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REGULAR ARTICLE

CXCL10/CXCR3 signaling mediates inhibitory action by interferon-gamma on CRF-stimulated adrenocorticotropic hormone (ACTH) release

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Abstract Secretion of hormones by the anterior pituitary gland can be stimulated or inhibited by paracrine factors that are produced during inflammatory reactions. The inflammation cytokine interferon-gamma (IFN-γ) is known to inhibit corticotropin-releasing factor (CRF)-stimulated adrenocorticotropin (ACTH) release but its signaling mechanism is not yet known. Using rat anterior pituitary, we previously demonstrated that the CXC chemokine ligand 10 (CXCL10), known as interferon-γ (IFN-γ) inducible protein 10 kDa, is expressed in dendritic cell-like S100β protein-positive (DC-like S100β-positive) cells and that its receptor CXCR3 is expressed in ACTH-producing cells. DC-like S100β-positive cells are a subpopulation of folliculo-stellate cells in the anterior pituitary. In the present study, we examine whether CXCL10/CXCR3 signaling between DC-like S100β-positive cells and ACTH-producing cells mediates inhibition of CRF-activated ACTH-release by IFN-γ, using a CXCR3 antagonist in the primary pituitary cell culture. We found that IFN-γ up-regulated *Cxcl10* expression via JAK/STAT signaling and proopiomelanocortin (*Pomc*) expression, while we reconfirmed that IFN-γ inhibits CRF-stimulated ACTH-release. Next, we used a CXCR3 agonist in primary culture to analyze whether CXCL10 induces *Pomc*-expression and ACTH-release using a CXCR3 agonist in the primary culture. The CXCR3 agonist significantly stimulated *Pomc*-expression and inhibited CRF-induced ACTH-release, while ACTH-release in the absence of CRF did not change. Thus, the present study leads us to an assumption that CXCL10/CXCR3 signaling mediates inhibition of the CRF-stimulated ACTH-release by IFN-γ. Our findings bring us to an assumption that CXCL10 from DC-like S100β-positive cells acts as a local modulator of ACTH-release during inflammation.

Keywords Anterior pituitary gland · ACTH · CXCL10 · CXCR3 · Interferon-gamma · S100β protein-positive cells

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Introduction

The anterior pituitary (adenohypophysis) consists in the anterior and intermediate lobes. The anterior lobe is composed of five types of hormone-producing cells, S100β-positive cells and fenestrated sinusoids (i.e., endothelial cells and pericytes). S100β-positive cells were first found in the brain (Moore 1965) and thereafter in several tissues. In the anterior lobe, they have a star-like appearance with extended cytoplasmic protrusions, which envelop hormone-producing cells or interconnect homophilically to form pseudofollicles and meshworks (Soji

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and Herbert 1989). S100β-positive cells in the anterior lobe are usually referred to as folliculo-stellate cells (Vila-Porcile 1972). It has been subsequently suggested that some S100β-positive cells act as stem cells or phagocytes, or regulate hormone release in the anterior lobe (Allaerts and Vankelecom 2005). Based on these histological and physiological features, S100β-positive cells have often been discussed as having functionally heterogeneous subpopulations. The heterogeneity was noted by the observation of different markers in four subpopulations: SOX2 or/and PROP1 (stem/progenitor cell-like) (Yoshida et al. 2009, 2011), glial fibrillary acidic protein or/and vimentin (astrocyte-like) and another population containing keratin (epithelial cell-like) (Höfler et al. 1984) or interleukin-6 (IL-6) (dendritic cell-like) (DC-like) (Allaerts et al. 1996). Recently, we found a simple and prompt method for isolating DC-like S100β-positive cells from the rat anterior lobe by use of their adhesive property to extracellular matrix (Horiguchi et al. 2014a). We found that DC-like S100β-positive cells expressed chemokine CXCL10 and its receptor CXCR3 was expressed in adrenocorticotropin (ACTH)-producing cells (Horiguchi et al. 2014b). We also showed that other CXCR3 ligands, CXCL9 and -11, were not expressed in rat anterior pituitary (Horiguchi et al. 2014b).

Chemokines are defined as small proteins (7–16 kDa) that induce chemotaxis and extravasation and modulate the functional properties of different leukocytes upon inflammation (Rot and Andrian 2004; Charo and Ransohoff 2006). Chemokines are grouped into four distinct families (CC, CXC, CX3C and C) according to the number and positions of the conserved cysteine residues in the N-terminus of these proteins (Zlotnik and Yoshie 2000). CXCL10, known as interferon-γ (IFN-γ) inducible protein 10 kDa, is secreted upon IFN-γ production by a wide variety of cell types, such as monocytes, leukocytes, endothelial cells and stromal cells (Luster and Ravetch 1987). It is well known that CXCL10 recruits Th1 lymphocytes to areas of inflammation and its expression is associated with Th1 immune responses (Khan et al. 2000; Lo et al. 2010). ACTH has a strong anti-inflammatory property and serves as a natural inhibitor of inflammatory responses (Zacharowski et al. 2006). In addition, IFN-γ is known to inhibit corticotropin-releasing factor (CRF)-stimulated ACTH-release through S100β-positive cells (Vankelecom et al. 1992) but its signaling mechanism is not yet known. We hypothesize that CXCL10/CXCR3 signaling between DC-like S100β-positive cells and ACTH-producing cells induces the inhibition of CRF-stimulated ACTH-release. In the present study, we examined whether the inhibition of CRF-stimulated ACTH-release was provoked by IFN-γ through CXCL10/CXCR3 signaling in primary cultures of rat anterior pituitary cells. Finally, we showed that it is the DC-like S100β-positive cells that play an important role in the regulation of CRF-stimulated ACTH-release during inflammation.

Materials and methods

Animals

S100β-GFP transgenic Wistar-crlj strain (S100β-GFP) rats were generated by integrating the reporter gene GFP driven by a rat S100β promoter (Itakura et al. 2007). Adult Wistar rats were purchased from Japan SLC (Shizuoka, Japan). Eight- to ten-week-old male rats weighing 250–300 g were given ad libitum access to food and water and housed under conditions of 12 h light and 12 h darkness. The rats were killed by exsanguination from the right atrium under deep Nembutal anesthesia and were then perfused with Hanks' balanced salt solution (Life Technologies, Carlsbad, CA, USA) for primary culture. The present study was approved by the Committee on Animal Experiments of the School of Agriculture, Meiji University and by Kyorin University, based on the NIH Guidelines for the Care and Use of Laboratory Animals.

