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Calcitonin Gene-Related Peptide Biases Langerhans Cells toward Th2-Type Immunity

Wanhong Ding,* Lori L. Stohl,* John A. Wagner,† and Richard D. Granstein2*

Langerhans cells (LC) are epidermal dendritic cells capable, in several experimental systems, of Ag-presentation for stimulation of cell-mediated immunity. LC have been considered to play a key role in initiation of cutaneous immune responses. Additionally, administration of donor T cells to bone marrow chimeric mice with persistent host LC, but not mice whose LC have been replaced by donor cells, exhibit marked skin graft-vs-host disease, demonstrating that LC can trigger graft-vs-host disease. However, experiments with transgenic mice in which regulatory elements from human langerin were used to drive expression of diphtheria toxin, resulting in absence of LC, suggest that LC may serve to down-regulate cutaneous immunity. LC are associated with nerves containing the neuropeptide calcitonin gene-related peptide (CGRP), and CGRP inhibits LC Ag-presentation in several models including presentation to a Th1 clone. We now report that CGRP enhances LC function for stimulation of Th2 responses. CGRP exposure enhanced LC Ag presentation to a Th2 clone. Upon presentation of chicken OVA by LC to T cells from DO11.10 chicken OVA TCR transgenic mice, pretreatment with CGRP resulted in increased IL-4 production and decreased IFN-γ production. CGRP also inhibited stimulated production of the Th1 chemokines CXCL9 and CXCL10 but induced production of the Th2 chemokines CCL17 and CCL22 by a dendritic cell line and by freshly obtained LC. Changes in production of these chemokines correlated with the effect of CGRP on mRNA levels for these factors. Exposure of LC to nerve-derived CGRP in situ may polarize them toward favoring Th2-type immunity. The Journal of Immunology, 2008, 181: 6020–6026.

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2 Abbreviations used in this paper: LC, Langerhans cell; CGRP, calcitonin gene-related peptide; KLH, keyhole limpet hemocyanin; cOVA, chicken OVA; EC, epidermal cell; eIC, LC-enchanced EC; pLC, purified LC; CRLK, calcitonin receptor-like receptor; CRCP, CGRP receptor component protein; Tg, transgenic; CM, complete medium.

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The experiments discussed in this paper evaluated the possibility that exposure of LC to CGRP may differentially modulate the ability of these cells to present Ag to Th1 and Th2 cells. The ability of CGRP to regulate the production of Th1 and Th2 chemokines, and thereby regulate the nature of a cutaneous immune response, was also evaluated. Some experiments were initially performed with XS106 cells [a dendritic cell line derived from neonatal BALB/c epidermis (16, 17)] as a surrogate for LC. All experiments other than Northern blotting were also performed with fresh, primary LC. The results discussed below strongly suggest that CGRP biases LC toward favoring Th2-type immune responses.

Materials and Methods

Mice

Six- to 12-wk-old female BALB/c (H-2\textsuperscript{b}), A/J (H-2\textsuperscript{k}), and DO11.10 chicken OVA (cOVA) TCR Tg mice on a BALB/c background [C.Cg-Tg(DO11.10)10Dlo/J] mice were purchased from The Jackson Laboratory and were kept in the animal facility of Weill Medical College of Cornell University on a 12-h light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee of the Weill Cornell Medical College.

Reagents

\(\alpha\)-CGRP and \(\alpha\)-CGRP\textsubscript{8-37} were purchased from Bachem. Conalbumin and keyhole limpet hemocyanin (KLH) were purchased from Sigma-Aldrich. A fragment of cOVA (cOVA\textsubscript{323-339}) was obtained from Peptides International.

Media and cell lines

Complete medium (CM) consisted of RPMI 1640 (Mediatech), 10% FCS (Gemini Biotech), 100 U/ml penicillin, 100 \(\mu\)g/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).
FIGURE 3. XS106 and murine LC express mRNA for all of the components of the CGRP receptor. a, Expression of mRNA in XS106 cells. Total RNA was extracted from XS106 cells and RT-PCR was performed using specific primers for the components of the CGRP receptor: Ramp1 (230 bp), Ramp2 (271 bp), Ramp3 (473 bp), CRCP (331 bp), and CRLR (514 bp). All amplified sequences were of the expected size. b, ECs were obtained from BALB/c mouse epidermis and fresh LC were isolated by FACS using anti-I-Ad mAbs. c, Expression of components of the CGRP receptor in LC. Total RNA was extracted from fresh LC and RT-PCR was performed using the same primers. All amplified sequences were of the expected size.

