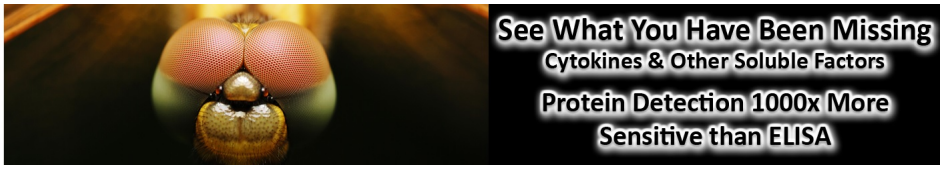

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Calcitonin Gene–Related Peptide–Exposed Endothelial Cells Bias Antigen Presentation to CD4$^+$ T Cells toward a Th17 Response

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Calcitonin gene–related peptide (CGRP) is a neuropeptide with well-established immunomodulatory functions. CGRP-containing nerves innervate dermal blood vessels and lymph nodes. We examined whether CGRP regulates the outcome of Ag presentation by Langerhans cells (LCs) to T cells through actions on microvascular endothelial cells (ECs). Exposure of primary murine dermal microvascular ECs (pDMECs) to CGRP followed by coculture with LCs, responsive CD4$^+$ T cells and Ag resulted in increased production of IL-6 and IL-17A accompanied by inhibition of IFN-γ, IL-4, and IL-22 compared with wells containing pDMECs treated with medium alone. Physical contact between ECs and LCs or T cells was not required for this effect and, except for IL-4, we demonstrated that IL-6 production by CGRP-treated pDMECs was involved in these effects. CD4$^+$ cells expressing cytoplasmic IL-17A were increased, whereas cells expressing cytoplasmic IFN-γ or IL-4 were decreased by the presence of CGRP-treated pDMECs. In addition, the level of retinoic acid receptor–related orphan receptor γt mRNA was significantly increased, whereas T-bet and GATA3 expression was inhibited. Immunization at the site of intradermally administered CGRP led to a similar bias in CD4$^+$ T cells from draining lymph node cells toward IL-17A and away from IFN-γ. Actions of nerve-derived CGRP on ECs may have important regulatory effects on the outcome of Ag presentation with consequences for the expression of inflammatory skin disorders involving Th17 cells. ***The Journal of Immunology*****, 2016, 196: 2181–2194.**

Neurologic status, including emotional state, influences immune function. The primary and secondary lymphoid organs, including the spleen, thymus and lymph nodes, are innervated, and dendritic cells and lymphocytes express receptors for peptide and nonpeptide products of nerves (1–5). In addition, many studies have demonstrated that stress can have immunoregulatory effects in humans and animals, and these effects are mediated, at least in part, by neuroendocrine pathways (3–14). There are also reports that stress might exacerbate psoriasis and atopic dermatitis (6–8) and an atopic dermatitis-like rash in an animal model (11). The importance of the nervous system to inflammatory skin disease is highlighted by the findings that psoriasis clears in denervated skin (15, 16) and that some animal models of psoriasiform dermatitis depend on innervation for their expression (17, 18).

Endothelial cells (ECs) line blood vessels within the dermis and contribute to cutaneous immunity and inflammation through many mechanisms. Among these mechanisms is the ability to release cytokines and chemokines and the expression of adhesion molecules involved in recruitment of inflammatory cells out of the vasculature and into the interstitium (19–23). In this regard, we have recently reported that the vasodilator and peptide neurotransmitter calcitonin gene–related peptide (CGRP) inhibits the stimulated expression of the chemokines CXCL8, CCL2, and CXCL1 by human dermal microvascular ECs (24). CGRP is a 37-aa neuropeptide generated by tissue-specific alternative processing of the calcitonin gene and is widely distributed in organs of the immune system and in the central and peripheral nervous systems (25). Of particular interest, in a murine model of psoriasiform dermatitis, in which the Tie2 receptor tyrosine kinase is overexpressed in keratinocytes, denervation of skin results in loss of the psoriasiform phenotype, but administration of CGRP to the animal inhibits this loss (17), suggesting a key role for CGRP in the phenotype observed. In this regard, it has been reported that in lesions of psoriasis ECs have CGRP on their surface (26). Furthermore, both sympathetic and sensory nerves are associated with dermal vessels (27, 28) and innervate lymph nodes (29). Moreover, recent evidence indicates that sympathetic neurotransmitters, including norepinephrine, regulate immune and inflammatory responses (30, 31).

Lymphocytes and APCs trafficking through the skin and exiting the vasculature to enter the interstitium of the dermis are closely associated with ECs during these processes. Furthermore, release of EC-derived factors on the abluminal side of vessels would be

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Abbreviations used in this article: ADM, adrenomodulin; BMDC, bone marrow–derived dendritic cell; CGRP, calcitonin gene–related peptide; CM, complete medium; cOVA, chicken OVA; DNFB, dinitrofluorobenzene; EC, endothelial cell; LC, Langerhans cell; pDMEC, primary murine dermal microvascular EC; RORγt, retinoic acid receptor–related orphan receptor γt; siRNA, short interfering RNA; SP, substance P; Tg, transgenic.

able to interact with immune cells in the interstitium, particularly those in a perivascular arrangement. Thus, we asked whether CGRP modulates the ability of ECs acting as bystanders, to regulate the outcome of Ag presentation by Langerhans cells (LCs) to $CD4^+$ T cells. LCs are dendritic APCs that reside in the epidermis that, depending on circumstances, can present Ag for induction or regulation of arms of the immune response (32, 33). They were chosen as APCs for this study because their function has been shown to be directly regulated by neuropeptides (34–40) and, when stimulated by Ag, they traffic through EC-lined lymphatics to regional lymph nodes (41). In addition, there is evidence that they can present Ag for generation of Th17 helper T cells (42, 43), believed to be important in the pathogenesis of certain inflammatory skin disorders including psoriasis (44, 45). In this regard, LCs are believed to play a role in some other inflammatory dermatoses (46).

We have examined the effects of adding CGRP-treated or untreated ECs to Ag presenting cultures of LCs and responding T cells, an environment perhaps similar to that in the dermis or regional lymph nodes during a local immune reaction, on the generation of Th cell subtypes.

Materials and Methods

Mice

Six- to twelve-week-old female BALB/c ($H\text{-}2^d$) and DO11.10 chicken OVA (cOVA) TCR transgenic (Tg) mice on a BALB/c background [C.Cg-Tg (DO11.10)10Dlo/J] mice were purchased from the Jackson Laboratory. The DO11.10 mice carry MHC class II–restricted, rearranged TCR-α and TCR-β chain genes that encode a TCR that recognizes a fragment of cOVA ($cOVA_{323–339}$) presented by $I\text{-}A^d$ (47, 48). All animal studies were approved by the Institutional Animal Care and Use Committee of the Weill Cornell Medical College.

Reagents

αCGRP was purchased from Bachem; a fragment of cOVA ($cOVA_{323–339}$) was obtained from Peptides International, anti-mouse CD3 mAb along with isotype controls was obtained from R&D Systems, and anti-mouse CD28 mAb from BD Biosciences. Mouse CGRP, $CGRP_{8–37}$, and substance P (SP) were purchased from Bachem. Mouse adrenomedullin (1-50) (ADM) was purchased from Phoenix Pharmaceuticals. Mouse recombinant IL-6 was purchased from R&D Systems.

Media and cell lines

Complete medium (CM) consisted of RPMI 1640 (Mediatech), 10% FBS (American Type Culture Collection), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 0.1 mM essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (all from Mediatech).

Primary murine dermal microvascular ECs (pDMECs) from BALB/c mice were obtained from Cell Biologics and maintained in complete EC medium [consisting of Endothelial Basal Medium-2 supplemented with hydrocortisone, human fibroblast growth factor, human vascular endothelial growth factor, human epidermal growth factor, ascorbic acid, gentamicin and amphotericin, L-glutamine, and 5% FBS (Endothelial Basal Medium-2 and supplement kits from Lonza)]. pDMEC were maintained in deplete EC medium (consisting of EBM-2 medium with 5% FBS and L-glutamine) overnight before the experiment performed.

The bEnd.3 cell line (49) was obtained from the American Type Culture Collection (Manassas, VA). This cell line is an EC line established from the cerebral cortex of BALB/c mice and has many characteristics of freshly isolated ECs including expression of von Willebrand factor (50), ICAM-1 (51), and VCAM-1. bEnd.3 cells were cultured in DMEM (Mediatech) supplemented with 10% heat-inactivated FBS (Gemini Bio-Products, Sacramento, CA), 100 U/ml penicillin (Mediatech), 100 μg/ml streptomycin (Mediatech), and 2 mM L-glutamine (Mediatech).

Preparation of LCs

ECs were prepared using a modification of a standard protocol (37). Truncal skins of mice were shaved with electric clippers and chemically depilated. Subcutaneous fat and panniculus carnosus were removed by blunt dissection. Skin was floated dermis-side down for 45 min in Ca^{2+}/Mg^{2+}-free PBS containing 0.5 U dispase/ml (BD Biosciences) and 0.38% trypsin (Mediatech). Epidermal sheets were collected by gentle scraping, washed, and dissociated by repetitive pipetting in HBSS (Mediatech) supplemented with 2% FBS. Epidermal cells were filtered through a 40-μm cell strainer (BD Biosciences) to yield ECs containing 2–3% LCs.

Epidermal cells were incubated with 5 μg/ml anti–$I\text{-}A^d$ mAb (BD Biosciences) for 30 min at 4°C. They were then incubated with goat anti-mouse IgG conjugated to magnetic microspheres (Dynabeads M-450; Invitrogen) for 10 min with continuous, gentle agitation. LCs were isolated by placing the tube in a magnetic particle concentrator (Invitrogen), discarding the supernatant and washing the bead-bound cells (up to five times) with HBSS containing 2% FBS. By FACS (using anti–$I\text{-}A^d$ mAb), this procedure yields a cell population of ~95% LCs.