Isolation of a subpopulation of S100β-positive cells

Anterior pituitary glands of S100β-GFP male rats were dispersed as described previously (Horiguchi et al. 2014a). Dispersed cells were separated into GFP-positive and -negative cells by a cell sorter (MoFlo XDP; Beckman Coulter, Fullerton, CA, USA). GFP-positive cells were plated onto 8-well glass chamber slides (1 cm^2/well; Nalge Nunc International, Rochester, NY, USA) with a coating of laminin substrate (10 μg/cm^2 of laminin; Millipore, Bedford, MA, USA) with Medium 199 with Earle's salts (Life Technologies), supplemented with 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 0.5 U/ml of penicillin and 0.5 μg/ml of streptomycin (Life Technologies). Isolation of DC-like GFP-positive cells was described in our previous report (Horiguchi et al. 2014a). The GFP-positive cells were cultured for 24 h. After that, the majority of GFP-positive cells showed flattened and markedly extended cytoplasmic processes (stellate-shape) but a small number of GFP-positive cells retained their round shape (round-shaped, DC-like S100β-positive cells). The round-shape cells were removed from the dish with gentle pipetting and collected by centrifuge from the retrieved medium followed by cultivation again for 24 h in 400 μl medium on laminin-coated 8-well glass. Fresh medium (400 μl) was added to the remaining adhering cells. The round-shape cells were then cultured for a total of 48 h at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air.

Immunocytochemistry

Cultured stellate- and round-shaped cells fixed with 4 % paraformaldehyde in 25 mM phosphate buffer for 15 min at room temperature were first immersed in PBS containing 0.5 % Triton X-100 for 5 min at room temperature. After that cells

were immersed in PBS containing 2 % normal goat serum for 20 min at 30 °C, then incubated overnight with anti-rat CXCL10 (1:200; Biorbyt, Cambridge, UK) at room temperature. After washing with PBS, cells were incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG diluted to 1:200 in PBS. Absence of an observable nonspecific reaction was confirmed using normal rabbit serum. Cells were scanned using a confocal laser microscope (FV500; Olympus, Tokyo, Japan). Each observation was performed in triplicate.

Cell culture

Dispersed cells of anterior pituitary from Wistar rats were plated onto 24-well multiplate (1.8 cm^2/well; Sumitomo Bakelite, Tokyo, Japan) at 4×10^5 cells/well in 600 μl with media as described above. Cells were then cultured for 48 h at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Thereafter, media were changed to a vehicle (medium containing dimethylsulfoxide, DMSO, at 0.1 %) for 24 or 27 h, IFN-γ (1000U/ml; Chemicon, Temecula, CA, USA) for 0, 1, 3, 24 and 48 h, CRF at 1×10^{-9} M for 0, 1, 3 and 24 h, IFN-g for 24 h and CRF at 1×10^{-9} M for 3 h (TOCRIS Bioscience, Avonmouth, UK), IFN-γ with Janus kinase 2 (Jak2) inhibitor (1,2,3,4,5,6-Hexabromocyclohexane; Selleckchem, Houston, TX, USA) at 1×10^{-5} M for 24 h, CXCR3 agonist (Stroke et al. 2006; (S)-N-((S)-1-((cyclohexylmethyl)amino)-5-guanidino-1-oxopentan-2-yl)-2-(4-oxo-4-phenylbutanoyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; Millipore, Billerica, MA, USA) at 1×10^{-6} M for 24 h, 1×10^{-7} M for 24 h, 1×10^{-8} M for 1, 3, 24 and 48 h, CXCR3 antagonist (Jenh et al. 2012; 4-cyano-N-(3-fluoro-4-(1H-tetrazol-5-yl)benzyl)-N-(2-fluorobenzyl)benzenesulfonamide; Millipore) at 1×10^{-7} M for 24 h, CXCR3 antagonist at 1×10^{-7} M for 24 h and CRF at 1×10^{-9} M for 3 h, CXCR3 agonist at 1×10^{-8} M for 24 h and CRF at 1×10^{-9} M for 3 h in 600 μl of Medium 199 with 0.1 % bovine serum albumin, respectively.

Quantification of mRNA levels by real-time PCR

Total RNA fractions were prepared with RNeasy Mini Kit (Qiagen, Hilden, Germany) from anterior pituitary tissue, isolated cells and cultured cells according to the manufacturer's instructions. They were incubated with RNase-free DNase Kit (Qiagen). Using 100 ng RNAs, cDNAs were synthesized in 20 μl by a Superscript III reverse transcription kit with oligo-$(dT)_{20}$ primer (Life Technologies). Quantitative real-time PCR (ABI PRISM 7500Fast; Life Technologies) was performed using gene-specific primers and Power SYBR Green PCR Master Mix (Life Technologies). The sequences of the gene-specific primers are listed in Table S1 of the Electronic Supplementary Material. For normalization, we also quantified β-actin (*b-actin*). The relative gene expression was calculated by comparing the cycle times for each target PCR. Cycle threshold values were converted to relative gene expression levels by using the $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$ method (Yoshida et al. 2011). The analysis was performed 3 times for each experimental group.

Measurement of ACTH

Cells were incubated for the predetermined time at the indicated condition in Medium 199 with 0.1 % bovine serum albumin (Sigma-Aldrich). Supernatants of culture medium were collected and frozen at −80 °C until evaluation of ACTH content. Concentration of ACTH was measured by a commercially available ELISA kit (Adrenocorticotropic Hormone ELISA Kit; USCN Life Science, Wuhan, China) according to the supplier's instructions.

