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UROCORTIN 2 SUPPRESSES HOST RESISTANCE TO *LISTERIA MONOCYTOGENES* INFECTION VIA UP-REGULATION OF IL-10

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Abstract It is well known that corticotropin releasing factor (CRF) modulates immune response during inflammation. We investigated the effect of CRF family peptides on host resistance to *Listeria monocytogenes* infection in mice. The numbers of *L. monoctyogenes* in the organs of Ucn2-treated mice were dramatically increased compared with CRF- or Ucn-treated mice. CRF receptor type 2 is involved in the suppressive effect of Ucn2 on *L. monocytogenes* infection. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α production were decreased and interleukin (IL)-10 production was significantly increased in the spleens of Ucn2-treated mice compared with those in Ucn2-untreated control mice. The effect of Ucn2 was canceled by depleting endogenous IL-10 using anti-IL-10 monoclonal antibody and in IL-10 deficient mice. The expression and activation of signal transducers and activators of transcription 3 (STAT3) were up-regulated and the expression and activation of STAT1 were down-regulated in the spleens from Ucn2-treated mice compared with vehicle-treated mice. Moreover, suppression of TNF-α production and augmentation of IL-10 production and expression and activation of STAT3 by Ucn2 treatment were observed in heat-killed *L. monocytogenes*-stimulated macrophages. These results suggested that Urn2 suppresses host resistance to *L. monocytogenes* infection via up-regulation of IL-10 production.

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Key words: Ucn2, *Listeria monocytogenes*, IL-10

Corticotropin releasing factor (CRF) is well known as a primary mediator of the mammalian stress response[1]. CRF plays an important role at the initial step of neuroendocrine system, e.g. control behavior and autonomic adaptive changes through hypothalamo-pituitary-adrenal (HPA) axis. Centrally produced CRF shows various effects including immunosuppression on the peripheral organs and tissues. CRF also activates the sympathetic nerve system. One of the effects of CRF to the central nervous system is the induction of anxiety and motor activity as well as the inhibition of food intake and sexual behavior. The effects of CRF are mediated by two types of receptors, CRF receptor type 1 (CRFR1) and CRFR2[2-4]. CRFR1 is the main receptor of CRF, thus it mediates the principal functions of CRF. CRF produced from hypothalamus acts through CRFR1 to stimulate the synthesis and release of adrenocorticotropic hormone (ACTH) by pituitary corticotropic cells[5,6]. ACTH stimulates the production of adrenocorticosteroids in adrenal glands[5]. Several studies showed that CRF is present in the synovium of patients with rheumatoid arthritis[7], colonic mucosa of patients with ulcerative colitis[8] and inflammatory thyroid lesions[9]. Peripherally produced CRF is also found in human placenta[10,11], ovary[12], endometrium[13] and peripheral nerves[14]. In human skin, peripheral CRF is produced on site, whereas peripheral CRF is produced from nerve endings in rodents, especially in C57BL/6 mice[15].

In late years, a second mammalian member of the CRF family peptide, urocortin (Ucn), which possesses characteristics of an endogenous ligand for CRFR2[16] was identified. Ucn shares

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a considerable degree of homology with CRF. Ucn binds to CRFR1 and also binds to CRFR2 with high affinity[16]. CRFR2 is abundant in the periphery, such as skeletal muscle, spleen, gastrointestinal tract, and heart[17]. Similar to CRF, Ucn is detected in synovium of patients with rheumatoid arthritis[18], human placenta[19], fetal membranes[19], circulating leukocytes[20] and skin[21]. These findings suggest that the peripheral presence of CRF and Ucn is involved in the modulation of local immune responses during inflammation.

Recently discovered CRF family peptide, Ucn2, can bind to only CRFR2[22,23]. Ucn2 is a 38-amino acid peptide. Central administration of Ucn2 showed the reduction of food intake in the similar degree of CRF[23]. Unlike CRF, Ucn2 treatment provoked no significant changes in gross motor activity[23]. However, a peripheral effect of Ucn2 is still unclear.

There have been studies on the involvement of CRF family peptides in controlling non-infectious inflammation. CRF and Ucn suppress experimental autoimmune encephalomyelitis[24], and Ucn can inhibit production of TNF-α induced by stimulation with bacterial lipopolysaccharide (LPS)[25]. However, the effect of CRF family peptides on microbial infections is still unknown. Therefore we investigated a role of CRF family peptides, CRF, Ucn and Ucn2 in *Listeria monocytogenes* infection. Host resistance to infection with *L. monocytogenes*, an intracellular-growing bacterium, is controlled by cell-mediated immunity. Various cytokines are reportedly involved in the regulation of host resistance to *L. monocytogenes*. *L. monocytogenes* infection promotes the induction of a host T-helper 1 (Th1) response including gamma interferon (IFN-γ, which is critical in host resistance to *L. monocytogenes*[26-28]. In contrast, IL-10 plays a regulatory role in *L. monocytogenes* infection including suppression of antilisterial resistance[29-31]. In this study, we demonstrate that Ucn2 dramatically enhances the susceptibility to a sublethal infection with *L. monocytogenes* in mice and that IL-10 mediates the suppressive effect of Ucn2 on antilisterial resistance.

