more effective, it is still laborious and carries substantial risk of adverse events. Regulatory dendritic cell (DC) therapy on the other hand is effective in mouse models of allergic disease, and has been shown to work with Th2 cells from atopic asthmatics.

Objective. Herein we assessed whether DC immunotherapy could reverse food allergen sensitivity in mouse models, in order to provide proof-of-concept relating to their use in the clinic.

Methods. We generated and characterized mature retinoic acid (RA)-induced DC (DC-RA), and assessed their abilities to reverse OVA or peanut allergies in mouse models, as well as their operative mechanisms.

Results. DC-RA displayed a mature, yet tolerogenic phenotype, expressing IL-10, TGFβ, IL-27, and aldehyde dehydrogenase 1A2, but not IL-12 or IL-35; IL-10 and TGFβ together drove their suppression of Th2 cell proliferation. Delivery of specific allergen-presenting DC-RA to half-maximally sensitized OVA- or peanut-allergic mice reduced anaphylactic responses to oral allergen challenge by 84-90%, as well as diarrhea, mast cell activation, and Th2 cytokine responses, and serum allergen-specific IgE/IgG1 levels. DC-RA expression of IL-27 was important to their induction of CD25<sup>+</sup>LAG3<sup>+</sup>CD49b Foxp3<sup>+</sup> regulatory T cells in vitro, such that Ebii<sup>−/−</sup> (i.e., IL-27-incompetent) DC-RA were ineffective in inducing food allergen tolerance.

Conclusion. Our data indicates that regulatory dendritic cell immunotherapy can be effective for food allergies, and suggests that induction of Foxp3<sup>−</sup> Treg may be a useful strategy for tolerance induction in this context. (252 words)

Key Messages
- Regulatory DC therapy can be used successfully to reverse food allergen sensitivity
- Mature RA-induced DC induce non-Tr1-type Foxp3<sup>−</sup> Treg via IL-27 secretion
Capsule Summary

We provide conceptual evidence that dendritic cell therapy could be an important approach to subjects with food allergies, wherein we have options regarding the types of regulatory T cell responses we induce.
INTRODUCTION

Dendritic cells (DC) are well known for their abilities 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 DC present innocuous aeroallergens while expressing low levels of MHCII and costimulatory molecules and higher levels of IL-10, and thereby induce naïve CD4+ T cells to differentiate into CD25+Foxp3+ Treg that mediate aeroallergen tolerance. In an analogous manner, intestinal CD103+ DC that present innocuous food or gut commensal antigens to naïve T cells in the gut-draining lymph node secrete TGFβ and retinoic acid while doing so, and thereby induce the differentiation of gut-homing (i.e., αβ7 integrin/CCR9-expressing) CD25+Foxp3+ Treg. Such regulatory DC (DCreg) can also be induced in vitro by exposure to an array of tolerogenic mediators (e.g., dexamethasone, IL-10) and indeed it has been reported by multiple laboratories that such DCreg can suppress the responsiveness of effector T cells in multiple settings (reviewed in ref. 3). We and others have similarly reported that IL-10-differentiated DC (DC10) can reverse the asthma phenotype in mouse models, where they induce Th2 cells to differentiate into CD25+Foxp3+ Treg in an IL-10-dependent fashion. While the tolerance so induced is progressive, long-lasting, and resistant to repeated challenge with physiologic levels of allergen, others have reported that Foxp3+ Treg immunotherapy in mice with colitis fails because the colitis-associated inflammation suppresses Foxp3 expression in these Treg, which subsequently convert into Th17 cells that exacerbate rather than ameliorate the colitis phenotype. This suggests that it could be important in the context of, for example, immunotherapy for inflammatory diseases to induce Foxp3− rather than Foxp3+ Treg. While induction of Foxp3+ Treg-driven tolerance can be effective in experimental asthma therapy, regulatory DC 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, allergen-induced inflammation in food-allergic individuals could also impact the phenotype of Foxp3+ Treg that might be present or induced in the gut.

Given the associations between intestinal retinoic acid and the induction of tolerogenic DC, we have been examining conditions that would lead retinoic acid-induced tolerogenic DC to foster differentiation of Foxp3-negative, as opposed to Foxp3-positive Treg responses. Herein we report...
that specific allergen-presenting retinoic acid-differentiated DC that have been exposed to strong
TLR4 signals during differentiation express high levels of IL-27 and thereby induce food allergen
tolerance in mouse models of OVA and peanut allergies, replacing allergen-specific CD4+ Th2
responses with CD25+LAG3+CD49b–Foxp3– Treg responses that suppress allergic responses to oral
allergen challenge.
METHODS

Animals. BALB/c, C57Bl/6, OT II and Ebi3-/- mice were purchased from Jackson Laboratory (Bar Harbor, ME); 6-12 wk-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 accord 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 (clone11-0042-81), CD69 (cloneH1-2F3), CD40 (cloneHM40-3), CD25 (cloneDC61.5), CD103 (clone2E7), CD276 (cloneM3.2D7), LAP (clone TW7-16B4), LAG3 (cloneBioC9B7W), CCR9 (cloneBioCW-1.2), OX40L (cloneRM134L), PDL1 (cloneMIH5), PDL2 (clone122) and isotype control antibodies were purchased from eBioscience (San Diego, CA), while anti-mouse CD86 (cloneGL1), CD54 (clone3E2), IgG1 and IgE were from BD Biosciences (San Jose, CA). Anti-CD4-specific magnetic sorting beads and columns were from Miltenyi Biotec (Auburn, CA). Staphylococcus aureus lipopolysaccharide (LPS), ovalbumin (OVA), and all-trans retinoic acid (RA) were purchased from Sigma-Aldrich (Oakville, ON), carboxyfluoryl succinylate ester (CFSE) from Invitrogen (Eugene, OR), crude peanut extract (CPE) from Greer Laboratories (Lenoir, NC), and 3H-thymidine 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, IFNγ and TGFβ were purchased from R&D Systems (Minneapolis, MN).