In situ hybridization

Dispersed cells of anterior pituitary from Wistar rats were plated onto 8-well multiplate coated with laminin (Millipore) at 1×10^5 cells/well in 400 μl with media as described above. Cells were then cultured for 72 h at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Thereafter, media were changed to a vehicle or IFN-γ for 3 h. Cells fixed with 4 % paraformaldehyde in 25 mM phosphate buffer for 5 min at room temperature were first immersed in PBS for 5 min. In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes, as described in our previous report (Horiguchi et al. 2014b). The following DNA fragments were amplified from rat pituitary cDNA by using PCR: *Cxcl10* with forward (5′-TGTCCGCATGTTGAGATCAT-3′) and reverse (5′-ATTTGCCATCTCACCTGGAC-3′) primers (Horiguchi et al. 2014b). A control experiment was performed and no specific signal was detected in sections processed with the DIG-labeled sense RNA probe. For fluorescent double-labeling of *Cxcl10* mRNA and S100-protein, an in situ hybridization signal was visualized by using a solution from the HNPP Fluorescent Detection Kit (Roche Diagnostics). After in situ hybridization, the sections were incubated with S-100 protein (1:1000; Dako, Glostrup, Denmark), Texas-Red-conjugated Avidin (Life Technologies) being used as fluorescent dye and Alexa-Fluor-488-conjugated anti-rabbit IgG (Life Technologies) being used as the secondary antibody. Nuclei were counterstained by mounting with Vectashield Mounting Medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Sections were scanned using a fluorescence microscope (cellSens® Dimension system; Olympus).

Statistical analysis

Data are presented as the mean±SEM for at least 3 rat preparations in each group. Student's *t* test after *F* test was used for

2-group comparisons and Dunnett's test was used for multiple comparisons. Differences were considered significant when P was less than 0.05.

Results

IFN-γ and CRF exert their role on *Pomc* expression and ACTH-release

Stimulation of both *Pomc*-expression and ACTH-release by CRF (Blumenfeld and Jaffe 1986; Jenks 2009) and inhibition of ACTH-release by IFN-γ (Vankelecom et al. 1992) were investigated. First, we identified whether *Ifng* and its receptors were expressed in the rat anterior pituitary and S100β-positive cells by real-time RT-PCR. The IFN-γ receptor (IFNGR) complex consists in IFNGR1 and IFNGR2 (Blouin and Lamaze 2013). We found that *Ifngr1* and *Ifngr2* were expressed in them but *Ifng* was not (Electronic Supplementary Figure S1). Next, to examine whether IFN-γ influences ACTH concentration and *Pomc*-expression in the presence of CRF using primary cultured cells, ACTH concentration was measured by ELISA after treatments with IFN-γ for 24 h and with CRF subsequently for 3 h (Fig. 1a). The concentration of ACTH with IFN-γ alone (IFN-γ 24 h+Vehicle 3 h) was the same level as that of the vehicle (Vehicle 27 h). On the other hand, treatment with CRF alone (Vehicle 24 h+CRF 3 h) significantly increased the ACTH concentration 2.35-fold. Combined treatment with IFN-γ and CRF (IFN-γ 24 h+CRF 3 h) induced a significant reduction of the CRF-stimulated ACTH concentration level 0.57-fold (Fig. 1a). It was the same level as that of the vehicle (Vehicle 27 h). Next, to examine whether IFN-γ and CRF affect *Pomc*-expression in the primary culture of the rat anterior pituitary cells, the time course of *Pomc*-expression was measured by real-time PCR after treatment with IFN-γ for 0, 1, 3, 24 and 48 h (Fig. 1b) or with CRF for 0, 1, 3 and 24 h (Fig. 1c). Treatment with IFN-γ for 24 h significantly ($P<0.05$) increased the *Pomc* mRNA level by 1.35 fold in comparison with that at 0 h (Fig. 1b). CRF also significantly ($P<0.05$) increased the *Pomc* mRNA level with treatment for 3 and 24 h by 1.28- and 1.82-fold, respectively (Fig. 1c). Next, involvement of CXCR3 in the increase of the *Pomc* mRNA level was examined using primary culture cells treated with a CXCR3 antagonist. Treatment with IFN-γ alone (IFN-γ 24 h+Vehicle 3 h), CRF alone (Vehicle 24 h+CRF 3 h) and IFN-γ/CRF (INF-γ 24 h+CRF 3 h) caused a significant increase of the *Pomc* mRNA level in comparison with that of vehicle (Vehicle 27 h). Furthermore, the increased-*Pomc* mRNA level with IFN-γ and CRF (IFN-γ 24 h+CRF 3 h) was significantly ($P<0.05$) decreased with CXCR3 antagonists (IFN-γ/CXCR3 ant. 24 h+Vehicle 3 h) (Fig. 1d). However, the treatment with CRF in cells pretreated with IFN-gamma/CXCR3 antagonists (IFN-γ/CXCR3 ant. 24 h+CRF 3 h) reversed the inhibitory effect of the CXCR3 antagonist alone (IFN-γ/CXCR3 ant. 24 h+Vehicle 3 h) (Fig. 1d).

CXCL10-expression in a subpopulation of anterior pituitary S100β-positive cells

We recently demonstrated that purified GFP-positive cell fractions from dispersed primary cell culture of the S100β-GFP rat anterior lobe can be separated into two subtypes, a stellate shape (adhesive, Fig. 2a–d) and a round shape (less-adhesive and DC-like cell type, Fig. 2e–h) in reference to the adhesiveness to the culture dish with gentle pipetting (Horiguchi et al. 2014a). Here, we examined an abundance of CXCL10 in either subtype of S100β-positive cells (round- and stellate-shaped). Immunocytochemistry for the two separated cell types showed that CXCL10 is present in the round-shaped DC-like S100β-positive cells (Fig. 2a–d) but not in the stellate-shaped (Fig. 2e–h). Furthermore, we observed whether IFN-γ affects the *Cxcl10*-expression cells in primary culture of the anterior pituitary cells on laminin-coated surfaces. We detected them by in situ hybridization of *Cxcl10* (Fig. 3c, g) and immunocytochemistry of S100 protein (Fig. 3d, h). Many *Cxcl10*-expression cells in S100 protein positive cells were observed in primary culture with IFN-γ (Fig. 4e–h). These data suggest that IFN-γ activate the *Cxcl10*-expression in DC-like S100β-positive cells.