The XS106 cell line is a dendritic cell line derived from neonatal A/J epidermis. It is capable of Ag presentation and has many of the phenotypic characteristics of LC (16, 17). XS106 cells were grown in CM with the addition of 2 ng/ml mouse rGM-CSF (Chemicon International), 10% NS cell supernatant (supernatant conditioned by a fibroblast-like cell line, known to support the growth of epidermal APC-derived cell lines (18)) and 5 × 10⁻⁴ M 2-ME (Sigma-Aldrich).

The HDK-1 cell line, a KLH-specific, I-A<sup>+</sup>-restricted Th1 clone (19), was maintained in CM supplemented with 5 × 10⁻³ M 2-ME, 10 ng/ml murine IL-2 (Chemicon International), and 10% FCS (Gemini Biotech). The D10.G4.1 cell line (ATCC) is a conalbumin-specific, I-A<sup>k</sup>-restricted Th2 clone (20). It was maintained in modified RPMI 1640 (American Type Culture Collection), supplemented with 0.05 mM 2-ME, 10 pg/ml mouse IL-1α (R&D Systems), 10% FCS (American Type Culture Collection), and 10% T-STIM with Con A (rat IL-2 culture supplement; BD Biosciences).

**Preparation of epidermal cells (EC), LC-enriched EC (eEC), and purified LC (pLC)**

EC were prepared using a modification of standard protocol (21). Briefly, the trunkal skins of mice were shaved and chemically depilated. The s.c. fat and carnosus panniculus were removed by blunt dissection. The skins were then floated dermis side down for 45 min in Ca²⁺- and Mg²⁺-free PBS containing 0.5 U of dispase/ml (BD Biosciences) and 0.38% trypsin (BD Biosciences). Epidermal sheets were collected by gentle scraping, washed, and purified LC (pLC) were prepared using a modification of standard protocol (21). Briefly, EC from BALB/c mice were obtained as described above and incubated with goat anti-mouse IgG conjugated to magnetic microspheres (Dynabeads M-450; Dynal Biotech) for 10 min with continuous, gentle agitation. The cells were then washed repeatedly (up to five times) to enrich LC, using a magnetic particle concentrator (Dynal Biotech) which adhered to the beads. By FACS analysis (using anti-I-Ad<sup>+</sup> mAbs), this procedure yields a cell population of ~95% LC.

**CGRP receptor mRNA detection by RT-PCR**

EC from BALB/c mice were obtained as described above and incubated with PE-conjugated anti-mouse I-Ad<sup>+</sup> (BD Biosciences). Then LC were sorted by FACS on a FACSscan (BD Biosciences). Total RNA was extracted from these freshly obtained LC as well as from XS106 cells using a total RNA extraction kit (Qiagen). RNase free DNase (Qiagen) was used to eliminate any contamination with genomic DNA. Then, 200 (LC) or 100 ng (XS106 cells) of RNA was reverse-transcribed into complementary DNA using 200 U of Superscript II reverse-transcriptase (Invitrogen) following the instructions of the manufacturer (Invitrogen). One-tenth of the synthesized CDNA was amplified by PCR using gene-specific primers made from published sequences for RAMP1, RAMP2, and RAMP3 (22). Primer sequences for calciitonin receptor-like receptor (CRLR) and CGRP-receptor component protein (CRCP) were designed from GenBank sequences for the mRNA of mouse CRLR and CRCP. CRCP, 5' TGGCGGAATAGGAGATAAGA-3' (forward) and 5' TGGCCGGAATAGGAGATAAAG-3' (reversed); CRCP, 5' AGACAGAAGGGACCGCATAA-3' (forward) and 5' CTACTATTTTCTGCTTCTTT-3' (reversed). The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and visualized with UV radiation.
In vitro Ag presentation to Th1 and Th2 clones