Flow cytometry. Cells were washed three times with FACS buffer (0.01M azide, 2% FBS, PBS), incubated with blocking antibody for 10 m at 4ºC, then labeled with marker-specific or isotype control fluorochrome-labeled antibodies for an additional 20 m at 4ºC. After washing two times with FACS buffer, the cells were analysed with an EPICS XL flow cytometer (Beckman Coulter, Mississauga, ON, Canada) and the FACS data analyzed using FlowJo software (Tree Star Inc., Ashland, OR).
**Generation of DC.** All DC were differentiated from bone marrow cells as noted by plating cells at $5 \times 10^5$ /mL in 6-well plates for 7 d in RPMI 1640 medium supplemented with 10% FBS, L-glutamine, and antibiotics; every 2-3 d 75% of the media in each culture was replaced. To generate tolerogenic retinoic acid-differentiated DC (DC-RA) we supplemented our standard DC differentiation cultures (20 ng/mL GM-CSF, 10 ng/mL IL-4) with 1 µM retinoic acid; immature DC-RA (iDC-RA) were used as is, while our standard mature DC-RA were also exposed to LPS (1 µg/ml) for the final 18 h of culture. It had been reported previously that immature retinoic acid-induced DC can induce the differentiation of Foxp3$^+$ Treg, but also that TLR4 signalling in dexamethasone-differentiated DC upregulates their expression of IL-10 and IL-27. Stimulatory DC-LPS were differentiated in 20 ng/ml GM-CSF (without IL-4 or RA) and pulsed with 1 µg/mL LPS for the final 18 h of culture. Differentiated DC were either pulsed with allergen (50 µg/ml) for the last 24 h before harvest or not so treated (control DC-RA).

**Quantitative real-time PCR assays.** RNA was isolated from cells using RNeasy (QIAGEN, ON) kits, while cDNAs were synthesized using qScript kits (Quanta Biosciences, MD), according to the manufacturer’s specifications. qRT-PCR reactions were performed using a PerfCTa SYBR Green FastMix (Quanta Biosciences, MD) with the appropriate primers in a C1000 Touch (BioRad, ON) thermocycler. Primers sequences are listed in Supplementary Table 1. Data was analyzed using Bio-Rad CFX Manager (BioRad, ON) software using β-actin as a normalizer.

**ELISA.** Concentrations of IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-27p28, IFNγ and TGFβ in biological samples were determined by ELISA using matched capture and detection antibodies and cytokine standards, as noted previously. The sensitivity of these assays was routinely 5-10 pg/ml. The mouse mast cell protease-1 (mMCP-1) ELISA reagents were purchased from eBioscience (San Diego, CA) and the assay carried out according to manufacture’s protocol. The concentrations of allergen-specific IgG1 was determined by sandwich ELISA, using plate-bound allergen to capture the antibodies from serum and biotinylated anti-IgG for detection, while the concentrations of allergen-specific IgE was determined by sandwich ELISA, using plate-bound anti-IgE to capture the antibodies from serum and biotinylated allergen for detection. Streptavidin alkaline phosphatase was used to visualize the captured biotinylated detection reagents. Serial dilutions of a pooled reference sera from mice immunized twice with 2 (OVA) or
Magnetic sorting of CD4+ cells and suppression assays. Spleen and lymph nodes cells were dispersed mechanically, then passed through a 70 µm cell strainer and the contaminating red blood cells lysed with hypotonic tris-ammonium chloride. CD4+ cells were purified using 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%.

Suppression assay. CD4+ Th2 cells were purified from the spleens and lymph nodes of OVA- or CPE-alum-immunized B6 mice or naive OT II mice and co-cultured with irradiated antigen-pulsed stimulatory DC (DC-LPS) for 48 hr in U-bottom 96-well plates (10^5 T cells and 3.7x10^3 DC-LPS/well), as noted^4; we determined in preliminary experiments that these conditions induced half-maximal T-cell proliferation. At 48 h, 0.5µCi ³H-thymidine was added to the cultures and the cells were harvested 24h later. The suppressive activities of putatively tolerogenic DC-RA or DC-RA-induced Treg was determined by titrating cells that had been irradiated into T-cell/DC-LPS co-cultures. In some experiments we also added neutralizing anti-IL-10 and/or anti-TGFβ antibodies into the cultures (10 µg/ml) to determine the roles of these cytokines in the regulatory activities of DC-RA or DC-RA-induced Treg.