Preparation of bone marrow–derived dendritic cells

Bone marrow–derived cells (BMDCs) were prepared from BALB/c mouse femur bones using modifications of standard procedures (52). Mice were euthanized and leg areas were shaved and sprayed with 70% ethanol. Skin was removed from legs, and the surrounding area and muscle and tendons were cut and scraped from the femur bones, which were subsequently cut from the body. Bones were washed in PBS in Petri dishes, and any remaining tissue was removed. Femur bones were placed in Petri dishes containing 70% ETOH for 2–3 min and then rinsed three times with fresh PBS. To remove bone marrow, PBS was flushed through both ends of the bone with a syringe and needle into a 50-mL conical tube. Cells were spun down at 250 × *g* for 10 min, washed once with PBS, and washed once with medium and resuspended in CM (RPMI 1640, 10% FBS, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES, 1 mM essential amino acids, 0.1 mM nonessential amino acids) containing 20–25 ng/ml GM-CSF (Peprotech). Cells were plated at 2.5–4×10^6 cells per 10 ml CM media in 10-cm Petri dishes and incubated at 37°C, 5% CO_2. Two days later, 10 ml fresh media was added to the plate. One to two days later, 10 ml was removed from each plate and placed in 50-ml conical tubes, and cells were spun down (1200 rpm [300 × *g*] for 8 min). Cells were resuspended in fresh media and returned to plates, and additional fresh media was added to plates to bring the total back to 10 ml. This feeding procedure was repeated every 2 d. BMDCs were harvested on day 8 or 9 and purified using binding to CD11c microbeads and MACS technology according to the manufacturer's instructions for resuspending and magnetic separation (Miltenyi Biotec). Nonadherent and loosely adherent proliferating DC aggregates were collected from plates, transferred to 50-ml conical tubes and spun at 250 × *g* for 8–10 min. Cells were washed once with MACS buffer (1× PBS, 1% FBS, 2 mM EDTA) and resuspended in 400 μl MACS buffer per 10^8 total cells; 100 μl CD11c microbeads were added per 10^8 total cells. The mixture was incubated for 15 min at 4–8°C, 10 ml MACs buffer was added, and cells were spun down as above and resuspended in 500 μl MACS buffer. Cells were loaded onto LS columns (Miltenyi Biotec), washed, and magnetically separated following manufacturer's procedures. Eluted cells were spun down, resuspended in 500 μl MACS buffer, and subjected to a second round of column purification. Eluted cells were spun down and washed once with PBS, twice with CM, and counted for use in coculture experiments.

Isolation of $CD4^+$ T cells from DO11.10 Tg mice

DO11.10 Tg mouse spleens were mechanically disrupted to yield a single-cell suspension, and erythrocytes were lysed. $CD4^+$ cells were isolated by depletion of nontarget cells. The nontarget cells were indirectly magnetically labeled with a mixture of biotin-conjugated mAbs (CD8a, Cd11b, CD11c, Cd19, CD45R (B220), CD49b (DX5), CD105, anti-MHC class II, and Ter-119) as primary labeling reagent, and anti-biotin mAbs conjugated to microbeads, as secondary labeling reagent. The magnetically labeled nontarget cells were on a MACS column in the magnetic field of a MACS separator, whereas the unlabeled $CD4^+$ T cells passed through the column (Miltenyi Biotec).

RNA interference

Ten thousand pDMECs per well were plated in 96-well round-bottom plates (BD Falcon) in antibiotic-free medium and incubated at 37°C until adherence. The appropriate amount of ON-TARGET plus SMARTpool Mouse IL-6 short interfering RNA (siRNA; Dharmacon) target sequences (5′-CCUAGUGCGUUAUGCCUAA-3′, 5′-UUACACAUGUUCUCUGGGA-3′, 5′-GGACCAAGACCAUCCAAUU-3′, and 5′-CUACCAAACUGGAUAUAAU-3′) or the corresponding ON-TARGET plus nontargeting pool were diluted in OptiMEM medium (Invitrogen) to obtain a final concentration of 100 nM. Lipofectamine 2000 (Invitrogen) was also diluted following the manufacturer's instructions in OptiMEM. The diluted siRNA and Lipofectamine 2000 were mixed and incubated for 20 min at room temperature for complex formation. Complexed siRNA (25 μl) was

added to each well. After overnight incubation at 37°C, the medium was replaced with depleted EC medium for treatment with CGRP. Under these conditions, siRNA pretreatment resulted in an ~67% reduction in release of IL-6 protein from muramyl dipeptide–stimulated bEnd.3 cells.

In vitro Ag presentation to DO11.10 T cells

Ten thousand pDMECs or bEnd.3 cells per well were plated in 96-well, round-bottom plates coated with 2% gelatin in depleted DMEM or depleted ECs medium, respectively, and then incubated overnight at 37°C. The following day, cells were treated with graded concentrations of CGRP or medium alone for 3 h at 37°C. Cells were then washed extensively, and 1×10^4 purified LCs (from BALB/c mice) and 2×10^5 purified $CD4^+$ T cells (from DO11.10 Tg mice) were added to each well in a total of 250 μl of CM containing 10 μM $cOVA_{323–339}$ (Peptides International). Supernatants were harvested 48 h later and analyzed with sandwich ELISA for cytokine content.

In some experiments, pDMECs were cocultured in graded concentrations of $CGRP_{8–37}$ during the period of culture in CGRP as above. In other experiments, no pDMECs or bEnd.3 cells were used; instead, graded concentrations of IL-6 were added to Ag-presenting cultures of LCs and $CD4^+$ T cells.

For experiments using Transwell plates, 1.25×10^4 ECs per well were plated in the lower chamber of 96-well Transwell plates (Corning Life Sciences) and 24 h later treated for 3 h with 100-nm CGRP or medium alone as above. Cells were then washed extensively, and 1.25×10^4 purified LCs (from BALB/c mice) and 2.5×10^5 purified $CD4^+$ T cells (from DO11.10 Tg mice) in CM were added to the upper chamber in a total of 275 μl of medium containing 10 μM $cOVA_{323–339}$ (Peptides International). Supernatants were harvested 48 h later and analyzed for cytokine content.

Cytokine determinations

Supernatant IL-17A, IL-6, IL-4, IFN-γ, and IL-22 levels were determined by sandwich ELISA following the manufacturer's instructions. IL-22 ELISA kits were purchased from Antigenix America; IL-17A, IL-4, and IL-6 kits were from (R&D Systems) and IFN-γ kits were from BD Biosciences.

Real-time PCR

For gene expression analysis, $CD4^+$ T cells from DO11.10 Tg and LCs from BALB/c mice were cocultured with CGRP-treated pDMECs in the presence of 10 μM $OVA_{323–339}$ for 24 h. T cells were gently collected from mixed culture wells after incubation for 24 h, and LCs still bound to beads were removed using magnetic capture. According to FACS analysis for CD3, the remaining cell populations were ~93% T cells. Total RNA was isolated from the remaining cells (primarily $CD4^+$ T cells) using the RNeasy Plus Mini Kit (Qiagen); DNA eliminator columns were used to eliminate any contamination with genomic DNA. cDNA was synthesized using a high-capacity RNA-to-cDNA kit according to the manufacturer's instructions (SuperScript VILO cDNA Synthesis Kit; Invitrogen). Real-time PCR for murine IL-17A (5′-GAGCTTCCCAGATCACAGAG-3′ forward; 5′-AGACTACCTCAACCGTTCCA-3′ reverse), IL-6 (5′-CAAGTGCATCATCGTTGTTCA-3′ forward; 5′-GATACCACTCCCAACAGACC-3′ reverse), IFN-γ (5′-GAGCTCATTGAATGCTTGGC-3′ forward; 5′-CAGCAACAACATAAGCGTCAT-3′ reverse), retinoic acid receptor–related orphan receptor γt (RORγt) (5′-TCCCACATCTCCCACATTG-3′ forward; 5′-AATGTCTGCAAGTCCTTCCG-3′ reverse), T-bet (5′-CAAGACCACATCCACAAACATC-3′ forward; 5′-TTCAACCAGCACCAGACAG-3′ reverse), IL-4 (5′-TCTTTAGGCTTTCCAGGAAGTC-3′ forward; 5′-GAGCTGCAGAGACTCTTTCG-3′ reverse), IL-22 (5′-AATCGCCTTGATCTCTCCAC-3′ forward; 5′-GCTCAGCTCCTGTCACATC-3′ reverse) and GATA3 (5′-GTCCCCATTAGCGTTCCTC-3′ forward; 5′-CCTTATCAAGCCCAAGCGAA-3′ reverse) expression was performed using PrimeTime primers (Integrated DNA Technologies) as shown above, power SYBR Green PCR Master Mix (Invitrogen), and an ABI 7900HT instrument (Invitrogen). Expression of each cytokine was normalized to glyceraldehyde 3-phosphate dehydrogenase (5′-GTGGAGTCATACTGGAACATGTAG-3′ forward; 5′-AATGGTGAAGGTCGGTGTG-3′ reverse).

CGRP receptor mRNA detection by RT-PCR

Total RNA was extracted from pDMECs of BALB/c mice using a total RNA extraction kit (Qiagen). A DNA elimination column (Qiagen) was used to eliminate any contamination with genomic DNA. Then 100 ng of RNA was reverse-transcribed into cDNA using SuperScript VILO cDNA Synthesis kit following the instructions of the manufacturer (Invitrogen). One twentieth of the synthesized cDNA was amplified by PCR using gene-specific primers for RAMP1, RAMP2, RAMP3, and calcitonin receptor-like receptor (CRLR) and CGRP-receptor component protein (CRCP). Primer sequences were designed from GenBank sequences for the mRNA of mouse RAMP1 (5′-TGTGACTGGGGAAAGACCATACAG-3′ forward; 5′-ATGAGCAGCGTGACCGTAATG-3′ reverse); RAMP2 (5′-CCCAGAATCAATCTCATCCCAC-3′ forward and 5′-AGCAGTTCGCAAAGTGTATCAGG-3′reverse); RAMP3 (5′-GGTTCAGATTGTCCATACTTTGC-3′ forward and 5′-TCAAGAAGGAGGTTCACGCTCTAC-3′ reverse); CRLR (5′-CTACTATTTTCTGCTTCTTT-3′ forward and 5′-TTTGTGCTTATTTTCTTTCC-3′ reverse); CRCP, (5′-TGGCGGAATAGGAGATAAGA-3′ forward and 5′-AGACAGAAGGGACCGCATAA-3′ reverse). The PCR products underwent electrophoresis in 1.5% agarose gel, staining with ethidium bromide, and visualization with ultraviolet radiation.

Sensitization of mice to dinitrofluorobenzene

BALB/c mice were divided into four groups of five. Mice were shaved on the dorsum with electric clippers and injected intradermally with 100 μl PBS containing 530 pmol CGRP or PBS alone. Fifteen minutes after injection, the mice were painted with 10 μl dinitrofluorobenzene (DNFB; 1% in acetone and olive oil [4:1]) epicutaneously at the injection site.