IFN-γ exerts its role on *Cxcl10*-expression

We examined whether IFN-γ affects the *Cxcl10*-expression in DC-like S100β-positive cells using primary culture of the rat anterior pituitary cells by real-time PCR (Fig. 4). The treatment of IFN-γ significantly ($P<0.01$) increased the *Cxcl10* mRNA level by over 150 times in comparison with that of the vehicle (Fig. 4a). In addition, the time course of *Cxcl10* mRNA levels was analyzed by real-time PCR in primary culture cells after treatment with IFN-γ for 0, 1, 3, 24 and 48 h. Treatment with IFN-γ for 1 h significantly increased *Cxcl10* mRNA levels, which reached its maximum after 3 h, followed by a decrease to 50 % of its value at 3-h (Fig. 4c). Since the IFN-γ signal is known to be mediated by intracellular Jak2 signaling (Watling et al. 1993; Qi et al. 2009), we also investigated the involvement of Jak2 signaling in the IFN-γ-stimulated *Cxcl10* expression using Jak2 inhibitor. Treatment with Jak2 inhibitor for 24 h significantly ($P<0.01$) decreased the IFN-γ-stimulated *Cxcl10* mRNA level (Fig. 4b). Then, we investigated whether the *Cxcl10* mRNA level is modulated with CRF. The *Cxcl10* mRNA level significantly ($P<0.05$) decreased by treatment with CRF alone for 3 h (Vehicle 24 h+CRF 3 h) by 0.46 fold in comparison with

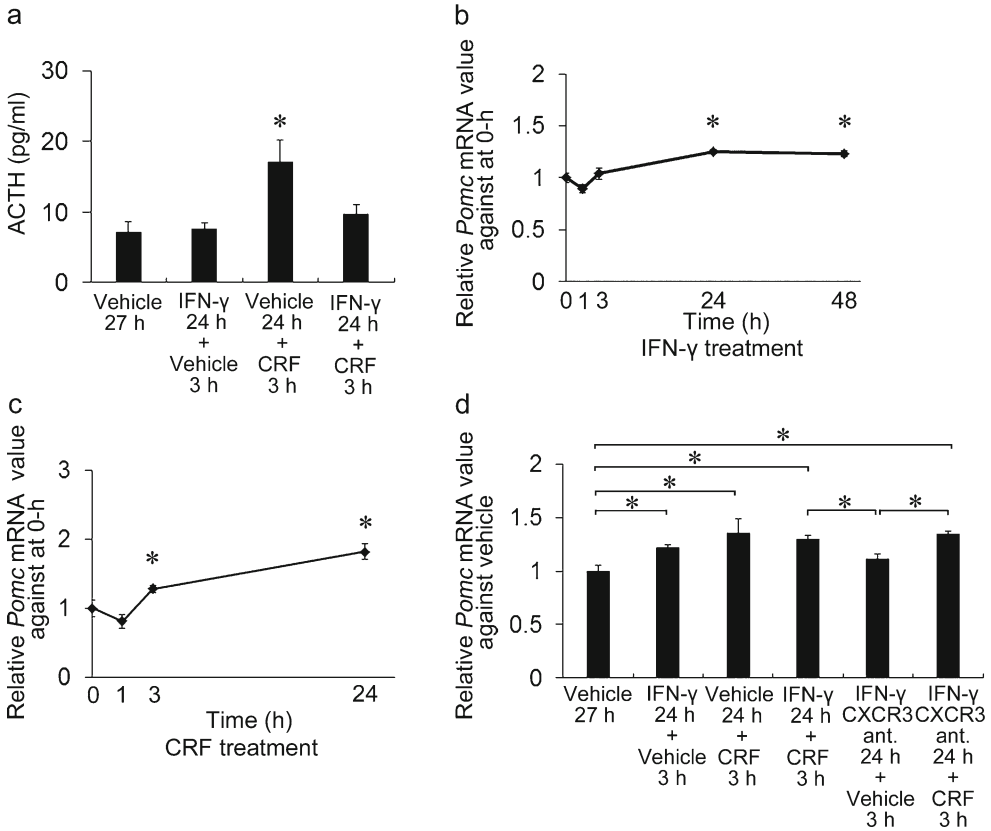

Fig. 1 Effect of IFN-γ on ACTH production. **a** Measurement of ACTH in media by ELISA with or without CRF for 3 h in media with IFN-γ. ACTH levels in primary culture were with a vehicle for 27 h (Vehicle 27 h), IFN-γ for 24 h and a vehicle for 3 h (IFN-γ 24 h+Vehicle 3 h), a vehicle for 24 h and CRF (1×10^{-9} M) for 3 h (Vehicle 24 h+CRF 3 h) and IFN-γ for 24 h and CRF for 3 h (IFN-γ 24 h+CRF 3 h) (mean±SEM, n=3). **b**–**d** *Pomc* mRNA levels were quantified by real-time PCR and were normalized with an internal control (*b-actin*) (mean±SEM, n=3). Their mRNA levels indicated relative values against that at 0 h (**b**, **c**) or a vehicle (**d**). The significance of the differences was determined against 0 h by Dunnett's test (**b**, **c**) or a vehicle value by the Bonferroni test (**d**). **b** *Pomc* mRNA levels during 48 h incubation (0, 1, 3, 24 and 48 h) with IFN-γ at 1000U/ml. **c** *Pomc* mRNA levels at different incubation times (0, 1, 3 and 24 h) with CRF at 1×10^{-9} M. **d** *Pomc* mRNA levels with a vehicle for 27 h (Vehicle 27 h), IFN-γ for 24 h and a vehicle for 3 h (IFN-γ 24 h+Vehicle 3 h), a vehicle for 24 h and CRF (1×10^{-9} M) for 3 h (Vehicle 24 h+CRF 3 h), IFN-γ for 24 h and CRF for 3 h (IFN-γ 24 h+CRF 3 h), IFN-γ and CXCR3 antagonist for 24 h and a vehicle for 3 h (IFN-γ CXCR3 ant. 24 h+Vehicle 3 h) and IFN-γ and CXCR3 antagonist for 24 h and CRF for 3 h (IFN-γ CXCR3 ant. 24 h+CRF 3 h). $^{**}P<0.01$, $^{*}P<0.05$