In vitro production of DC-RA-induced Treg. Magnetically-sorted CD4+ Th2 cells from OVA-alum-immunized mice were co-cultured for 5 d with DC-RA or, as a negative control, DC-LPS (10^5 T cells and 3x10^4 DC/well)in 96-well U-bottom plates, after which the induced putative CD4+ Treg from these cultures were purified by magnetic sorting. In some assays the Th2 cells were pre-labeled with CFSE to assess their proliferation responses to allergen-presenting DC-RA or DC-LPS in these cultures, while in other assays the putatively induced Treg from these cultures were analysed by FACS for the indicated markers or were assessed for their abilities to suppress DC-LPS-induced Th2 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 i.p. twice, two weeks apart, with 200 µl of either OVA-alum (2 µg OVA/2 mg alum) or with crude peanut extract (CPE)-alum (20 µg
CPE/mg alum). Beginning two weeks later the mice were gavaged every second day with 2 mg OVA or 50 mg peanut butter, respectively (with 3 hr 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 anaphlaxis were first observed during the sensitization phase, >3 additional challenges would most often lead to lethal outcomes. We employed a standard 5-point scoring system for anaphylactic responses wherein: 0 = no clinical symptoms; 1 = repetitive vigorous nose/ear scratching; 2 = lethargy, puffy eyes/mouth; 3 = periods of motionless for >1 m, lying prone; 4 = no response to whisker stimuli, reduced or no response to prodding; and 5 = tremor, convulsion, death; for humane reasons, animals that attained a clinical score of 4 were euthanized immediately. Since our purpose was to evaluate the impact (positive or negative) of dendritic cell treatments on the anaphylaxis phenotype, we titrated our OVA model by adjusting the numbers of pre-experiment challenges to yield half-maximal mean scores (i.e., ±2). Most animals that displayed allergen sensitivity also experienced an explosive diarrhea approximately 30-40 m after allergen challenge.

**Regulatory DC treatment of food-allergic animals.** In order to ensure that our treatment DC were not exposed to a potentially confounding inflammatory environment *in situ* after delivery, we allowed two weeks after the animals had achieved full allergen sensitivity before beginning DCregr immunotherapy, and then assessed the impact of the therapy on anaphylactic responses to challenge four weeks later. For treatment each animal was injected i.p. with 1x10^6 treatment or control DC, or an equal volume of saline, and 4 wk later they received one more oral challenge with OVA or peanut butter, as appropriate. The mice were observed over the ensuing 40 m for clinical demeanor and incidence of diarrhea, while at 50 m a blood sample was taken from each mouse for mMCP-1 analysis. The next day the mice were sacrificed, and blood, peritoneal lavage fluids and various tissues were collected.

**Statistical Analysis.** In experiments with only two study groups Student’s t-tests were performed, while in all other experiments One-way ANOVAs were used with Tukey’s *post-hoc* testing to compare pairs of experimental groups. All analysis was performed using GraphPad Prism software (La Jolla, CA). As the occurrence of diarrhea in our study was scored as its incidence, we could not undertake meaningful statistical analyses with this parameter.
RESULTS

Characterization of mature retinoic acid-differentiated DC. DC differentiated in the presence of retinoic acid (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 MHCII, CD54, CD80 and CD86, but also PDL1, ICOSL, and uniquely higher levels of PDL2 than DC-LPS (Fig. 1A). They expressed modest levels of TGFβ, high levels of IL-27 (IL-27p28/Ebi3) and the retinoic acid-metabolizing enzyme Aldh1A2, little IL-10 and only background levels of IL-12, as determined by IL-12p35 qRT-PCR and IL-12p70 ELISA assays. This latter outcome indicates also that DC-RA also do not express significant levels of the other IL-12p35-containing heterodimer, IL-35 (IL-12p35/Ebi3). Fully mature DC-LPS also strongly expressed MHCII, CD54, CD80, PDL1, and IL-12, but essentially only background or modest levels of TGFβ, Aldh1A2 or IL-27 (Fig 1B).

When titrated into co-cultures of allergen-presenting immunostimulatory DC-LPS and either OVA TCR-transgenic (i.e., OTII) T cells or Th2 cells from OVA-allergic C57/BL6 mice, OVA-loaded DC-RA suppressed the 3-day T cell proliferative responses in a dose-dependent fashion (Fig. 2A). This suppression occurred whether the DC-RA 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. 2B). We assessed whether expression of *Ebi3*-containing heterodimeric cytokines (i.e., IL-27 [p28/Ebi3] or IL-35 [p35/Ebi3]) by DC-RA was important for their suppression of effector T cell proliferation in response to DC-LPS activation; as noted, our data suggests that DC-RA do not express discernible levels of IL-35, although they do secrete ample IL-27 (Fig. 1B). We also confirmed that loss of Ebi3 in DC-RA did not impact other parameters relevant to their tolerogenic activities, including expression of Aldh1A2, IL-10, IL-12, and TGFβ, as well as MHC I and II and an array of costimulatory markers, chemokine receptors and inhibitory receptors (Supplementary Fig. S1). Both WT and *Ebi3<sup>−/−</sup>* DC-RA were equally able to inhibit T cell proliferation, suggesting that IL-27 was dispensable for DC-RA suppression of Th2 cell responses in these 3-day cultures (Fig. 2C, left panel). In further assessing what drives the abilities of DC-RA to inhibit Th2 proliferative responses, we found that neutralizing IL-10 significantly reduced the cells’ inhibitory
activities (p<0.05), while simultaneous inhibition of IL-10 and TGFβ fully reversed the DC-RA-dependent suppression of Th2 cell activation by DC-LPS (p<0.001; Fig. 2C, right panel).