To examine the effect of CGRP on LC Ag presentation to Th1 and Th2 clones in vitro, EC or pLC were prepared from A/J or BALB/c mice and plated in 96-well round-bottom plates at 1 × 10^5 cells/well (EC) or 1 × 10^6 cells/well (pLC) in CM. They were then incubated with varying concentration of CGRP (0.1, 1, 10, and 100 nM) at 37°C. After 2.5 h, cells from A/J were exposed to conalbumin and cells from BALB/c were exposed to KLH at a final concentration of 100 μg/ml, still in the presence of CGRP. After an additional 2.5 h, cells were extensively washed with CM. Then, A/J cells were cocultured with D10.G4.1 cells (Th2) (10^6 cells/well) and cells from BALB/c mice were cocultured with HDK cells (Th1) (10^6 cells/well). Supernatants were collected after 24 h or 72 h (Th1), respectively. IL-4 production by D10.G4.1 cells was measured by a sandwich ELISA using purified rat anti-mouse IFN-γ monoclonal capture Abs, biotinylated rat anti-mouse IFN-γ monoclonal detection Abs, avidin–HRP (1/100 dilution), and ABTS substrate, read at 405 nm (all reagents BD Biosciences).

Ag presentation to DO11.10 Tg T cells

Spleens were isolated from DO11.10 Tg mice and mechanically disrupted to yield a single cell suspension. Erythrocytes were then lysed by brief exposure to hypotonic medium. Then, splenocytes were passed through a nylon-wool column to yield a suspension of cells enriched for T cell content (“T cells”). pLC from BALB/c mice were cultured for 3 h in CM containing 10 nM CGRP or medium alone. Then, cells were washed three times and cocultured in 96-well round-bottom plates with DO11.10 T cells. Ten thousand CGRP treated or untreated pLC were cultured in each well with 1 × 10^5 T cells (for IFN-γ assay) or 2 × 10^5 T cells (for IL-4 assay) in 200 μl of CM along with varying concentrations of cOVAΔ23–33, in triplicate. Supernatants were harvested 48 h later, and content of IL-4 and IFN-γ was assessed by sandwich ELISA kits (R&D Systems).

Preparation of culture supernatants

XS106 cells and pLC were cultured at a concentration of 0.5 × 10^6 cells per well in 6-well plates, stimulated with or without 100 ng/ml IFN-γ in the presence or absence of varying concentration of CGRP. Supernatants were collected at different times and CXCL9, CXCL10, CCL17, and CCL22 content was assessed by sandwich ELISA kits (R&D Systems) according to the manufacturer’s protocol.

RNA extraction and Northern blot analysis

XS106 cells were cultured at a concentration of 5 × 10^5 cells per 100 mm tissue culture dish and cultured with or without 100 ng/ml IFN-γ in the presence or absence of CGRP (10 nM) for 12 h. Cells were collected and total cellular RNA extracted by TRIzol (Invitrogen), according to the manufacturer’s protocol. Then, 10 μg of total RNA was electrophoresed on 1.5% agarose-formaldehyde gels, transferred in 20× SSC onto a Hybond-N+ membrane (Amersham Biosciences), and cross-linked to the membrane using UV light.

Probes for murine chemokines and GAPDH were generated by RT-PCR using specific primers for CXCL9 (23), CXCL10 (24), CCL17
Oligonucleotides were end-labeled with $^{32}$P-dCTP (PerkinElmer) by using Klenow fragment of DNA polymerase I (Invitrogen). The RNA-containing membranes were prehybridized for 24 h at 42°C and hybridized for 24 h at 45°C with labeled probes [106 cpm] in Hybrisol-1 buffer (Millipore). The membranes were then washed twice in 2XSSC containing 0.1% SDS (20 min; 25°C) and once with 0.1% SSC containing 0.1% SDS (10 min; 55°C). The membranes were then exposed to x-ray film (Kodak). The intensity of the transcript was digitized and quantified using a phosphor imaging system (Typhoon Trio; GE Healthcare) and then normalized to the intensity of GAPDH mRNA with results expressed as fold-increase over the level obtained with medium alone. One representative experiment of three is shown.