Preparation of supernatants conditioned by $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28

Three days after immunization, mice were sacrificed and draining lymph nodes (axillary and inguinal) were removed. Lymph nodes were mechanically disrupted and passed through a 70-μm nylon mesh to yield a single-cell suspension. $CD4^+$ T cells were isolated as described above. Ninety-six–well flat-bottom plates were treated with 10 μg/ml anti-mouse CD3 mAb in PBS overnight and washed. T cells were cultured (3×10^5 cells/well) in 250 μl CM containing 2 μg/ml anti-mouse CD28 mAb. Supernatants were collected 72 h after stimulation, and cytokine contents were determined.

Flow cytometry

pDMECs were treated with 100 nM CGRP or medium alone for 3 h, washed four times, and then cocultured with LCs and $CD4^+$ T cells (from DO11.10 Tg mice) in the presence of 10 μM $OVA_{323–339}$ for 48 h. For the last 5 h of coculture, cells were stimulated with 50 ng/ml PMA and 750 ng/ml ionomycin (Sigma-Aldrich). After 1 h, Golgi Stop (BD Biosciences) was added to block cytokine secretion. LCs still bound to beads were then removed using magnetic capture. $CD4^+$ T cells were surface stained for 20–30 min at 4°C with PerCP-Cy 5.5-labled anti-CD4 mAb (BD Biosciences) in PBS supplemented with 0.1% BSA and 0.1% sodium azide. After fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences), cells were stained with Alexa Fluor 647-labeled anti–IL-17A and FITC-labeled anti–IFN-γ (clone XMG1.2; BD Biosciences), PE or Alexa Fluor 647-lableled anti–IL-17A (clone TC11–18H10; BD Biosciences), and anti–IL-4 (clone 11B11; BD Biosciences) mAbs. Analysis was performed on a FACSCalibur (BD Biosciences). Data analysis was conducted using CellQuest Pro software (BD Biosciences).

Biostatistics

Differences in average cytokine levels under different treatments at varying cOVA concentrations were analyzed using ANOVA. Data were log transformed before analysis to satisfy the underlying model assumptions. Average cytokine levels under each cOVA concentration were then compared between CGRP treatment and control groups; p values were adjusted by controlling for the false discovery rate.

For assessment of mRNA levels, effects of intradermal administration of neuropeptides and effects of anti–IL-6 mAb on Ag-presenting cultures, a linear mixed effects model was used to estimate the average level of the biomarkers under different treatments. This model accounts for variations for each treatment both within and between plates. Data were log transformed prior to analysis to satisfy the underlying model assumptions. Differences in the average level of the biomarker under pairs of experimental conditions of interest were evaluated using simultaneous tests for general linear hypotheses. The p values were again adjusted for multiple comparisons by controlling the false discovery rate.

Results

Pretreatment of pDMECs with CGRP biases Ag presentation toward enhanced IL-17A and IL-6 responses with reduced IFN-γ, IL-22, and IL-4 responses

Initial experiments examined the ability of ECs to present Ag to responsive T cells. ECs were cocultured with $CD4^+$ T cells from

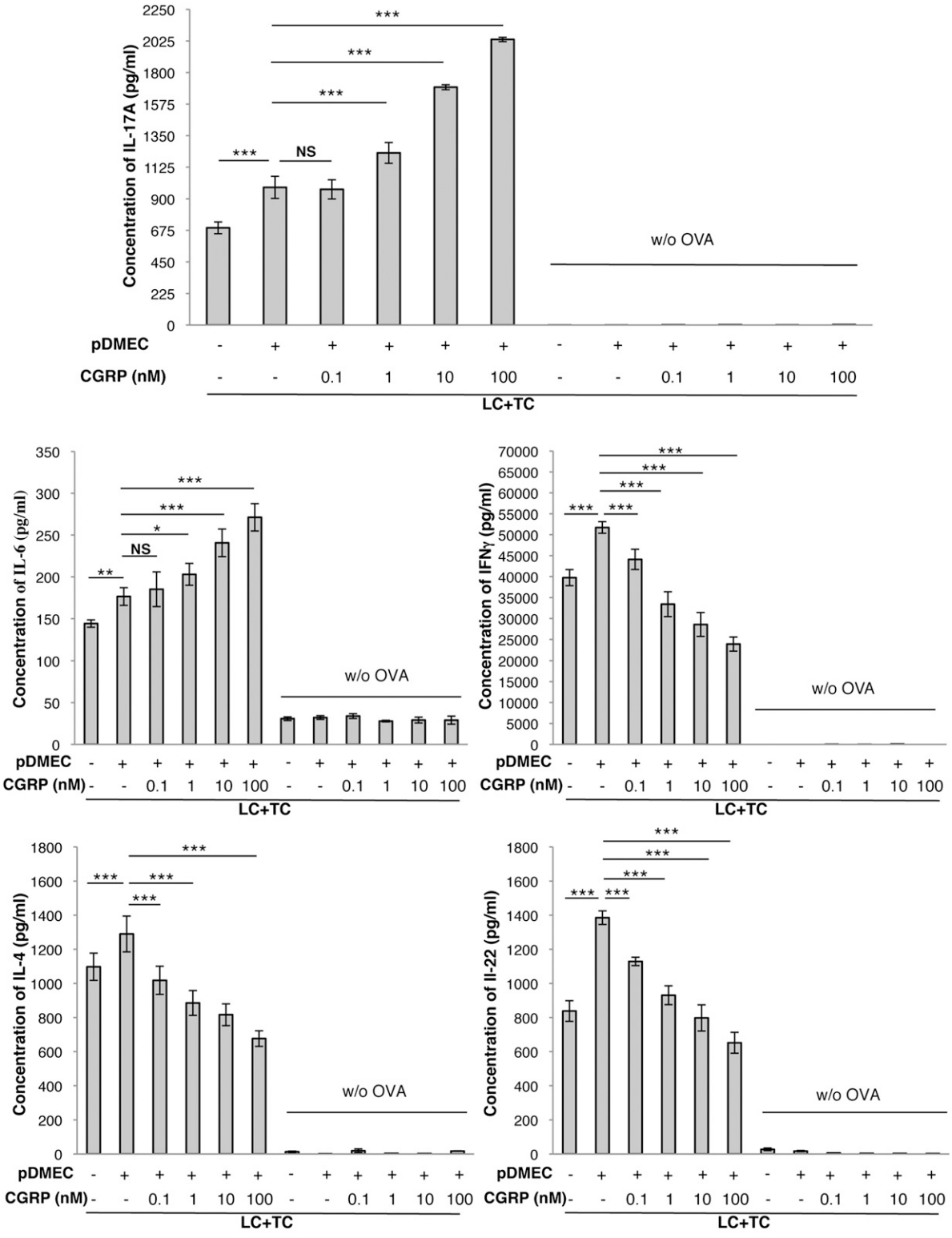

FIGURE 1. CGRP pretreatment of pDMECs biases LC Ag presentation to T cells toward an enhanced IL-17A and IL-6 response with a reduced IFNγ, IL-22, and IL-4 response. Medium- or CGRP-treated (0.1, 1, 10, and 100 nM concentrations) pDMECs were added to cultures of LCs, T cells (from DO11.10 Tg mice), and $cOVA_{323–339}$. After 48 h, supernatants were assessed for cytokine content. The addition of medium-treated pDMECs slightly but significantly enhanced IL-17A, IL-6, and IL-4 production alone with more substantial and significant increases in IFN-γ and IL-22. The addition of CGRP-treated pDMECs led to a much larger increase in IL-17A and IL-6 production, but largely eliminated the increased production of IFN-γ and IL-22 with significant reductions in IL-4 production compared with that seen in wells with the addition of medium-treated pDMECs. $n = 3$ experiments, all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