We also asked whether LPS-dependent maturation of retinoic acid-induced regulatory DC was a critical parameter in the induction of their regulatory activities. Thus, we titrated immature DC-RA (iDC-RA), LPS-matured DC-RA (DC-RA) or, as a control, DC-LPS into cultures of stimulatory DC-activated CD4+ T cells magnetically sorted from OVA-sensitized (Effector T cells) or unsensitized (Naïve T cells) OTII mice, and assessed OTII T cell proliferation (Fig. 3A). The LPS-matured DC-RA were effective in attenuating the responses of both naïve and sensitized Th2 cells to allergen-presenting stimulatory DC, but the immature DC-RA were significantly less able to suppress the Th2 responses, although they did have significant regulatory activities on both accounts (Fig. 3A). DC-LPS had no such regulatory activities. While the mature DC-RA and the iDC-RA expressed equivalent levels of Aldh1A2 and TGFβ, the mature cells expressed substantially more IL-10 and IL-27p28, as determined by qRT-PCR (Fig. 3B).

**Specific allergen-presenting DC-RA suppress allergen-induced anaphylaxis in OVA and peanut models of food allergies.** We established OVA-induced anaphylaxis in mice as noted in the materials and methods section, then rested the mice for 2 wk, treated them i.p. with either saline or 1x10^6 control or allergen-pulsed DC-RA, and 4 wk later we challenging them again by gavage with allergen and assessed their responses over the next 24 h (Fig. 4A). As noted, we had titrated our model to achieve half-maximal anaphylaxis responses to this final allergen challenge (i.e., a score of ±2). In our OVA model, the saline-treated mice (sal.) developed substantial nasal and ear urticaria, facial edema and lethargy following allergen challenge, but also displayed an explosive diarrhea response. They had high serum levels of OVA-specific IgE and IgG1, such that they developed a robust mast cell activation response to allergen challenge, as determined by circulating levels of mMCP-1 (Fig. 4B, C). As a surrogate measure of gut Th2 sensitivity we assessed the levels of IL-4, -5, -9, and -13, but also IFNγ in peritoneal wash fluids 24 h after allergen-challenge. Each of the Th2 markers was upregulated in the anaphylactic mice, while very modest levels of IFNγ were detected. Normal unsensitized control mice (norm.) displayed no discernible indications of food allergies or allergen-specific IgE or IgG1 antibodies in response to oral administration of allergen 24 h beforehand (Fig. 4D).
Anaphylaxis phenotype mice that had been treated with 1x10^6 OVA-presenting DC-RA (OVA-DC-RA) displayed significant decreases in clinical scores, mast cell activation, IgE and IgG1 levels, and the levels of peritoneal wash fluid IL-4, -5, -9, and -13, but not IFNγ, relative to the saline-treated mice (p≤0.05 to 0.001; Fig. 4B-D). In order to confirm the allergen-specificity of these effects, we also assessed the impact of treating the mice with cells that had not be exposed to allergen (DC-RA), and found that these cells had no impact on any gut-related anaphylaxis events (clinical scores, diarrhea, mMCP-1, Th2 cytokines or IFNγ levels) or serum IgG1 levels (each, p>0.05 versus saline-treated mice), although for reasons that are not clear the OVA-specific IgE levels were statistically significantly lower in these animals relative to saline-treated mice (p≤0.05). However, taken together, our data indicate that, like previous reports of the allergen-specificity of murine and human DC10-5, 6, 13, the tolerogenic effects of DC-RA 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 had the saline-treated OVA-allergic mice, potentially related to the higher doses of allergen we had used for sensitization of these mice 14. Nevertheless, here too we found that four wk after treatment, CPE-presenting DC-RA had substantially reduced clinical scores (p≤0.01), mast cell degranulation (serum mMCP-1 levels; p≤0.05)(Fig. 5A), and circulating peanut allergen-specific IgG1 (p≤0.01) and IgE (p≤0.05) levels (Fig. 5B). Interestingly, while the mean peritoneal Th2 cytokine values were lower and IFNγ values higher in CPE-pulsed DC-RA- versus saline-treated mice at 24 hr post-challenge, these did not achieve statistical significance, although IL-10 levels were significantly increased (p≤0.05; Fig. 5C).

**DC-RA induce Th2 cells to adopt an IL-27-dependent CD25^+LAG3^+CD49b^+Foxp3^ regulatory T cell phenotype.** We next asked whether our tolerogenic DC-RA were inducing the development of regulatory T cell responses among the Th2 cells of allergic animals. We co-cultured OVA-presenting DC-RA or DC-LPS and CFSE-labelled splenic CD4^+ T cells from OVA-sensitized mice for 5 d, and then assessed the proliferative responses that had occurred in these
cultures (CFSE dilution assay; Fig. 6A) and expression of Treg markers, as determined by FACS of gated CD4^+ T cells. As expected, immunostimulatory DC-LPS strongly induced T cell proliferation, while 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. 6B). The numbers of CD4^+ T cells recovered from the DC-RA/Th2 cocultures were equivalent to the numbers of CD4^+ cells placed into the cultures, and we observed no changes in the FACS forward 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/Th2 cell co-cultures had no impact on this T cell response (data not shown), further indicating that these cells were not simply anergic. When we magnetically sorted the CD4^+ T cells from these DC-RA cocultures and assessed their suppressive activity in our standard DC-LPS/OTII Th2 cell assay, we found that the DC-RA-induced T cells displayed potently regulatory T cell activity, suppressing the OTII T cell proliferation in a dose-dependent fashion (Fig. 6C; WT DC-RA bars). These induced Treg strongly expressed LAG3 and CCR9, but no CD49b or Foxp3 (Fig. 6B), which distinguishes them from classical LAG3^+CD49b^+Foxp3^-Tr1 cells or CD25^-Foxp3^-Treg.