**Results**

**CGRP enhances LC Ag presentation to a Th2 clone while inhibiting Ag presentation to a Th1 clone**

The ability of eEC and ~95% pLC to present conalbumin to the responsive Th2 clone D10.G4.1 (27) was examined. These cells respond to presentation of conalbumin by I-A$^b$. As shown in Fig. 1a, CGRP treatment of eEC (upper panel) and pLC (lower panel) dose-dependently increased their ability to present conalbumin to the Th2 clone D10.G4.1. In contrast, as shown in Fig. 1b, CGRP dose-dependently induced a decrease in the response of the Th1 clone HDK-1 (28) to presentation of KLH by eEC.
DO11.10 Tg mice carry a MHC class II-restricted rearranged TCR 
upon presentation of cOVA

ability of CGRP to inhibit the stimulated release of CXCL9 and 
was confirmed at the mRNA level by Northern blotting (Fig. 4
these chemokines and production of each chemokine was reduced 
by pLC stimulated with IFN- 

were cultured with DO11.10 T cells in the presence of varying concentra-
tured with DO11.10 T cells and LC in the same manner (data not shown). 
No effect was observed on the D10.G4.1 cell response under these conditions (data not shown). 
With regard to treatment of respond-
ing Th1 cells with CGRP, we have already reported that treatment of a Th1 T hybridoma with CGRP did not affect its response to Ag 
presented by LC (5).

CGRP treatment of pLC enhances the IL-4 response while 
diminishing the IFN-γ response of T cells from DO11.10 cOVA

Tg mice upon presentation of cOVA

DO11.10 Tg mice carry a MHC class II-restricted rearranged TCR transgene and react to cOVA_{323-339} (29, 30). The transgene contains rearranged TCR α-chain and TCR β-chain genes and is expressed in the majority of T cells. These rearranged genes encode a COVA-specific MHC class II (I-A^b)-restricted TCR. We used T cells from these mice as a second measure of the ability of CGRP to bias LC toward presenting Ag for a Th2 response. BALB/c pLC were cultured in CGRP or medium alone, washed, and then cocul-
tured with DO11.10 T cells in the presence of varying concentrations of cOVA_{323-339}. After 48 h, conditioned supernatants were harvested and assayed for IL-4 and IFN-γ content by ELISA. As shown by the data in Fig. 2a, exposure to CGRP significantly enhanced the IL-4 response of DO11.10 Tg T cells. In contrast, as shown in Fig. 2b, exposure of pLC to CGRP diminished the IFN-γ response.

The dendritic cell line XS106 and epidermal LC express all of the components of the CGRP receptor

Both the dendritic cell line XS106, derived from neonatal A/J epidermis, and primary LC isolated by FACS from BALB/c EC were examined for expression of mRNA for the CGRP receptor by RT-PCR. As shown by the data in Fig. 3, both XS106 cells and primary fresh LC were found to express all of the components of the CGRP receptor.

CGRP inhibits the stimulated production of CXCL9 and CXCL10 by XS106 cells and pLC

We next examined the ability of CGRP to modulate the production of Th1 chemokines. XS106 cells were stimulated with 100 ng/ml IFN-γ in the presence or absence of various concentrations of CGRP. After 72 h of culture, supernatants were harvested and CXCL9 and CXCL10 content assayed by ELISA. As shown by the data in Fig. 4a, exposure to IFN-γ led to significant production of these chemokines and production of each chemokine was reduced in a dose-dependent fashion by the presence of CGRP. This finding was confirmed at the mRNA level by Northern blotting (Fig. 4b). CGRP also inhibited the production of both CXCL9 and CXCL10 by pLC stimulated with IFN-γ in a similar manner (Fig. 4c). The ability of CGRP to inhibit the stimulated release of CXCL9 and CXCL10 from XS106 cells could be blocked by the presence of the type 1 CGRP receptor antagonist CGRP_v8_37 (data not shown).

CGRP induces production of CCL17 and CCL22 by XS106 cells and pLC

We also evaluated the ability of CGRP to induce release of the Th2 chemokines CCL17 and CCL22. XS106 cells were cultured in medium containing 0–100 nM CGRP or medium alone. Supernatants were collected after 72 h of culture and assayed for CCL17 and CCL22 content by ELISA. As shown by the data in Fig. 5a, CGRP dose-dependently increased production of CCL17 and CCL22 by XS106 cells. This effect could also be observed at the mRNA level by Northern blotting (Fig. 5b). CGRP treatment of pLC in the same manner also increased CCL17 and CCL22 production (Fig. 5c). Interestingly, this effect of CGRP could not be inhibited by CGRP_v8_37 (data not shown). Thus, it is likely that this latter effect is not mediated by the CGRP1 receptor. CGRP is known to also activate the calcitonin-like-receptor when associated with RAMP3 in transfected cell models (31) and it is likely acting on XS106 cells and LC in the same manner.