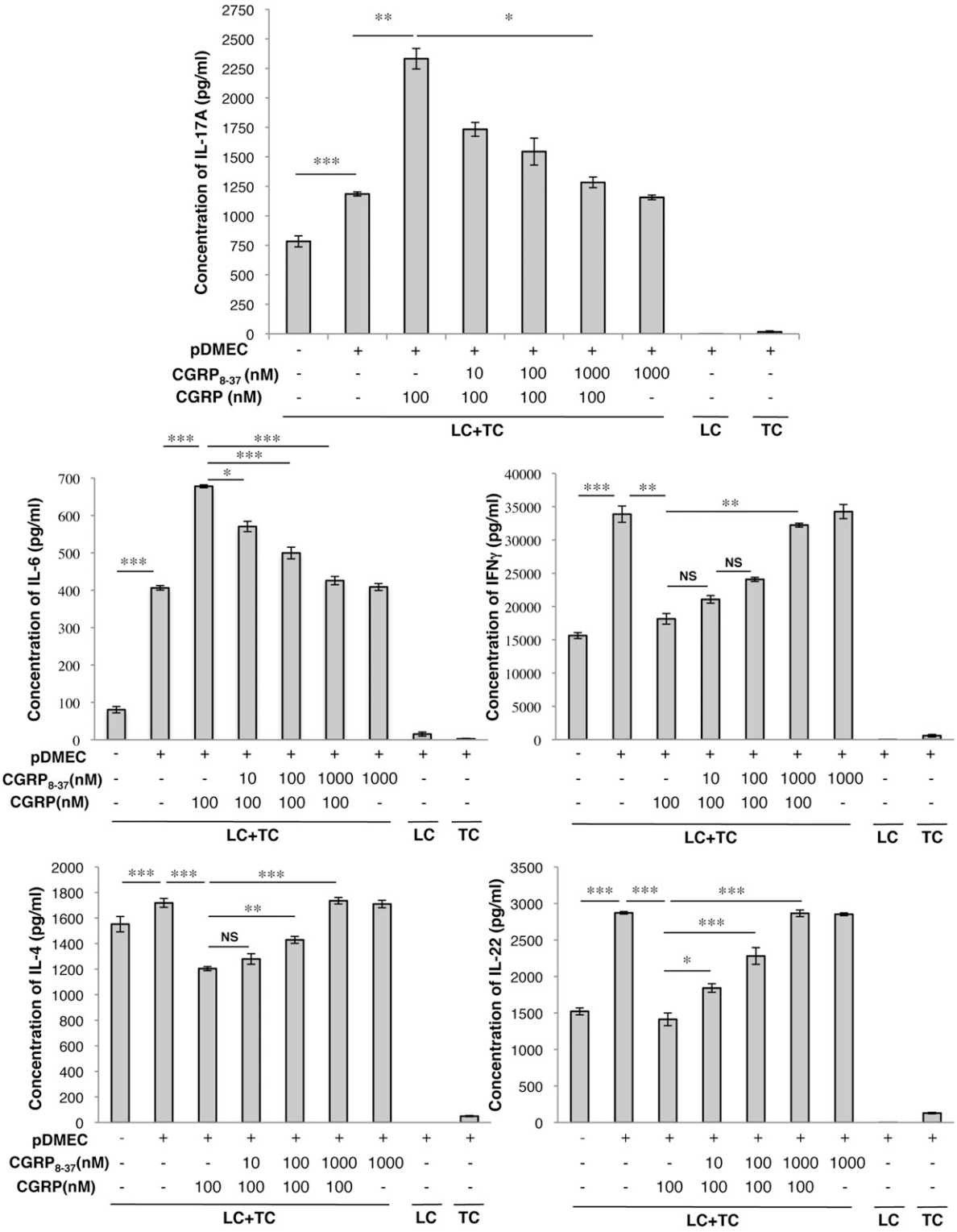

FIGURE 2. The effects of CGRP pretreatment of pDMECs on LC Ag presentation to T cells can be blocked with $CGRP_{8–37}$. pDMECs were treated with graded concentrations of $CGRP_{8–37}$ or medium alone during exposure to CGRP followed by washing and addition to cultures of LCs, T cells (from DO11.10 Tg mice), and $cOVA_{323–339}$. After 48 h, supernatants were assessed for cytokine content. The effects of CGRP on the production of IL-17A, IL-6, IFN-γ, IL-4, and IL-22 were all significantly inhibited by $CGRP_{8–37}$ in a dose-dependent manner. $n = 3$ experiments, all groups. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DO11.10 Tg mice in the presence of a fragment of chicken OVA ($cOVA_{323–339}$). Forty-eight–hour supernatants were harvested and assessed for cytokine content by ELISA. No significant presentation was observed as assessed by production of IFN-γ, IL-6, IL-4, or IL-17A (data not shown). Because murine ECs do not express MHC class II molecules in the steady-state, this was not unexpected (53). We next performed experiments in which ECs were added to Ag-presenting cultures containing LCs and T cells.

pDMECs were cultured in CGRP or medium alone for 3 h followed by extensive washing to remove CGRP. pDMECs were then cocultured with LCs and $CD4^+$ T cells from DO11.10 Tg mice in the presence of $cOVA_{323–339}$. As shown in Fig. 1, the presence

of medium-treated pDMECs resulted in an Ag-dependent and significant increase in release of IL-17A, IL-6, IFN-γ, and IL-22, with a small increase in IL-4. However, pretreatment of pDMECs with CGRP led to a dose-dependent and much greater increase in IL-17A and IL-6 production, whereas the increase in IFN-γ and IL-22 was completely reversed and production of IL-4 was significantly reduced. Similar results were obtained substituting cells of the BALB/c-derived EC line bEnd.3 for pDMECs in these experiments (Supplemental Fig. 1).

The specificity of the CGRP effect was shown by additional experiments comparing CGRP with SP (which colocalizes with CGRP in sensory nerves) and ADM (which binds to the CGRP receptor but with much lower avidity than to the ADM receptor). Although SP and ADM may have a very small amount of activity at inducing IL-17A bias, CGRP is dramatically more effective at biasing the outcome of Ag presentation toward IL-6 and IL-17A production and away from IFN-γ, IL-22, and IL-4 release (Supplemental Fig. 2). Furthermore, the addition of the CGRP receptor inhibitor $CGRP_{8–37}$ blocked the effects of CGRP on the outcome of Ag presentation (Fig. 2). Similar results were seen when bEnd.3 cells were substituted for pDMECs in these types of experiments (Supplemental Fig. 3). Consistent with these findings, using RT-PCR, pDMECs were found to express the components of the CGRP receptor RAMP1 and CLR as well as RAMP2, RAMP3, CRLR, and CRCP (Supplemental Fig. 4).

Pretreatment of pDMECs with CGRP enhances differentiation of T cells with intracellular IL-17A and reduces differentiation of T cells with intracellular IL-4 or IFN-γ

Additional experiments were created in the same manner with medium-pretreated or CGRP-pretreated pDMECs, and isolated $CD4^+$ T cells were studied using FACS analysis after 48 h of culture. The addition of medium-treated pDMECs to wells somewhat enhanced the proportion of cells expressing IL-17A or IFN-γ while slightly decreasing the numbers of cells expressing IL-4. However, the addition of CGRP-treated pDMECs further enhanced the proportion of cells expressing intracellular IL-17A, but the percentages of cells expressing intracellular IFN-γ or IL-4 were greatly reduced (Fig. 3). Few cells expressing intracellular IL-22 were observed, presumably because of the sensitivity of the assay (data not shown).

Pretreatment of pDMECs with CGRP yields T cells with increased levels of mRNA for IL-17A, IL-6, and RORγt, accompanied by decreased levels of IFN-γ, IL-22, T-bet, and GATA3

Additional experiments were created as described above, and $CD4^+$ cells were isolated with a magnetic Ab technique after 24 h of culture. Total RNA was extracted, and real-time RT-PCR was performed to assess IL-6, IFN-γ, IL-17A, and IL-22 mRNA levels. The presence of medium-treated pDMECs during Ag presentation led to significantly enhanced levels of IL-17A, IL-6, IFN-γ, and IL-22 mRNA, whereas the IL-4 mRNA level was little changed (Fig. 4A). The presence of CGRP-treated pDMECs during Ag presentation led to a much more substantial increase in IL-17A and IL-6 mRNA levels, whereas the increase in IFN-γ and IL-22 mRNA levels was completely blocked (Fig. 4A). The level of IL-4 mRNA showed a strong trend toward being decreased by the presence of CGRP-pretreated pDMECs ($p = 0.07$; Fig. 3A). Similarly, the level of mRNA for the transcription factor RORγt (Th17 cells) was slightly but significantly elevated by the presence of medium-treated pDMECs and much more substantially elevated by the presence of CGRP-treated pDMECs (Fig. 4B).

The expression of mRNA for cytokines was also reflected in the expression of key transcription factors. T-bet mRNA was slightly

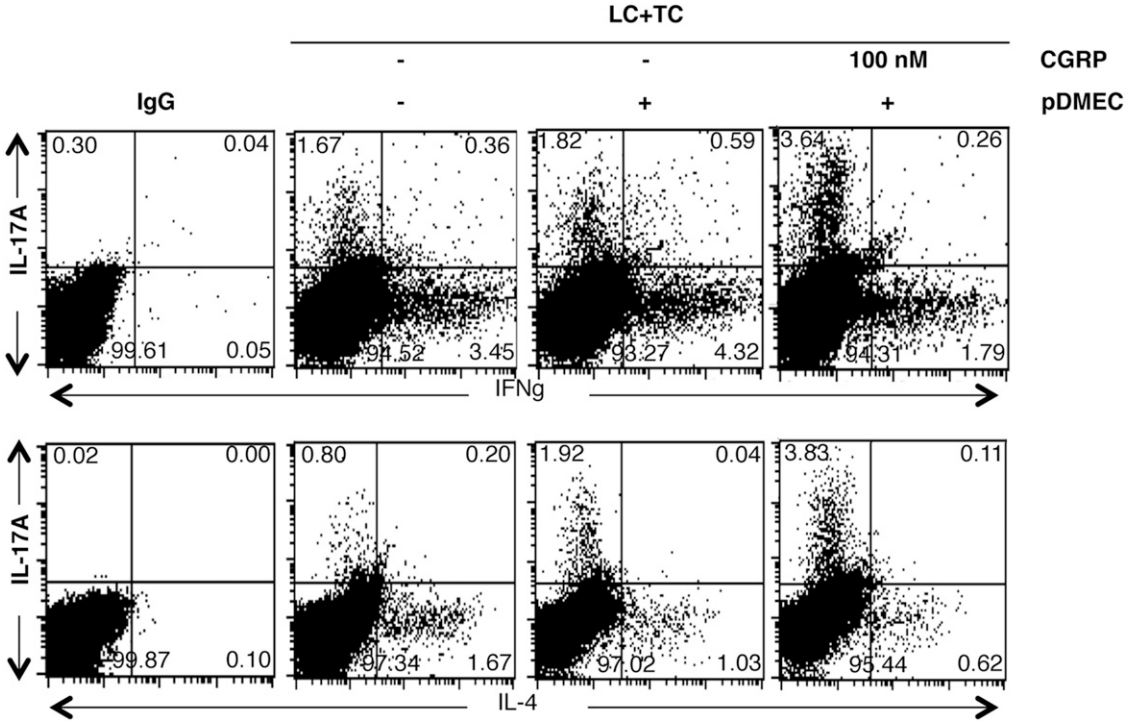

FIGURE 3. CGRP pretreatment of pDMECs enhances differentiation of T cells containing intracellular IL-17A or IL-4 while decreasing expression of IFN-γ. pDMECs pretreated with CGRP or medium alone were added to cultures of LCs, $CD4^+$ T cells from DO11.10 Tg mice, and $cOVA_{323–339}$. Forty-eight hours later, cells were harvested, and $CD4^+$ cells were examined using FACS for intracellular IL-17A, IFN-γ, or IL-4 content. The addition of medium-treated pDMECs somewhat enhanced the proportion of cells expressing IL-17A or IFN-γ while slightly decreasing the numbers of cells expressing IL-4. The addition of CGRP-treated pDMECs further enhanced the proportion of cells expressing intracellular IL-17A, whereas the proportions of cells expressing intracellular IFN-γ or IL-4 were greatly reduced. Data are representative of four experiments.