Given the role that IL-27 can play in induction of Treg and the strong expression of this cytokine by DC-RA, we queried whether IL-27 was important to the regulatory phenotype of DC-RA. Thus, we repeated our 5-day Th2 cell/DC-RA co-culture experiment, but used DC-RA generated from the bone marrow of WT or Ebi3^-/-mice. We again magnetically sorted the CD4^+ T cells from these co-cultures and confirmed that, unlike the T cells from the WT DC-RA cocultures, the CD4^+ T cells from the Ebi3^-/- DC-RA/Th2 co-cultures had no discernible regulatory activity in this assay (Fig. 6C); we noted above that WT DC-RA produce IL-27 (i.e., IL-27p28/Ebi3), but not IL-35 (IL-12p35/Ebi3). Since IL-27 induces IL-10 expression in T cells and Foxp3^-Tr1 cells employ IL-10 as a primary regulatory mediator, we next assessed whether IL-10 had a role in the regulatory activities of our DC-RA-induced Treg. We confirmed that mesenteric lymph node (Fig. 6D, inset) or splenic (data not shown) T cells from DC-RA-treated OVA-allergic mice did in fact secrete IL-10 (<50 pg/3x10^6 cells over 48 h; p≤0.05 versus saline-treated anaphylaxis mice). We then assessed the impact of neutralizing anti-IL-10 antibodies on DC-RA-induced Treg suppression of Th2 proliferative responses in DC-LPS cocultures. We titrated DC-RA-induced Treg into these cultures and added either isotype control or neutralizing anti-IL-10 antibodies, but found that
neither of these treatments had any impact of the regulatory activities of DC-RA-induced Treg (Fig. 6D), even though these levels of anti-IL-10 had reduced the abilities of DC-RA to suppress Th2 cell proliferation (Fig. 2D).

We next examined the expression by these Treg of a panel of markers, comparing their expression with that of DC-LPS-activated effector Th2 cells from asthmatic OTII mice and of naïve CD4+ OTII T cells (Fig. 7). The DC-RA-induced Treg expressed substantial levels of LAG3 and PDL-1, although no more so than the comparator Teff cells, but negligible PD1, CTLA4, PDL-2 or neurophilin-1. We also found that our DC-RA-induced Treg expressed only low levels of IL-10, TGFβ, c-maf or the ligand-activated transcription factor aryl hydrocarbon receptor (AhR) (Supplementary Fig. S2). Taken together, this data suggests that DC-RA induce IL-10-independent CD25+LAG3+CD49b−Foxp3− Treg in an IL-27-dependent manner.

**Induction of tolerance to allergen by DC-RA is critically-dependent on DC-m expression of IL-27.** In order to confirm that secretion of IL-27 by DC-RA is important to their tolerogenic activities, we next assessed the abilities of OVA-presenting Ebi3−/− versus WT DC-RA to suppress allergic responses in our OVA anaphylaxis model (Fig. 8). As above, the WT DC-RA reduced clinical scores, mast cell activation, serum OVA-specific IgG1 and IgE levels, and peritoneal Th2 cytokine responses in this model, while OVA-presenting Ebi3−/− DC-RA had no discernible impact on clinical scores, mast cell activation, or peritoneal IL-4, 5 or -9 levels. Interestingly, the IgG1, IgE and IL-13 levels in the mice treated with Ebi3−/− DC-RA were not significantly different from those in the WT DC-RA-treated animals. Taken together, however, this data suggests that IL-27 expression by DC-RA is critical for their induction of a robust tolerance to anaphylaxis in food-allergic animals.
DISCUSSION

In the gut, DC that differentiate locally under the influence of retinoic acid and TGFβ expressed by intestinal epithelial cells foster tolerance to harmless commensal bacteria and food antigens. We found that DC differentiated in the presence of retinoic acid and exposed to LPS maturational signals (DC-RA) express markers consistent with retinoic acid-induced gut dendritic cells (CD103, CCR9, Aldh1A2) and some found on fully mature DC (MHCII, CD40, CD54, CD80, OX40L), but at the same time numerous markers consistent with a tolerogenic phenotype. These specific allergen-presenting DC-RA suppressed Th2-type T cell responses in an IL-10- and TGFβ-dependent fashion, but it was their secretion of IL-27 that was critical to their induction of the Foxp3+ Treg that suppressed allergen-induced anaphylactic responses in vivo. This data provides proof-of-principle that regulatory DC can be used therapeutically for food allergies in mouse models, wherein they induce a novel phenotype of CD4+Foxp3+ Treg. As far as we are aware, this is the first such demonstration of successful implementation of DC immunotherapy in the context of intestinal allergic inflammation. These Treg were neither classical CD25+Foxp3+ Treg or Tr1 (i.e., IL-10-dependent CD25+Maf+AhR+LAG3+CD49b+Foxp3+) cells; 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 regulatory DC immunotherapy that could potentially be useful in the face of intestinal inflammatory diseases, which have the capacity to suppress Foxp3 expression in classical Foxp3+ Treg and thereby also their tolerogenic influence.