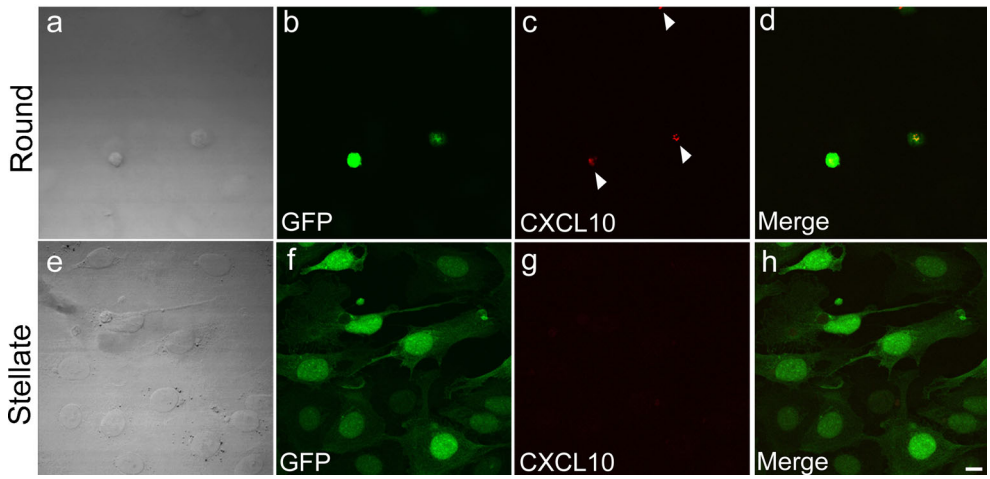

Fig. 2 Expression of CXCL10 in a subpopulation of S100β-positive cells. Phase-contrast images (**a**, **e**), GFP images (*green*, S100β-positive cells) (**b**, **f**), immunocytochemistry of CXCL10 (*red*) (**c**, **g**) and the merged images (**d**, **h**) of the round-shaped (*Round*) (**a**–**d**) and the stellate-shaped (*Stellate*) (**e**–**h**) S100β-positive cells, respectively. *Arrowheads* indicate CXCL10-positive cells in the round-shape. *Scale bar* 10 μm

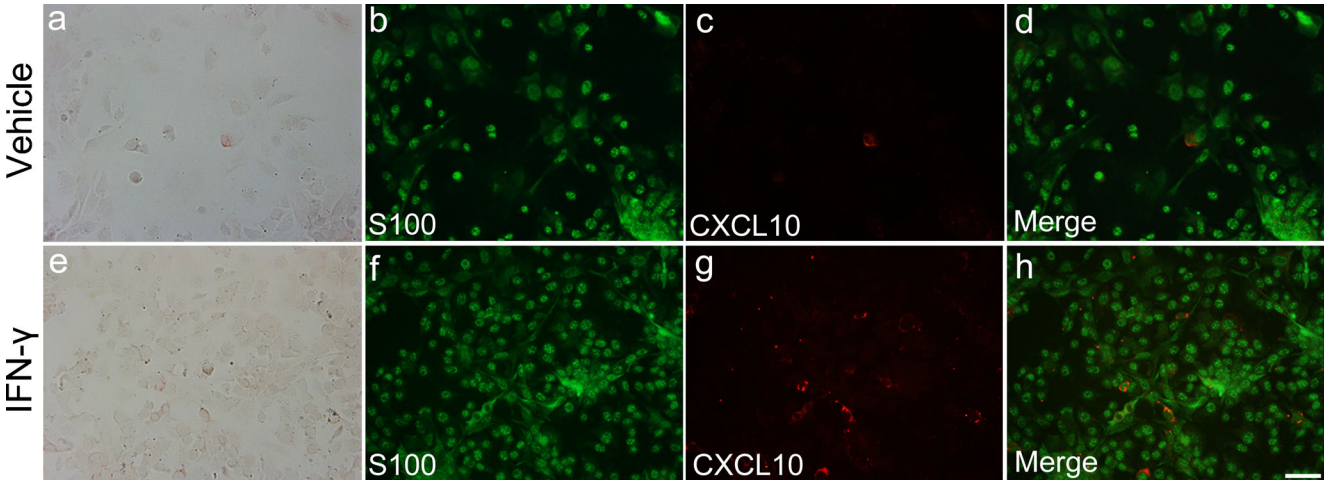

Fig. 3 Expression of CXCL10 in primary culture of the anterior pituitary cells with or without IFN-γ. Phase-contrast images (**a**, **e**), immunocytochemistry of S100 protein (*green*, S100β-positive cells) (**b**, **f**), in situ hybridization of *Cxcl10* (*red*) (**c**, **g**) and the merged images (**d**, **h**) of the vehicle (**a**–**d**) and IFN-γ treatment (**e**–**h**), respectively. *Scale bar* 50 μm

the vehicle for 27 h (Vehicle 27 h). In addition, combined treatment with IFN-γ and CRF (IFN-γ 24 h+CRF 3 h) did not show any apparent difference from the IFN-γ-stimulated *Cxcl10* mRNA level (IFN-γ 24 h+Vehicle 3 h) (Fig. 4d).