Discussion

LC reside in the suprabasilar portion of the epidermis and have been shown to be capable of presenting hapten, immunogenic peptides, and tumor Ags for T cell-dependent immune responses (32–35). As mentioned above, LC have been considered to be professional APC that play an important role in the initiation of cutaneous immunity. However, several lines of evidence have challenged that view. In experimental models of leishmaniasis, LC are not believed to be responsible for induction of immunity and, instead, may induce tolerance (36). Similarly, in murine models of herpes simplex infection, LC do not appear to be responsible for induction of immunity (37, 38). Of particular interest, as mentioned above, Kaplan and collaborators (4) used Tg mice in which regulatory elements from human langerin were used to drive expression of diphtheria toxin resulting in elimination of epidermal LC. In this system, elimination of LC resulted in enhanced contact hypersensitivity responses, suggesting that in situ LC down-regulate the expression of contact hypersensitivity (4).

We have hypothesized that the observation that LC are frequently anatomically associated with epidermal nerves containing CGRP may be relevant to the possibility that in the steady-state LC subserve a primarily down-regulatory or immunosuppressive role, perhaps to prevent unwanted immune reactivity against self-Ags or commensal microorganisms. We and others (5, 7) have previously shown that CGRP treatment of LC in vitro inhibits their ability to present Ag in several assays in vitro. Additionally, treatment of CGRP ex vivo inhibits their ability to used to immunize naive mice upon s.c. injection and immunization of mice with a contact sens-
sitizer applied to a site injected intradermally with CGRP leads to a suppressed degree of sensitivity (6). We have now extended these observations to demonstrate that CGRP biases LC toward favoring functioning for Th2-type immunity.

We found that CGRP treatment of LC augmented their ability to present Ag to a Th2 clone while inhibiting Ag presentation to a Th1 clone. Similarly, treatment of LC with CGRP led to a decreased ability to present cOVA_{323-339} to DO11.10 Tg T cells for a Th1 response (IFN-γ production) while augmenting a Th2 response (IL-4 production). CGRP also induced production of the Th2 chemokines CCL17 and CCL22 by XS106 cells and pLC. In the case of the Th1 chemokines CXCL9 and CXCL10, CGRP treatment resulted in inhibition of the stimulated production of these chemokines. With XS106 cells, we examined mRNA levels of these chemokines and the mRNA levels correlated with the
effects of CGRP on expression of each of the chemokines at the protein level. To examine the possibility that any of these effects could be due to changes in viability of LC or T cells induced by exposure of LC to CGRP, we examined the viability of pLC in culture over time in 0, 0.1, 1, 10, and 100 nM CGRP. No significant differences in viability were observed (data not shown). To examine possible differences in viability of HDK-1 cells when Ag presentation is performed with LC treated with CGRP, we set-up experiments where LC were used with and without pretreatment with CGRP before being used to present KLH to HDK-1 cells. At the end of 72 h, HDK-1 cells were enumerated by performing FACS analysis for CD3. No significant difference in the number of HDK-1 cells was observed between the two conditions (data not shown).

It is likely that exposure of LC to CGRP from associated nerves in situ in the epidermis maintains LC in a state that favors activation of Th2 mechanisms over Th1 mechanisms and this effect may account, at least in part, for the immunosuppressive activities of LC with regard to Th1-type immunity. It has long been known that culture of EC in vitro leads to enhanced LC Ag presenting capability for some asays of APC function (39). Although exposure to GM-CSF and other stimulatory cytokines in culture (from contaminating keratinocytes) says of APC function (39). Although exposure to GM-CSF and other stimulatory cytokines in culture (from contaminating keratinocytes) may account for some of this maturation, removal from an environment containing CGRP may also play a role. As a whole, these results support the concept of a loop of interaction between the nervous system and the immune system within the skin by which the nervous system is able to regulate the character of the cutaneous immune response. Further studies might suggest practical new avenues for manipulation of cutaneous immunity through activation or inhibition of CGRP receptors.

Disclosures

The authors have no financial conflict of interest.

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