Within 4 wk of administering a single dose of specific allergen-presenting DC-RA, the treatment had reduced anaphylaxis clinical scores by 84-90%, and IgE/IgG1 levels by 65-90%. We expected that reductions in mast cell activation in this model would lag behind those for serum IgE or IgG1 because the half-life of mast cell FceRI-bound IgE is in the order of 4-6 wk in mice, even after serum levels of IgE have become indiscernible. We know that in mouse models of asthma the therapeutic effects of DC10 immunotherapy are progressive in nature, with the IgE and IgG1 responses lagging somewhat behind the DC10-induced corrections of the Th2 responses. Nevertheless, within 12 wk of a single DC10 treatment IgE levels are at background in this model, and remain there for at least another 4 months, while four biweekly doses bring the asthma phenotype to near background within 2 months. We did not assess the effects of delivering DC-
RA multiple times in our anaphylaxis models, but would anticipate similarly accelerated outcomes with repeated DC-RA immunotherapy.

DC-RA suppression of in vitro Th2 proliferative responses did not require direct DC-RA/Th2 cell-cell contact, although the DC-RA strongly expressed the PD-L2 inhibitory receptor for PD1, which was reciprocally expressed by the Th2 cells in this study. PDL2 has been shown to be important to the induction of oral tolerance in mouse models. Other clues that our DC-RA might be tolerogenic could be found in their expression of Aldh1A2 and TGFβ. As noted above, intestinal CD103+ DC that develop under the influence of TGFβ and retinoic acid secreted by the intestinal epithelium (which in turn secrete TGFβ and retinoic acid themselves) induce the differentiation of gut-homing (i.e., α4β7- and CCR9-expressing) Foxp3+ Treg. While our DC-RA-induced Treg were CCR9+, we did not assess their expression of α4β7 integrin, but did find them to be Foxp3− cells; these Treg did effectively induce food allergen tolerance, suggesting that they would have had some affinity for the gut mucosal compartment. TGFβ and RA are both important to the development of the gut-homing Treg phenotype, inasmuch as neutralization of TGFβ in co-cultures of human monocyte-derived (immature) retinoic acid-derived DC and T cells inhibits α4β7, but not CCR9 expression by the T cells, while addition of a retinoic acid receptor antagonist by itself inhibits expression of both α4β7 and CCR9. But CD103+ dendritic cells are also found in the lungs, where expression of retinoic acid and TGFβ by these cells is also important to their local induction of Foxp3+ Treg and tolerance. While our DC-RA expressed high levels of Aldh1A2 and modest amounts of TGFβ, we did not assess their production of retinoic acid. However, our data indicates that both IL-10 and TGFβ were jointly responsible for the DC-RA-dependent suppression of Th2 cell proliferation we observed in our 3-day cultures. Others have reported that IL-10 and TGFβ cooperate in suppressing alloantigen-driven mixed lymphocyte reactions, with neither IL-10 nor TGFβ alone being sufficient to realize full hyporesponsiveness, 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, but secretion of IL-10 and TGFβ by DC-RA would likely mask an IL-10-associated influence of IL-27 in such DC-RA/Th2 cell cultures, such that any
effect of removing IL-27-induced Th2 cell IL-10 secretion (i.e., by use of Ebi$^{−/−}$ DC-RA) would not be apparent in Th2 cell inhibition assays.

While IL-27 did not discernibly affect Th2 proliferative responses in our 3-d cultures, it was expressed at high levels by our DC-RA and was important in their inducing Th2 cells to differentiate into Foxp3$^{−}$ Treg across five days in our model. Immature retinoic acid-induced DC reportedly in turn induce the differentiation of Foxp3$^{+}$ Treg$^8$, but Toll-like receptor (TLR) ligands, such as we used with our DC-RA, can regulate IL-27 expression. For example, TLR2 or TLR4 signalling in DC 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 DC induces expression of IL-10 and IL-27, and fosters the differentiation of Foxp3$^{+}$ Treg$^{28}$, but Th3 cells induce DC to secrete IL-27, IL-10 and TGFβ, which in turn induce the development of Foxp3$^{−}$ Tr1 cells$^{29}$. However, Th3 cell-induced regulatory DC express more TGFβ and substantially less IL-27 than our DC-RA$^{29}$, suggesting perhaps that the relative levels of IL-10, TGFβ and IL-27 expression by regulatory DC may be a factor in their induction of Foxp3$^{−}$ versus Foxp3$^{+}$ Treg. It has been reported that IL-27 and TGFβ synergize in induction of c-Maf$^{30}$, a critical component in IL-10 induction within Tr1 cells, although others reported that IL-27 induced c-Maf, IL-21 and ICOS expression are all critical to the induction of Tr1 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 promotor$^{32}$. Our DC-RA-induced Foxp3$^{−}$ Treg expressed only very low levels of c-Maf or AhR, suggesting that DC-RA 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, is LAG3, which was strongly expressed on our DC-RA-induced Treg and has been reported to play a significant role in inflammatory bowel disease tolerance$^{33}$. Thus, while DC-RA-induced Foxp3$^{−}$ Treg share some features in common with Tr1 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 IL-10-differentiated DC (DC10) 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 asthma$^{4-6,34,35}$, albeit via mechanisms distinct from the DC-RA in the present study.
DC10 abrogate airway hyperresponsiveness within 3 wk of treating asthmatic mice and progressively diminish allergen-specific Th2 responses for up to 8 months. Moreover, DC10-induced tolerance is resistant to repeated challenge with physiological levels of allergen (i.e., levels that induce early and late responses in asthmatic individuals). Mouse DC10 induce allergen-specific Th2 cells to transdifferentiate into CD25+Foxp3+ Treg that can passively transfer asthma tolerance, while semi-mature human DC10 generated from atopic asthmatic individuals can induce autologous Th2 cell tolerance and Th2 cell conversion into LAG3+CTLA4+ILT2+ CD25+Foxp3+ Treg. As noted, in contrast to our LPS-exposed DC-RA, retinoic acid-DC that have not been exposed to LPS induce the differentiation of CD25+Foxp3+ Treg. Our rationale herein for choosing differentiation conditions that lead to induction of CD25+Foxp3− Treg relates to the report that Foxp3+ Treg lose expression of Foxp3 when introduced into mice suffering from colitis and subsequently convert into Th17 cells that exacerbate rather than ameliorate colitis pathology. We query whether gut inflammation *per se*, such as might occur in food allergen-induced anaphylaxis or in food-allergic patients with colitis, could similarly suppress Foxp3 expression within nascently-induced Foxp3+ Treg and thereby reduce their therapeutic effectiveness. We hypothesize that Foxp3− Treg could be more resistant to these types of inflammation and might therefore 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 Tr1 cells, expanding our options as we diversify the array of indications for regulatory DC immunotherapy.
ACKNOWLEDGEMENTS