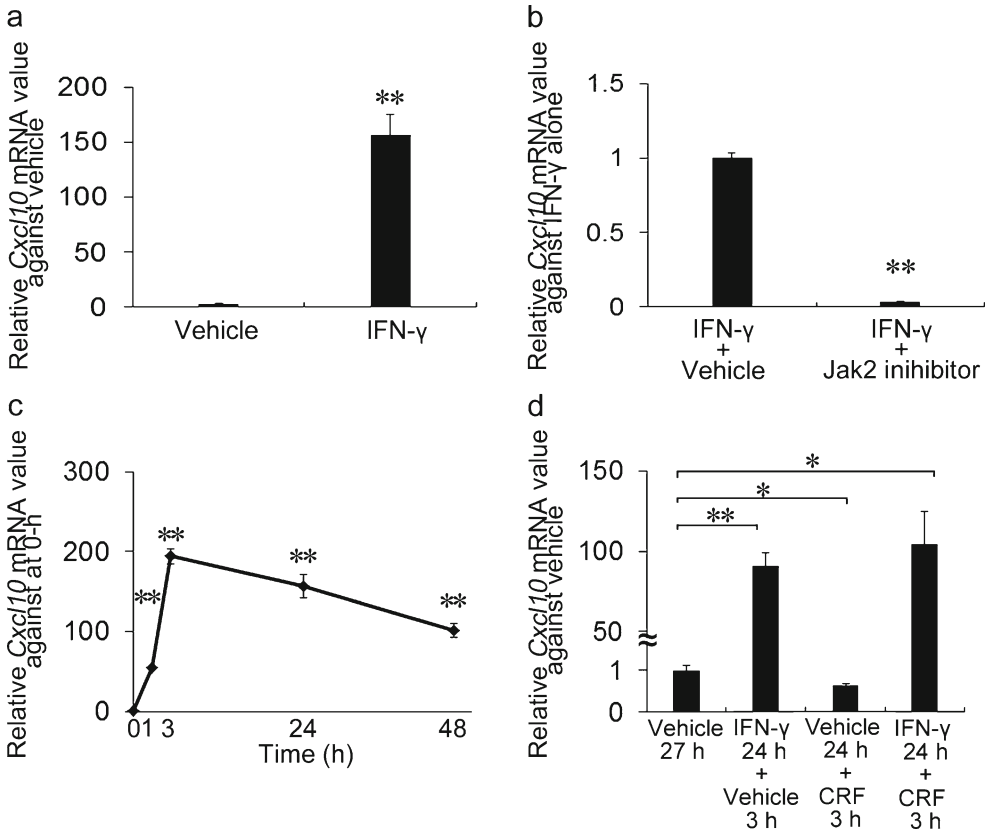

Fig. 4 Changes in *Cxcl10*-expression by IFN-γ. **a**–**d** *Cxcl10* mRNA levels were quantified by real-time PCR and their data were normalized with that of *b-actin*, an internal control (mean±SEM, $n=3$). **a** *Cxcl10* mRNA level in primary culture with IFN-γ for 24 h (IFN-γ) was calculated as a ratio against that of the vehicle value (Vehicle). **b** *Cxcl10* mRNA level with IFN-γ and Jak2 inhibitor (IFN-γ+Jak2 inhibitor) was calculated as a ratio against that of the vehicle value (IFN-γ+Vehicle). **c** *Cxcl10* mRNA levels during 48 h incubation (0, 1, 3, 24 and 48 h) with IFN-γ were measured and their levels indicated relative values against that at 0 h. The significance of the differences was determined against 0 h by Dunnett's test. **d** *Cxcl10* mRNA level was with IFN-γ for 24 h and a vehicle for 3 h (IFN-γ 24 h+Vehicle 3 h), a vehicle for 24 h and CRF (1×10^{-9} M) for 3 h (Vehicle 24 h+CRF 3 h) and IFN-γ for 24 h and CRF for 3 h (IFN-γ 24 h+CRF 3 h). Data are indicated as a ratio against that of the vehicle (Vehicle 27 h). The significance of the differences was determined by Dunnett's test. $^{**}P<0.01$, $^{*}P<0.05$

CXCL10/CXCL3 signaling mediates the effect of INF-γ

First, involvement of CXCR3 in the *Pomc*-expression was examined with different doses and time of the CXCR3 agonist. *Pomc* mRNA levels with the CXCR3 agonist were significantly ($P<0.05$) higher than that of the vehicle and 0 h, respectively (Fig. 5a, b). However, in the same conditions, the CXCR3 agonist did not produce significant changes for the mRNA levels of other anterior pituitary hormones in the primary cell culture (Fig. 5c). Then, the involvement of CXCR3 in the CRF-stimulated *Pomc*-expression was examined. Treatment with the CXCR3 agonist for 24 h and CRF for 3 h showed a significant ($P<0.05$) increase in *Pomc*-expression. The treatment with the CXCR3 agonist