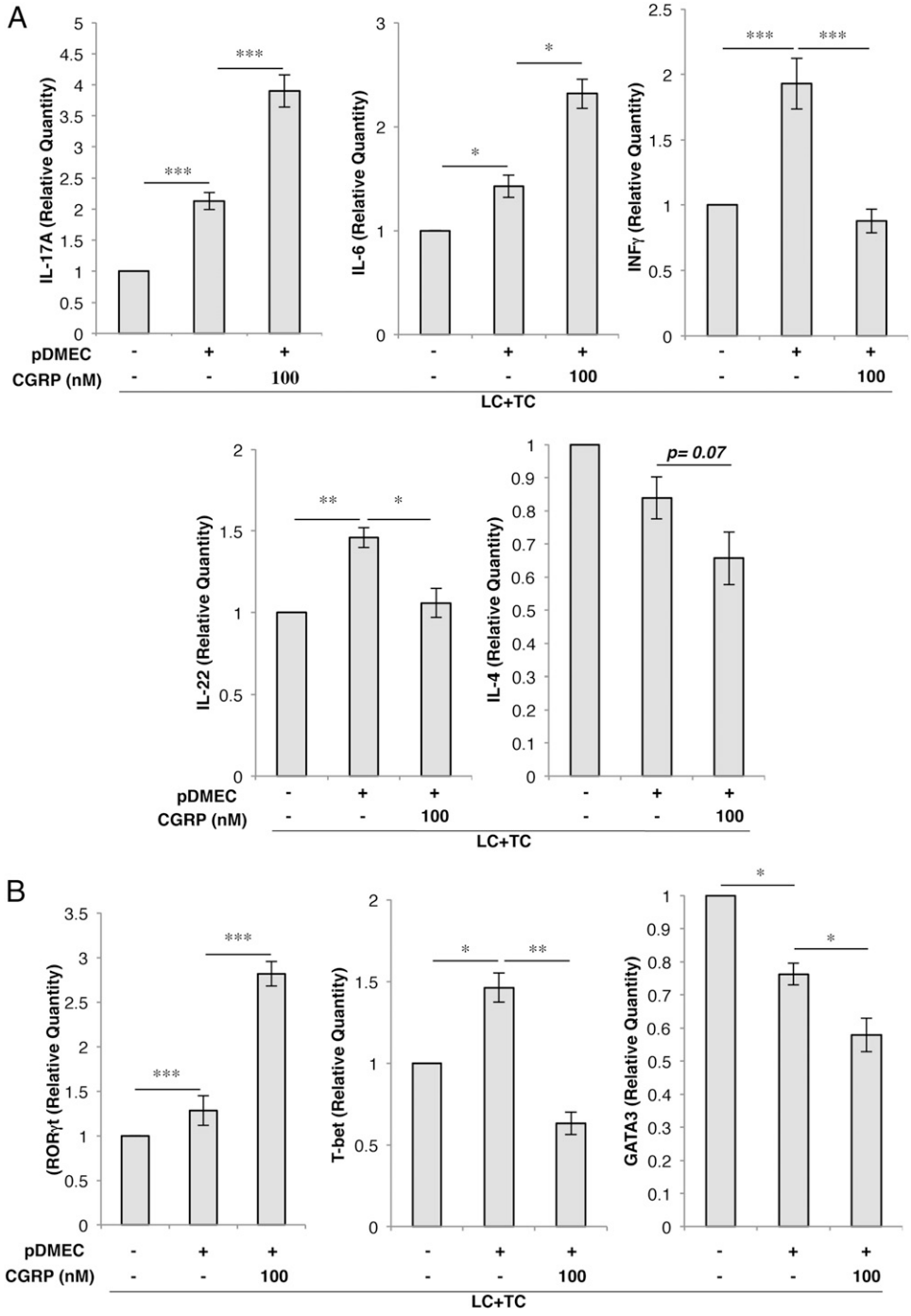

FIGURE 4. (**A**) CGRP pretreatment of pDMECs induces T cells with increased levels of mRNA for IL-17A and IL-6 accompanied by decreased levels of mRNA for IFN-γ, IL-22, and IL-4. pDMECs pretreated with CGRP or medium alone were added to cultures of LCs and $CD4^+$ T cells from DO11.10 Tg mice. Twenty-four hours later, LCs still bound to beads were removed by magnetic capture, and total RNA was isolated from the remaining cells. By RT-PCR, cultures containing medium-treated pDMECs had significantly increased IL-17A, IL-6, IFN-γ, and IL-22 levels, whereas a small and not significant decrease in the IL-4 mRNA level was observed. The addition of CGRP-treated pDMECs led to a substantial and significant further increase in IL-17A and IL-6 mRNA levels accompanied by a loss of the increase in IFN-γ and IL-22 mRNA seen with the addition of medium-treated pDMECs. The level of IL-4 was further decreased compared with wells containing medium-treated pDMECs with $p = 0.07$. (**B**) CGRP-pretreated pDMECs led to enhanced mRNA levels of RORγT accompanied by decreased levels of T-bet and GATA3. The addition of medium-treated pDMECs led to a small but significant decrease in GATA3 mRNA levels. The addition of CGRP-treated pDMECs resulted in a large increase in the level of RORγt mRNA, the loss of the increase in T-bet observed, and a further small but significant decrease in the level of GATA3 mRNA compared with wells containing medium-treated pDMECs. $n = 6$ experiments for all except T-bet, for $n = 7$. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

but significantly elevated by the presence of medium-treated pDMECs, and this elevation was completely abolished by the presence of CGRP-treated pDMECs (Fig. 4B). A small but statistically significant decrease in the level of GATA3 mRNA was seen with the addition of medium-pretreated pDMECs, whereas the addition of CGRP-pretreated pDMECs led to a further and significant reduction in the mRNA level (Fig. 4B).

Pretreatment of pDMECs with siRNA to IL-6 prior to exposure to CGRP inhibited their ability to bias Ag presentation

To determine whether IL-6 is involved in the process by which CGRP induces ECs to bias the outcome of Ag presentation, we treated pDMECs with IL-6 siRNA to knockdown IL-6 production prior to treatment with CGRP and coculture with LCs and T cells. As shown in Fig. 5, pretreatment of pDMECs with either siRNA to IL-6 or nontarget siRNA without CGRP exposure appeared to enhance production of IL-17A, IL-6, IFN-γ, and IL-22, whereas IL-4 release was slightly decreased. Pretreatment of pDMECs with IL-6 siRNA prior to CGRP exposure significantly inhibited IL-6 release and IL-17A production in these cultures while substantially and significantly reducing the inhibition of IFN-γ production (Fig. 5). Only a slight, but significant, reversal of the effect of CGRP exposure by siRNA treatment of pDMECs was seen on the inhibition of IL-22 mRNA, and no effect was seen on suppression of IL-4 mRNA (Fig. 5).

Regulatory effects of CGRP-treated pDMECs do not depend on cell-cell contact

To determine whether ECs must touch LCs or responding T cells, additional experiments were created in which Transwell inserts

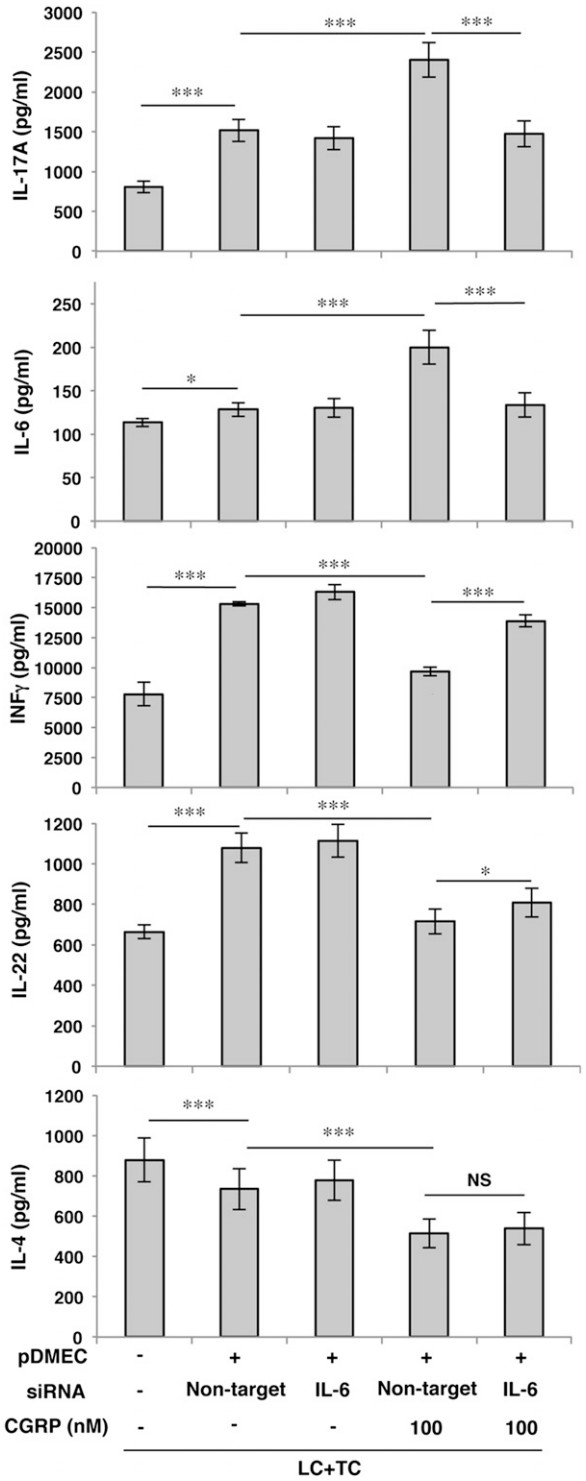

FIGURE 5. Pretreatment of pDMEC with siRNA to IL-6 inhibited the ability of CGRP-treated pDMECs to bias Ag presentation. Treatment of pDMECs with non-target siRNA prior to culture of pDMECs in medium alone followed by the addition to cultures of LCs, $CD4^+$ T cells from DO11.10 Tg mice and $cOVA_{323–339}$ led to small but significant increases in supernatant content of IL-17A and IL-6 and a substantial increase in IFN-γ and IL-22 production, along with a small but significant decrease in IL-4 production. Treatment of pDMECs with nontarget siRNA prior to culture in CGRP and the addition to cultures led to a significant and substantial increase in IL-17A and IL-6 production compared with wells containing nontarget, siRNA-treated pDMECs exposed to medium alone, along with a significant decrease in IFN-γ, IL-22, and IL-4 production. The addition of pDMECs treated with IL-6 siRNA prior to exposure to CGRP and addition to culture wells led to a loss of the increase in IL-17A and IL-6 production observed with the addition of nontarget siRNA-treated pDMECs exposed to medium alone, accompanied by a significant increase in IFN-γ production and a small but significant increase in IL-22 production, but no change in IL-4 production. $n = 3$ experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

were used to separate ECs spatially from LCs, and T cells and supernatant content of cytokines were assessed. The changes seen in these experiments were similar, but not identical to the other experiments (Fig. 1). As seen in the previous experiments, CGRP-treated pDMECs increased the expression of IL-6 and IL-17 and reduced the expression of IFN-γ and IL-22 (Fig. 6). However, in the absence of pDMEC contact, medium-treated pDMECs greatly increased the expression of IL-4, and this effect was decreased when the pDMECs had been exposed to CGRP (Fig. 6), suggesting that the regulation of IL-4 expression differs from the regulation of the other four cytokines we studied.