We thank Mark Boyd for his assistance in FACS analysis.

KEY MESSAGES

- Specific allergen-presenting mature retinoic acid-skewed dendritic cells (DC-RA) can induce food allergen tolerance via induction of LAG3⁺CD49⁺Foxp3⁻ Treg in mouse models

- IL-27 secretion by DC-RA drives Th2 cell differentiation into Foxp3⁻ Treg
REFERENCES


FIGURE CAPTIONS

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

Figure 2. DC-RA inhibit immunostimulatory DC-induced T cell proliferation in an IL-10- and TGFβ-dependent but IL-27- and contact-independent manner. (A) OVA-presenting DC-RA were titrated into cultures of OVA-presenting DC-LPS (3.7 x 10^3 cells/well) and T cells (1x10^5 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-LPS, or immature DC alone. Note that the scales for the TCR-transgenic OTII cell (100% OVA-specific T cells) and the B6 mouse Th2 cells are different. (B) Assessment of whether direct DC-RA - T cell contact was required for this suppression. (C) Left panel. The role of IL-27 expression by DC-RA in their suppression of Th2 responses, as determined using DC-RA generated from WT or IL-27^-/- mice. Right panel. The impact of neutralizing anti-IL-10 and/or TGFβ antibodies on DC-RA suppression of Th2 cell proliferation. NS, *, ** or *** indicate p≥0.05, ≤0.05, ≤0.01 or 0.001, respectively, versus wells containing DC-LPS and T cells alone.

Figure 3. LPS maturation of DC-RA enhances their suppressive ability. DC-LPS or either immature (no LPS exposure; iDC-RA) or LPS-matured DC-RA were (A) titrated into co-cultures of OVA-presenting DC-LPS and T cells obtained from either naïve or OVA-immunized (effector T cells) OT II mice in order to assess their abilities to suppress T cell proliferative responses. (B) Expression of mRNA encoding the indicated tolerance-associated markers, as determined by qPCR. Bars represent the mean +/- SEM of three independent experiments. *, **, or NS indicate p≤0.05 or 0.01 or p>0.05, respectively.
Figure 4. Specific allergen-presenting DC-RA suppress anaphylactic responses to oral allergen challenge in a mouse model of ovalbumin-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 wk before being given 1x10^6 otherwise untreated DC-RA, OVA-pulsed DC-RA or an equal volume of saline. All mice were challenged four weeks 4 w later by OVA gavage and assessed over the next 24 h. (B) At 30 m post-challenge the clinical scores and incidence of diarrhea were assessed, while at 50 m serum was collected for analysis of mouse mast cell protease-1 (mMCP-1), as a surrogate marker of intestinal mast cell activation. At 24 h (C) serum was collected for analysis of OVA-specific IgG1 and IgE, while (D) peritoneal lavage fluids were assayed for the indicated cytokines. NS, *, **, or *** indicate p≥0.05, or ≤0.05, 0.01 or 0.001, respectively. The results are the mean of two independent experiments (n=9-10 mice/group).

Figure 5. DC-RA also suppress anaphylactic responses to allergen challenge in a mouse model of peanut-induced anaphylaxis. Mice were left unsensitized (control) or sensitized using crude peanut extract (CPE) for initial exposure followed by repeated gavage with peanut butter until they displayed overt anaphylactic responses. They to the oral challenge were then rested for 2 wk before being given 1x10^6 CPE-presenting DC-RA or an equal volume of saline, as in Fig. 4. Four weeks later all mice were given an oral peanut butter challenge. (A) Clinical scores, diarrhea and mMCP-1 were assessed as in Fig. 3, (B) as were serum peanut allergen-specific IgG1 and IgE. (C) At 24 h peritoneal lavage fluids were collected and assayed for the indicated cytokines by ELISA. NS, *, **, or *** indicate p≥0.05, or ≤0.05, 0.01 or 0.001, respectively. The results are the mean of two independent experiments (n=10 mice/group).