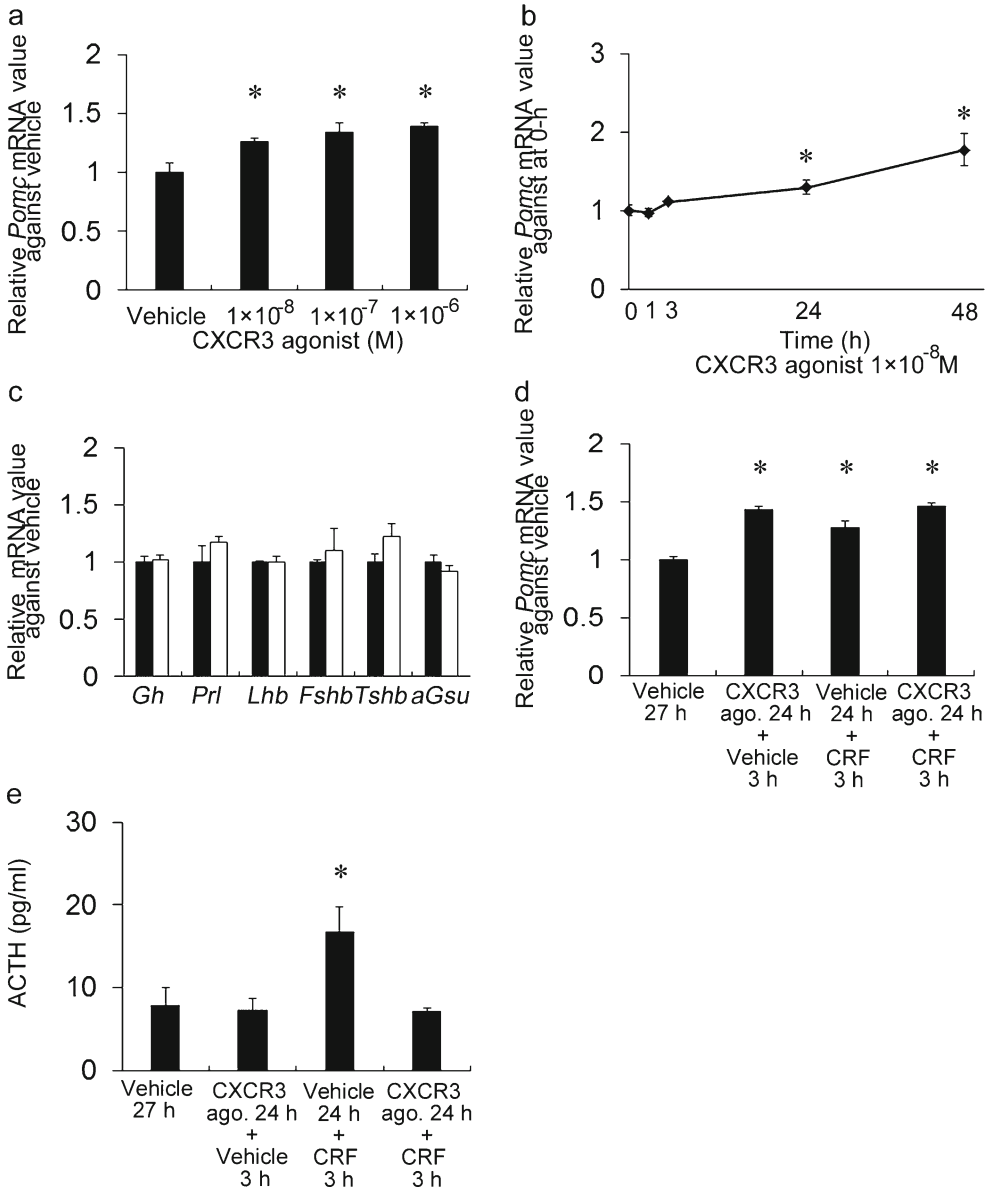

Fig. 5 Effect of the CXCR3 agonist on ACTH production. *Pomc* mRNA levels, as determined by real-time PCR, was normalized with an internal control *b-actin* (mean±SEM, $n=3$). Their levels were calculated as ratios of the vehicle (**a**, **c**, **d**) or 0 h (**b**) value. The significance of the differences was determined against the vehicle (**a**, **d**) or 0 h (**b**) value by Dunnett's test. **a** *Pomc* mRNA levels at different concentrations of the CXCR3 agonist at 1×10^{-6} M, 1×10^{-7} M and 1×10^{-8} M. **b** *Pomc* mRNA levels in incubation time (0, 1, 3, 24 and 48 h) with the CXCR3 agonist at 1×10^{-8} M. **c** mRNA levels of growth hormone (*Gh*), prolactin (*Prl*), luteinizing hormone beta subunit (*Lhb*), follicle-stimulating hormone beta subunit (*Fshb*), thyroid-stimulating hormone beta (*Tshb*) and glycoprotein hormone alpha-subunit (*aGsu*) in the presence (*open bars*) and absence (*closed bars*) of the CXCR3 agonist at 1×10^{-8} M. **d** *Pomc* mRNA levels after treatment with a vehicle for 27 h (Vehicle 27 h), CXCR3 agonist (1×10^{-8} M) for 24 h and a vehicle for 3 h (CXCR3 ago. 24 h+Vehicle 3 h), a vehicle for 24 h and CRF for 3 h (Vehicle 24 h+CRF 3 h), CXCR3 agonist for 24 h and CRF for 3 h (CXCR3 agon. 24 h+CRF 3 h). **e** ACTH concentration in cell culture media after treatment of a vehicle for 27 h (Vehicle 27 h), CXCR3 agonist (1×10^{-8} M) for 24 h and a vehicle for 3 h (CXCR3 agon. 24 h+Vehicle 3 h), a vehicle for 24 h and CRF for 3 h (Vehicle 24 h+CRF 3 h), CXCR3 agonist for 24 h and CRF for 3 h (CXCR3 agon. 24 h+CRF 3 h). *$P<0.05$

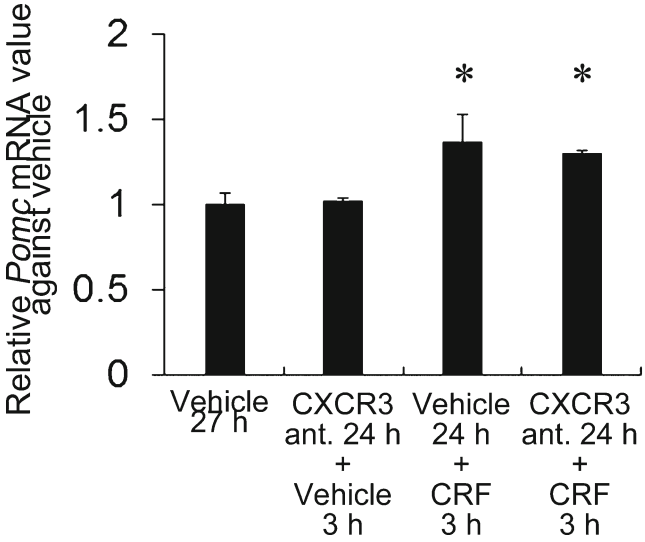

Fig. 6 Effect of CXCR3 antagonist on *Pomc*-expression. *Pomc* mRNA levels were quantified by real-time PCR and their data were normalized with that of *b-actin*, an internal control (mean±SEM, $n=3$). *Pomc* mRNA levels after treatment with CXCR3 antagonist for 24 h (CXCR3 ant. 24 h) and CRF (1×10^{-9} M) for 3 h (CRF 3 h) or a vehicle (Vehicle 27 h), respectively. *$P<0.05$

for 24 h followed by CRF for 3 h (CXCR3 ago. 24 h+CRF 3 h) did not increase with increments by single treatment (Fig. 5d). The concentration of ACTH was examined by treatment with the CXCR3 agonist and/or CRF. Treatment with the CXCR3 agonist for 24 h (CXCR3 agon. 24 h+ Vehicle 3 h) did not increase ACTH release but that with CRF significantly ($P<0.05$) increased it (Vehicle 24 h+ CRF 3 h) (Fig. 5e). Combined treatment with the CXCR3 agonist and CRF (CXCR3 agon. 24 h+CRF 3 h) induced a significant reduction of the CRF-stimulated ACTH concentration level (Fig. 5e).

CXCL10/CXCR3 signaling and CRF independently increase *Pomc* mRNA

Finally, involvement of CXCR3 in the increase of the *Pomc* mRNA level was confirmed using the CXCR3 antagonist. Treatment with the CXCR3 antagonist alone did not increase the *Pomc* mRNA level (CXCR3 ant. 24 h+Vehicle 3 h) but that with CRF alone significantly did so (Vehicle 24 h+CRF 3 h) (Fig. 6). The prior exposure of the cells to the CXCR3 antagonist did not change the subsequent response to CRH (Fig. 6).

Discussion

The present study examined whether CXCL10/CXCR3 signaling mediates a role of IFN-γ to inhibit the CRF-stimulated ACTH release. The present data showed that mediation with CXCL10/CXCR3 signaling participates in the pathway between IFN-γ and inhibition of CRF-stimulated ACTH-release (Fig. 7).