Given the evidence that IL-6 expression by pDMECs is important for biasing of Ag presentation in this system and that cell-cell contact is not needed, we performed experiments to determine whether adding IL-6 to cultures of LCs, responding T cells, and Ag in the absence of pDMECs could similarly bias the outcome of Ag presentation. As shown in Fig. 7, the addition of IL-6 to wells in the absence of pDMECs reproduced most of the findings of co-culture with CGRP-treated pDMECs, including a significant increase in IL-17A production with a significant decrease in IFN-γ and IL-4 production. However, IL-22 release was unchanged by the addition of IL-6.

BMDCs also respond to CGRP-treated pDMECs but with less magnitude

pDMECs were cultured in CGRP or medium alone for 3 h followed by extensive washing to remove CGRP. pDMECs were then cocultured with BMDCs and $CD4^+$ T cells from DO11.10 Tg mice in the presence of $cOVA_{323–339}$. The presence of medium-treated pDMECs and Ag resulted in a dose-dependent and significant increase in release of IL-17A, IL-6, IFN-γ, and IL-22 with a significant decrease in IL-4 (Fig. 8). However, pretreatment of pDMECs with CGRP led to a greater increase in IL-17A and IL-6 production, whereas the increase in IFN-γ and IL-22 was reversed and production of IL-4 was perhaps somewhat reduced (a significant reduction was seen only with the 10-nm CGRP concentration).

Intradermal CGRP biases the $CD4^+$ lymph node cell response to epicutaneous immunization toward an IL-17A and IL-4 response while inhibiting IL-22 and IFN-γ responses

To determine whether CGRP can modulate the immune response in vivo, groups of BALB/c mice were injected intradermally with CGRP or medium alone. Fifteen minutes later, mice were immunized by topical application of DNFB at sites of injection. Three days later, draining lymph nodes were harvested, and a single-cell suspension of lymphocytes was stimulated in culture with anti-CD3 and anti-CD28. After 72 h, supernatants were assayed for cytokine content. Lymphocytes from mice treated with CGRP produced significantly more IL-17A and IL-4, but significantly less IL-22 and IFN-γ compared with cells from control mice (Fig. 9).

Discussion

ECs are uniquely positioned to allow for products of nerves to influence immune reactions within the skin. First, blood vessels and probably lymphatics within the skin are surrounded by both sensory and sympathetic nerves (27, 28, 54). Second, immune cells, both APCs and lymphocytes, are closely associated with ECs as

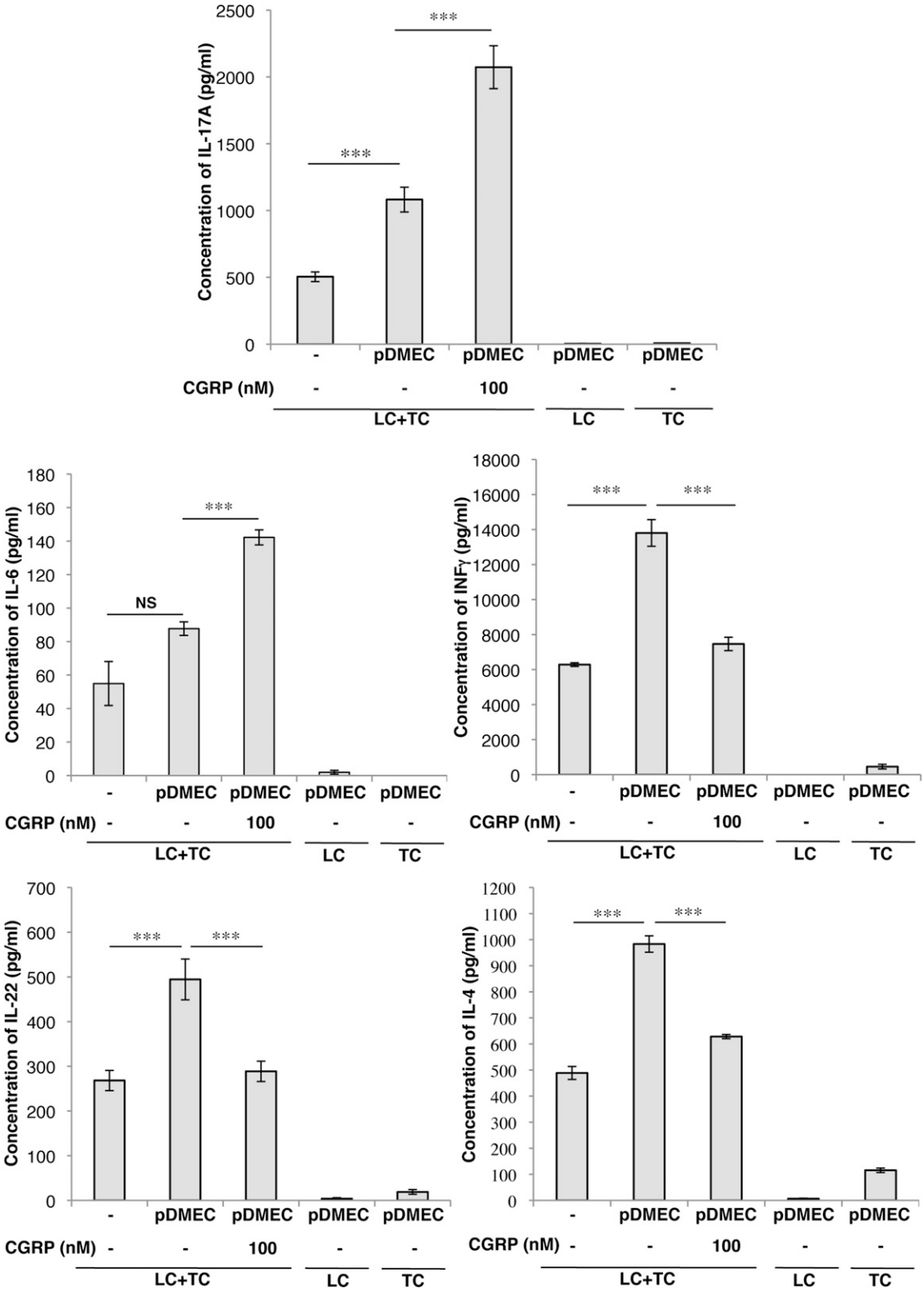

FIGURE 6. Biasing of Ag presentation by CGRP-treated pDMECs does not require contact between pDMECs and LCs or $CD4^+$ T cells. Medium- or CGRP-treated pDMECs were added to the lower chamber of wells in 96-well Transwell plates with LCs, and $CD4^+$ T cells from DO11.10 Tg mice added to the upper chambers in the presence of $cOVA_{323–339}$. The upper chamber of some control wells contained only LCs or only T cells. After 48 h, supernatants were assessed for cytokine content. The addition of cells not exposed to CGRP significantly enhanced IL-17A, IFN-γ, IL-22, and IL-4 concentrations. Pre-exposure of added pDMECs to CGRP led to a much larger increase in IL-17A and IL-6 production, but it largely eliminated the increased production of IFNγ, IL-22, and IL-4 seen in wells with the addition of medium-treated pDMECs. $n = 3$ experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

they exit vessels at sites of immunologic reactions or, conversely, leave the skin to enter lymphatic or blood vessels. Many inflammatory and immunologic disorders of the skin are characterized by perivascular T cells along with APCs, including, in the case of psoriasis, LCs (45, 46). Thus, there is an anatomic basis by which nerves can release transmitters, such as CGRP, which then bind to