Figure 6. DC-RA expression of IL-27 expression is critical to in vitro induction of the Foxp3 regulatory T cells that suppress Th2 responses. (A) CD4^+ cells from OVA-alum-immunized mice were labeled with CFSE and co-cultured for 5 d with DC-RA or DC-LPS, after which and their proliferative responses were assessed using a CFSE dilution assay. (B) CD4^+ cells from the end of the 5 d DC-RA cultures (panel A) were assessed by FACS for their expression of the indicated markers. (C) CD4^+ cells sorted from 5-day WT or IL-27^−/− DC-RA/Th2 cell co-cultures
were titrated into secondary co-cultures containing $10^5$ OT II cells and $3.7 \times 10^3$ OVA-pulsed DC-LPS/well, and OTII 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 was assessed as in panel C. As a control, we also included wells with no DC-RA-induced Treg (0:1). (Inset). IL-10 secretion over 48 h by CD4 cells from the mesenteric lymph nodes (mLN) of mice taken 24 h after allergen-induced anaphylaxis. NS, *, **, or *** indicate $p \geq 0.05$, or $\leq 0.05$, 0.01 or 0.001, respectively. The results are representative of three independent experiments (n=10 mice/group).

Figure 7. FACS characterization of DC-RA-induced Treg. CD4$^+$ T cells from OVA/alum immunized OTII mice were cocultured with either DC-RA or DC-LPS. At the end of the 5 d cultures, naïve OTII CD4$^+$ T cells or CD4$^+$ T cells from the co-cultures were assessed for expression of the indicated markers (black line); isotype control antibody-stained cells (solid gray) were also examined. FACS plots are gated on CD4$^+$ cells. The results are representative of three independent experiments.

Figure 8. IL-27 is also critical to DC-RA reversal of the anaphylactic phenotype following food allergen challenge. Mice were sensitized to OVA, treated with OVA-presenting DC-RA and challenged with OVA allergen as in Fig. 4, but using either WT or IL-27$^{-/-}$ DC-RA. (A) Clinical scores, incidence of diarrhea, serum mMCP-1 levels, (B) serum OVA-specific IgG1 and IgE levels, and (C) peritoneal lavage fluid Th2 cytokine levels were assessed as in Fig. 4. NS, *, **, or *** indicate $p \geq 0.05$, or $\leq 0.05$, 0.01 or 0.001, respectively. The results are the mean of two independent experiments (n=10-12 mice/group).
Supplementary Table SI. Sequences of primers used for qRT-PCR analysis of regulatory dendritic cell markers.

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* F, forward primer; R, reverse primer
A

OTII T cells

B6 Th2 cells

B contact-depend.

C

Prolif. (cpm × 10^3)

No. DC-RA/well

Prolif. (cpm × 10^3)

No. DC-RA/well

Prolif. (cpm × 10^3)

No. WT DC-RA (x10^3)

DC-RA (x10^3)

DC-RA only

DC-RA & TGF

DC-RA & antibody
Dawicki et al, Fig. 4

A

wk: 0  2  4  10  14

↑ allergen-alum (i.p.)  ↑ allergen gavage (6-9x)  ↑ DC-RA (i.p.)  ↓ oral chall.

B  

clin. score  diarrhea  mMCP-1

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C  

IgG1  IgE

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D  

IL-4  IL-5  IL-9  IL-13  IFNγ

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** NS  ***

Gavage (6-9x)
Peanut anaphylaxis

A) Clinical score

B) IgG1 and IgE

C) Cytokine levels (pg/ml)

Diarrhea (incidence)
mMCP-1 (ng/mL)

IL-1
IL-10
IL-5
IL-9
IL-13
IFNγ
Dawicki et al, Fig. 6

**A**

No. cells
CFSE

DC-RA

No. cells
CFSE

DC-LPS

**B**

CD25
FoxP3

LAG3

CD49b

F

A

B

**C**

Prolif. (cpm x 10^{-3})

16
12
8
4
0

1:8 1:4 1:2 1:1

WT DC-RA IL-27^{+} DC-RA

DC-RA

Naive OT2

1:8 1:4 1:2 1:1

**D**

Prolif. (cpm x 10^{-3})

15
10
5
0

0:1 1:1 1:2 1:4 1:8

isotype anti-IL-10

mLN IL-10

OT2 reg Treg APC
Dawicki et al, Fig. 7
**Cytokine (pg/ml)**

- **IL-4**
  - untreated DCra
  - DCra IL27-/- unsensitised

- **IL-5**
  - untreated DCra
  - DCra IL27-/- unsensitised

- **IL-9**
  - untreated DCra
  - DCra IL27-/- unsensitised

- **IL-13**
  - untreated DCra
  - DCra IL27-/- unsensitised

**Clin. score**

- Diarrhea (incidence)
  - untreated DCra
  - DCra IL27-/- unsensitised

**mMCP-1 (ng/mL)**

- untreated DCra
  - DCra IL27-/- unsensitised

**IgG1 (x10^3 EU/mL)**

- untreated DCra
  - DCra IL27-/- unsensitised

**IgE (x10^3 EU/mL)**

- untreated DCra
  - DCra IL27-/- unsensitised
CD54
D
ata
CD40
CD40L
MHC I
CD103
MHC II
CCR7
CD80
CCR9
CD86
PDL1
PDL2
ICOS-L
OX40L

Expression (mRNA)

WT
Ebi3/

MFI

CD54
CD40
CD40 L
MHC I
CD103
MHC II
CCR7
CD80
CCR9
CD86
PDL1
PDL2
ICOS L
OX40 L
Expression

IL-10

AhR

TGFβ

naive  w.t.  IL-27−/−
naive  w.t.  IL-27−/−
naive  w.t.  IL-27−/−
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**Supplementary table 1. Sequences of forward and reverse qRT-PCR primers**

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