Secretion of ACTH in the anterior lobe is modulated by several factors. CRF is the most important physiologic secretagogue for ACTH, although inflammatory cytokines such as interleukin-1 (IL-1), IL-6, tumor necrosis factor and IFN-γ from immune cells, particularly monocytes, macrophage and lymphocytes, also affect its release (Karalis et al. 1991). IFN-γ is known to inhibit the CRF-stimulated ACTH release (Vankelecom et al. 1990) and to induce *Cxcl10*-expression via the JAK2/STAT signaling pathway in immune cells (Qi et al. 2009). Recently, we revealed that DC-like S100β-positive cells express CXCL10 and its receptor CXCR3 was expressed in ACTH-producing cells (Horiguchi et al. 2014b). It is also

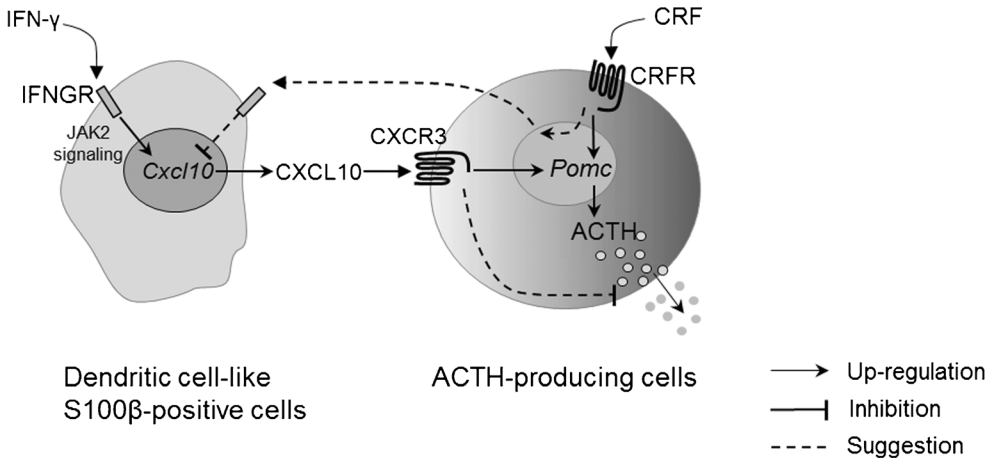

Fig. 7 Model for CXCL10/CXCR3 signaling between DC-like S100β-positive cells and ACTH-producing cells. *Solid lines* indicate the correlations by data of the present study. *Dotted lines* indicate the suggestion by data of the present study. CRF stimulates *Pomc*-expression and ACTH secretion in the ACTH-producing cells through its membrane receptor. IFN-γ induces *Cxcl10*-expression via intracellular signaling by Jak2 in DC-like S100β-positive cells. CXCL10 produced by DC-like S100β-positive cells is recognized with its receptor CXCR3. It is suggested that CXCL10/CXCR3 signaling inhibits ACTH release in ACTH-producing cells. It is also suggested that CRF down-regulates *Cxcl10*-expression in the DC-like S100β-positive cells with paracrine action

well known that S100β-positive cells in the anterior lobe play an important role in the maintenance of the functional anterior lobe by supplying inflammatory cytokines to hormone-producing cells (Vankelecom et al. 1989) and that IL-6 from S100β-positive cells inhibit ACTH production during inflammation (Gloddek et al. 2001; Horiguchi et al. 2014c). In addition, Vankelecom et al. (1992) reported that S100β-positive cells are also mediators of IFN-γ to inhibit CRF-activated ACTH-release. These observations provided us with a hypothesis that IFN-γ inhibits CRF-activated ACTH-release by mediating CXCL10/CXCR3 signaling. Hence, we examined how IFN-γ functions under CRF influence by analyzing ACTH peptide and *Pomc* mRNA levels, using primary cultured cells treated with IFN-γ or the CXCR3 agonist/antagonist and subsequent incubation with CRF. The results showed that IFN-γ induced *Cxcl10* expression via the JAK2/STAT pathway in DC-like S100β-positive cells and consequently inhibited CRH-stimulated ACTH-release by CXCR3 signaling (Fig. 7). These phenomena were similarly produced by treatment with the CXCR3 agonist. Although these data support our hypothesis, CRF-stimulated *Pomc*-expression and inhibition of ACTH-release by CXCL10/CXCR3 signaling had inconsistent results, unless ACTH production and/or release was also inhibited. We consider that CXCR3 does not increase the intracellular cAMP and Ca^{2+} concentration through the cAMP-protein kinase A pathway, which is known to induce ACTH-release (Jenks 2009). Leukemia inhibitory factor (LIF) is known to only stimulate *Pomc*-expression without increasing the cAMP concentration (Blumenfeld and Jaffe 1986). Furthermore, Sui et al. (2006) made a noteworthy report that CXCL10/CXCR3 signaling induces Ca^{2+} dysregulation in neural cells. The present findings led us to suspect that CXCL10/CXCR3 signaling inhibits the increase of intracellular cAMP and Ca^{2+} concentrations caused by CRF. We suggest that crosstalk on the cAMP-protein kinase A pathway is possibly the responsible mechanism for CXCL10/CXCR3 signaling. Nevertheless, further study is necessary to elucidate how CXCL10/CXCR3 signaling inhibits CRF-activated ACTH-release.

In the present study, we additionally examined *Cxcl10*-expression in the presence of CRF. The results showed that CRF decreases the *Cxcl10* mRNA level more than the vehicle (Fig. 4d). CRF might indirectly act on the DC-like S100β-positive cells, because the CRF receptor is mainly expressed on ACTH-producing cells and LH/FSH-producing cells (Kageyama et al. 2003). We observed that CRF-induced down-regulation of *Cxcl10* can be blocked by IFN-γ (Fig. 4d). From these data, we hypothesized that CRF-stimulated ACTH-producing cells secrete some paracrine factors that influence S100β-positive cells to inhibit *Cxcl10*-expression in the presence of endogenous CXCL10 (Fig. 7).

We recently revealed that CXCL10 is expressed in DC-like S100β-positive cells and suggested that CXCL10 inhibits CRF-activated ACTH-release for neighboring ACTH-producing cells as a paracrine factor in the anterior lobe (Horiguchi et al. 2014b). Successively, we also demonstrated that DC-like S100β-positive cells are responsive to sense increasing extracellular protons during inflammation and suppress the up-regulation of the *Pomc*-expression in response to the production of IL-6 (Horiguchi et al. 2014c). In the present study, we showed that IFN-γ inhibited CRF-stimulated ACTH-release via the CXCL10/CXCR3 axis. Taken together, our current results suppose that DC-like S100β-positive cells have a role as local modulators of hormone production by releasing various cytokines at the inflammation phase in the anterior lobe.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest that might prejudice the impartiality of this research.

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