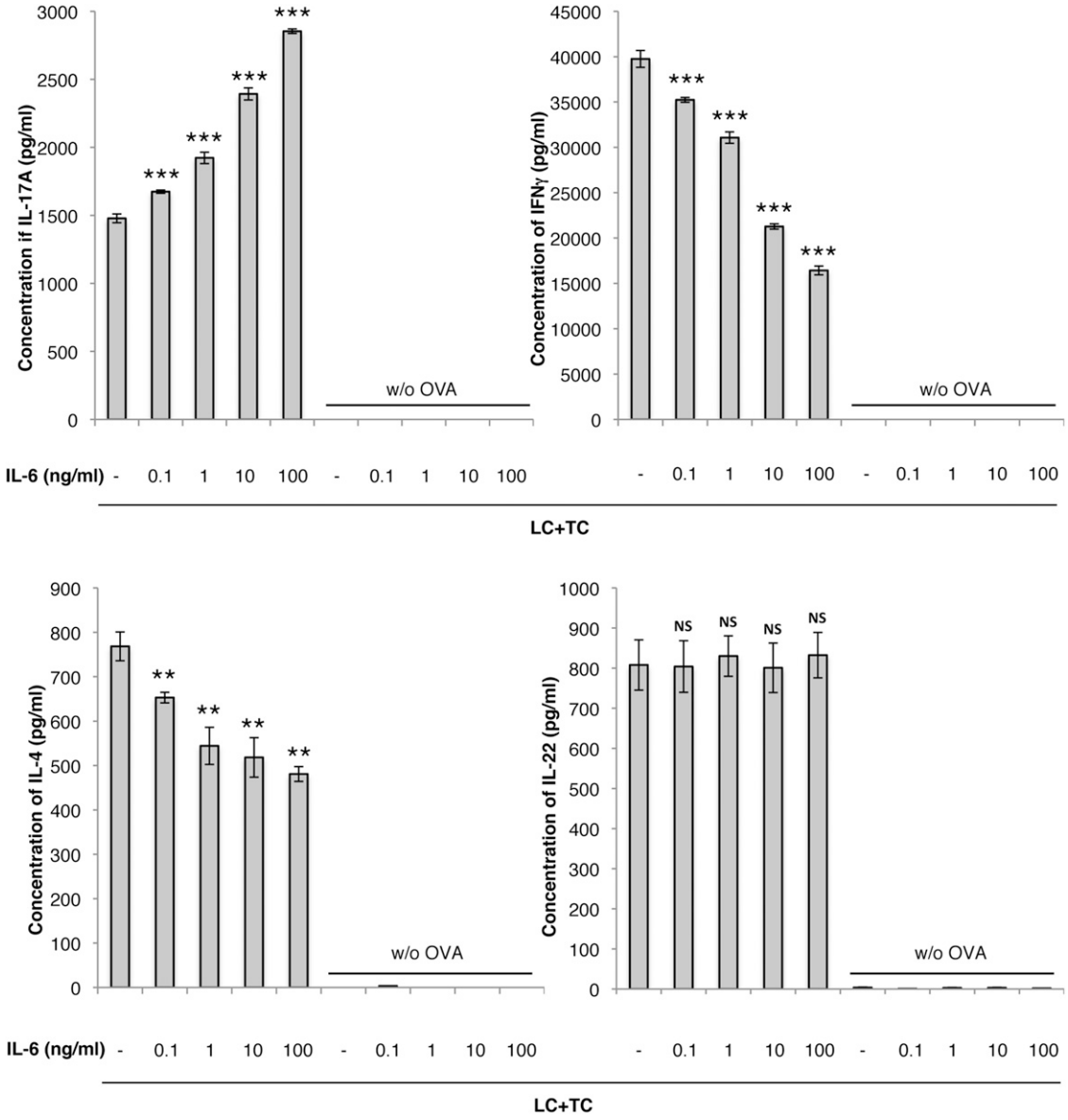

FIGURE 7. IL-6 biases LC Ag presentation to T cells toward an enhanced IL-17A response with a reduced IFN-γ and IL-4 response. IL-6 was added to cultures of LCs, T cells (from DO11.10 Tg mice) and $cOVA_{323–339}$. After 48 h, supernatants were assessed for cytokine content. The addition of IL-6 significantly enhanced IL-17A production with a significant decrease in IL-4 production. IL-22 production was not affected by IL-6. $n = 2$ experiments, all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with no IL-6.

receptors on ECs that are then able to bias the outcome of Ag presentation toward the generation of cells producing IL-17A and away from cells producing IFN-γ and IL-4.

T cells that predominantly produce IL-17A and other members of the IL-17 family of cytokines have been termed Th17 cells (55). These cells play a physiologic role in protection against extracellular organisms (55). They also appear to be involved in the pathophysiology of a number of skin disorders marked by abnormal immune reactivity, notably psoriasis (56). In the mouse, these cells also produce IL-22, and some do so in humans (57). However, there are also cells that produce IL-22 but not IL-17 (56). Interestingly, our work indicates that exposure of ECs to CGRP reduces IL-22 expression.

Our results demonstrate that CGRP can regulate the outcome of Ag presentation through effects on ECs acting as bystanders. As shown above, CGRP-exposed pDMECs lead to markedly enhanced IL-17A and IL-6 production with decreased release of IFN-γ, IL-22, and IL-4 accompanied by enhanced generation of Th17 cells. In addition, the CGRP effect could be blocked by treating ECs in the presence of the CGRP receptor blocker $CGRP_{8–37}$. Interestingly, blocking of IL-6 expression by pDMECs through the use of siRNA for IL-6 inhibited the effect on all cytokines except IL-4, although the effect on suppression of IL-22 was small. Experiments substituting the addition of IL-6 to Ag-presenting culture for the addition of CGRP-treated pDMECs produced similar results, skewing of the results of Ag presentation with increased IL-17A accompanied by decreased IFN-γ and IL-4 production, but no effect was observed on IL-22 release. Thus, the effects of CGRP-treated ECs on IL-22 production in Ag-presenting cultures appear to be mediated by factors other than IL-6 alone. Although the addition of IL-6 to Ag-presenting cell cultures in the absence of ECs did result in decreased IL-4 production, the treatment of pDMECs with siRNA for IL-6 did not inhibit the effect of CGRP-treated pDMECs on IL-4 production in our assay. Furthermore, the effects of CGRP-treated pDMECs in the system did not depend on cell-cell contact, with the interesting exception that the

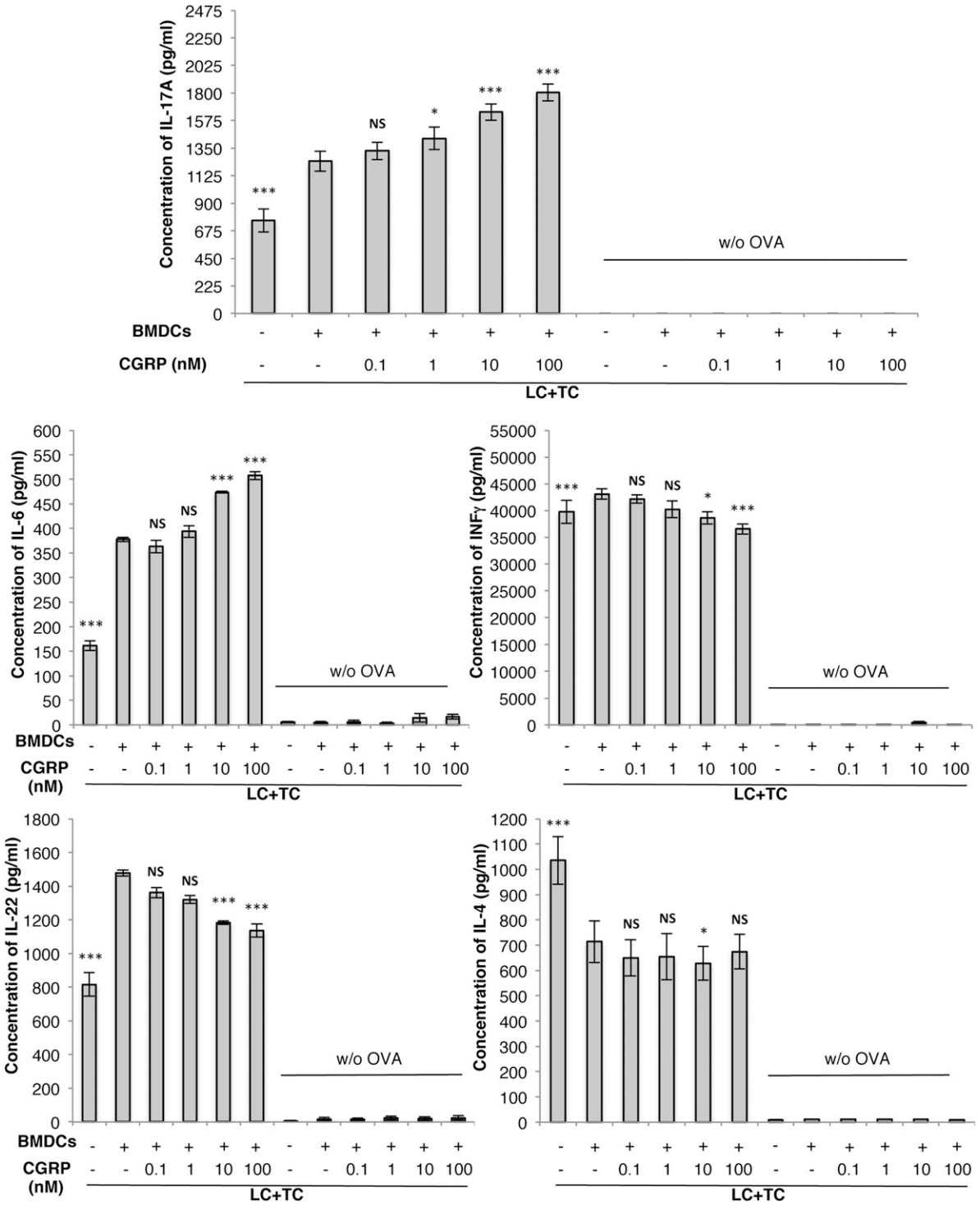

FIGURE 8. CGRP pretreatment of pDMECs biases BMDC Ag presentation to T cells toward an enhanced IL-17A and IL-6 response with a reduced IFN-γ and IL-22 response. Medium- or CGRP-treated pDMECs were added to cultures of BMDCs, T cells (from DO11.10 Tg mice) and $cOVA_{323–339}$. After 48 h, supernatants were assessed for cytokine content. The addition of medium-treated pDMECs slightly but significantly enhanced IL-17A and IFN-γ (very slightly) production alone with more substantial and significant increases in IL-6 and IL-22. A significant decrease in IL-4 production occurred. The addition of CGRP-treated pDMECs led to a significant increase in IL-17A and IL-6 production and a small but significant decrease in production of IFN-γ and IL-22, with little or no reduction in IL-4 production compared with that seen in wells with addition of medium-treated pDMECs. $n = 3$ experiments, all groups. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

presence of medium-treated pDMECs greatly enhanced IL-4 release in the Ag-presenting cell cultures when cell-cell contact did not take place; however, this was not seen when cell-cell contact was possible. Thus, the role of IL-6 in the effect we have observed for IL-4 production with CGRP-treated pDMECs in our assay requires further study. In contrast to the effect seen with the addition of IL-6 to Ag-presentation cultures, contact between pDMECs and LCs and/or responding T cells appears to be important for the alteration of the IL-4 response in Ag-presenting cultures seen in the presence of CGRP-treated ECs.

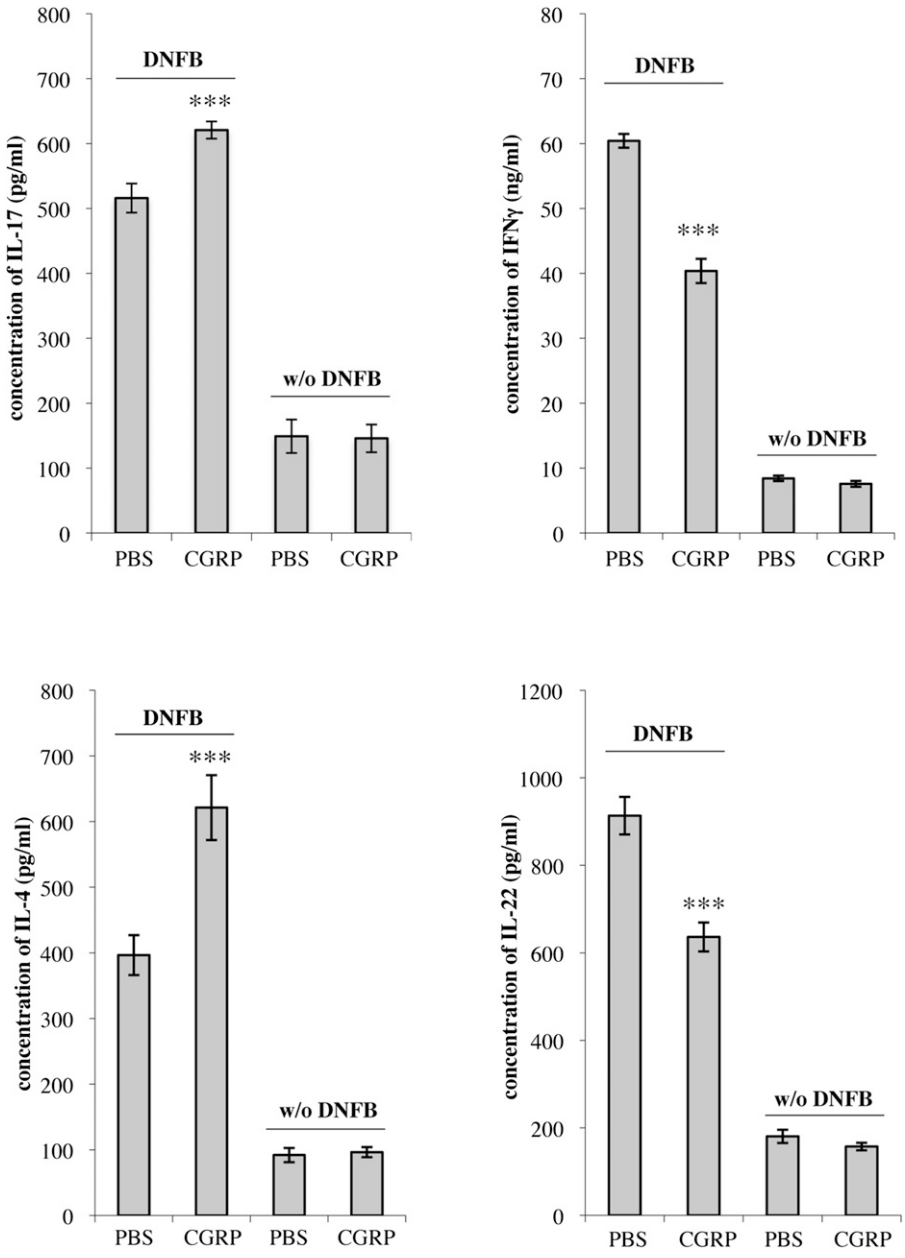

FIGURE 9. CGRP administered intradermally can modulate the immune response in vivo. Groups of BALB/c mice were injected intradermally with medium alone or CGRP. They were then immunized by application of DNFB at sites of injection. Three days later, draining lymph nodes were harvested, and a single-cell suspension of $CD4^+$ lymphocytes was stimulated in culture with anti-CD3 and anti-CD28 mAbs. After 72 h, supernatants were harvested and cytokine content assessed by ELISA. Production of IL-17A and IL-4 were significantly increased in lymphocytes obtained from mice immunized at CGRP-treated sites compared with those at medium-treated sites, whereas production of IFN-γ and IL-22 was significantly decreased. $n = 10$ mice per group for all groups except IL-17A, for which $n = 15$ mice per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

It should also be mentioned that in control wells containing non–CGRP-treated pDMECs, IL-4 expression was increased in some experiments but not in others. In one set of experiments, GATA3 mRNA levels showed a small reduction in responding T cells from wells containing non–CGRP-treated pDMECs (Fig. 4B). The reasons for these discrepancies are not clear, but the various experiments performed differed in experimental protocol and endpoints measured, and these differences might account for some of the variations. These findings might also relate to the apparently more complex nature of regulation of T cell IL-4 production in our system, compared with the other cytokines examined, as described in the paragraph above. However, in all experiments using LC Ag presentation to T cells, IL-4 expression was decreased when pDMECs were pretreated with CGRP.

The peptides SP and ADM did not replicate the effects of CGRP in this system demonstrating the specificity of the CGRP effects, although some effects were seen on some cytokine responses. The failure of ADM to have a large effect strongly indicates that CGRP is working through actions at the CGRP receptor rather than the ADM receptor. The role of these other peptides in modifying the outcome of Ag presentation remains to be elucidated fully.

One caveat to these results is that the population of pDMECs may have a small contaminating population of other cell types. To control for the possibility that the effects we have observed relate to a contaminating cell type, experiments were also performed with the clonal bEnd.3 cell line substituted for pDMECs. Similar results were observed (Supplemental Figs. 1 and 3), minimizing the possibility that CGRP was acting through a contaminating population in our pDMECs preparations.

The effects of CGRP-treated pDMECs in Ag-presenting cultures were similar when BMDCs were used as APCs in place of LCs. Whether this is a general phenomenon with regard to a large, diverse range of APCs is an important question that should be examined.

These findings could have important implications for regulation of immune processes within the skin, both physiologically and pathophysiologically, by the nervous system. Psoriasis is a disorder in which Th17 cells and IL-17A are believed to have key roles (58).

Of particular interest, it has long been known that denervation of skin bearing psoriasis leads to an improvement or clearing of the psoriasis (15, 16). Thus, products of nerves would appear to play an important role in the pathogenesis of this disorder. The key role of IL-17A in the pathophysiology of psoriasis is highlighted by the recent reports that an Ab against IL-17A and its receptor is highly efficacious in treating psoriasis (59). A limitation to this hypothesis, however, is that there is considerable evidence that IL-22 is also involved in the pathophysiology of psoriasis (60). It induces acanthosis in several models, and enhanced levels of IL-22 are found in psoriatic skin (60–62). IL-22 was originally described as a significant product of Th17 cells. However, more recent work has shown that IL-22 in the skin is also derived from $CD8^+$ T cells, $ROR\gamma t^+$ innate lymphocytes, dermal γδ T cells (which also produce IL-17A), and $CD4^+$ T cells that produce IL-22 but not IL-17 (Th22 cells) (57, 63, 64). Indeed, there is some evidence in animal models that γδ T cells are necessary for psoriatic plaque formation (65). Thus, it may be that IL-22 in psoriasis is not necessarily derived from $CD4^+$ IL-17A–producing cells. Thus, CGRP might play a role in the pathogenesis of psoriasis via effects on ECs despite inducing decreased IL-22 production from $CD4^+$ T cells. Of interest, development of an Ab against IL-22 for treatment of psoriasis was discontinued in 2011 after a phase 2 study; there are no publications resulting from these trials, and the results of the psoriasis trial were reportedly not promising (66, 67).

Experiments examining the ability of intradermally administered CGRP to alter the outcome of the T cell response to immunization with a hapten strongly suggest that our in vitro observations have in vivo relevance. Administered CGRP resulted in changes in the generation of Th cell subtypes similar to that seen in the in vitro experiments in which CGRP-exposed ECs were added to Ag-presenting cultures. The direction of change in cytokine expression from $CD4^+$ T cells obtained from lymph nodes draining the sites of immunization pretreated with CGRP were the same in direction as seen in the in vitro experiments with the exception of IL-4. In this regard, we have previously reported that exposure of LCs to CGRP enhances their Ag-presenting ability to stimulate IL-4 responses (37). Thus, in our in vivo experiments, multiple cell types, including both EC and LCs, are likely affected. Thus, a direct effect of CGRP on LCs, inducing them to present Ag for an IL-4 response, might account for the disparate effect seen on production of this cytokine in the in vivo experiments. An effect of CGRP on biasing of the immune response from effects on dermal ECs is consistent with our IL-17A, IFN-γ, and IL-22 results. Nonetheless, other putative targets cannot be excluded from this type of experiment, and the actual loci of action of the CGRP (including possible cell types and signals involved) cannot be stated with certainty.

The in vivo effect of CGRP on biasing the response toward the Th17 pole was relatively modest. However, these results might understate the actual physiologic effects of this pathway in vivo. CGRP was administered only once and at a single concentration. The half-life of CGRP in the circulation is only 7–10 min (68), and it is unlikely to be much longer in the skin. Presumably, a putative physiologic effect of this pathway would depend on the local concentration at ECs in dermal vessels, and it is likely that under some conditions CGRP is released chronically or repetitively by nerves in close proximity to ECs. Definitive experiments to determine the in vivo relevance of CGRP signaling at the level of the EC, although beyond the scope of this report, can be pursued in the future through the use of inducible, conditional knockout animals lacking CGRP receptors in ECs.

The observation that CGRP-treated ECs exert their effects in the absence of contact with T cells excludes the possibility that under the influence of factors in the Ag-presentation milieu they become APCs themselves that contribute to the outcomes observed. This observation is significant because human ECs have been shown to present Ag, especially after stimulation with T cell products, most notably IFN-γ (69, 70). Thus, it appears that the effect is mediated by the production of soluble mediators, including, at least in part, IL-6. In this regard, it should be mentioned that CGRP has been reported previously to induce AP-1 activity in pre-B cells (71) and c-fos and IL-6 mRNA transcription in bone marrow–derived macrophages (72). These pathways might account for the induction of IL-6 production by pDMECs exposed to CGRP in our experimental system.

Interestingly, there may be other pathways by which nerves can influence the expression of psoriasis. We have shown previously that exposure of LCs to vasoactive intestinal polypeptide or pituitary adenylate-activating peptide biases LC Ag presentation toward the generation of Th17 cells in vitro (73). Thus, if this occurs in vivo and Th17 cells indeed play a role in the pathophysiology of psoriasis, these neuropeptides might have an important role. Similarly, the application of imiquimod to murine skin induces a psoriasis-like inflammation. A subset of sensory neurons expressing the ion channels TRPV1 and Nav1.8 has been shown to be required for this inflammatory response, and imaging indicated that a large fraction of dermal dendritic cells are in close contact with these nociceptors (74). Evidence was presented suggesting that the nerves influence dermal dendritic cells to release IL-23 and then induce γ-δ T cells to produce IL-17, driving the inflammation observed. If this mechanism is operant in psoriasis, this pathway might also be important in the pathogenesis of the disorder.

Overall, the results of our investigations strongly suggest that a novel pathway exists by which the nervous system can regulate immunity through actions on ECs. Future work will determine the physiologic, pathophysiologic, and therapeutic importance of these findings.

Disclosures

The authors have no financial conflicts of interest.

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