Data were expressed as mean±SD in bacterial numbers, cytokine titers and relative expression of STATs. Six to 9 samples were used in each figure. One-way ANOVA was performed to determine the significance of the differences of bacterial counts, cytokine titers and relative expression of STATs in the organs between the control and experimental groups, followed by Scheffe's F post hoc test. Chi-square test was carried out to determine the significance of the difference of survival rate among CRF-, Ucn- or Ucn2-treated and vehicle-treated group. At first we investigated the effect of administration of CRF family peptides on the susceptibility to a sublethal infection with *L. monocytogenes*. C57BL/6 mice and IL-10 deficient ($^{-/-}$) mice on a C57BL/6 background were used in this study. C57BL/6 mice were purchased from CLEA Japan, Inc., Tokyo, Japan. IL-10$^{-/-}$ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were used at 6- to 8-wk-old. Animals were cared for under specific-pathogen-free conditions in the Institute for Animal Experiments, Hirosaki University School of Medicine. All animal experiments in this paper were conducted in accordance with the Animal Research Ethics Committee, Hirosaki University School of Medicine and followed the Guidelines for Animal Experimentation, Hirosaki University. *L. monocytogenes* 1b 1684 cells were prepared as described previously[32]. CRF and Ucn were purchased from Peptide Institute, Osaka, Japan. Mouse Ucn2 and antisauvagine-30 (AS-30) were synthesized by Asahi Techno Glass, Chiba, Japan. Mice were administered ip with 2.5μg of CRF, Ucn or Ucn2 in PBS or PBS only 30 min before intravenous infection with 5×10^5 CFU of *L. monocytogenes*. The spleens and livers of infected animals were homogenized in PBS or

Table 1. Ucn2 suppresses host resistance to a sublethal infection with *L. monocytogenes*[a].

	Control	CRF	Ucn	Ucn2
Spleen	4.17 ± 0.42	4.04 ± 0.53	4.23 ± 0.72	6.51 ± 0.32*
Liver	4.31 ± 0.22	4.30 ± 0.61	4.47 ± 0.61	6.46 ± 1.08*

[a]Mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with 2.5 ìg of CRF, Ucn or Ucn2 30 min before infection. The numbers of viable bacteria in the spleens and livers of infected mice were determined 5 d after infection. Each result represents the mean and SD for a group of 6 to 8 mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P < 0.01$).

Table 2. The effect of Ucn2 is dose-dependent and blocked by CRFR2 antagonist[a].

Ucn2 (μg/mouse)	0	0.025	0.25	2.5	2.5	0
AS-30 (μg/mouse)	0	0	0	0	2.5	2.5
Spleen	4.43 ± 0.58	4.71 ± 0.46	5.21 ± 0.38	7.71 ± 0.21*	5.35 ± 0.31	5.42 ± 0.25
Liver	4.52 ± 0.32	5.11 ± 0.52	5.82 ± 1.61	8.21 ± 0.35*	4.71 ± 0.36	4.80 ± 0.53

[a]Mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with various doses of Ucn2 30 min before infection. Mice were injected ip with 2.5 mg of AS-30 30 min before infection. The numbers of viable bacteria in the spleens or livers were determined 5 d after infection. Each result represents the mean and SD for a group of 6 mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P < 0.01$).

1% 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfate (CHAPS, Wako Pure Chemical Industries Ltd., Osaka, Japan). The numbers of viable bacteria in the organs of infected animals were counted by plating serial 10-fold dilutions of organ homogenates on tryptic soy agar (BD Diagnosis Systems, Sparks, MD). Colonies were routinely counted 24 h later. At day 5 after infection, the bacterial numbers in the organs of mice treated with Ucn2 showed significant increment compared with those of PBS-injected mice (Table 1, $P<0.01$).

In contrast, the treatment with CRF or Ucn showed no significant change in bacterial numbers (Table 1). The survival rate of Ucn2-treated mice during *L. monocytogenes* infection was dramatically decreased compared with PBS-injected mice (Figure 1A, $P<0.05$). Again, no change was shown in survival rates when mice were treated with CRF or Ucn before infection (Figure 1A). These results showed that the treatment with Ucn2 dramatically enhanced the susceptibility to a sublethal infection with *L. monocytogenes*. Then we assessed the kinetics of *L. monocytogenes* in the organs of mice treated with Ucn2 or PBS. The numbers of bacteria were continuously increased during 5 d after infection in the spleens and livers of Ucn2-treated mice, whereas the bacterial numbers were increased up to 3 d after infection and then decreased in the organs of PBS-injected mice (Figure 1B). These results suggested that the treatment with Ucn2 suppressed host resistance to *L. monocytogenes* infection.

Next, we investigated the dose-dependency of the effect of Ucn2 on host resistance to *L. monocytogenes* infection. Mice were administered ip with various doses of Ucn2 30 min before infection. The numbers of viable bacteria in the organs of mice were counted 5 d after infection (Table 2).

As the dose of Ucn2 was decreased, the numbers of bacteria in the organs of mice were decreased. Mice were injected ip with 2.5 μg/

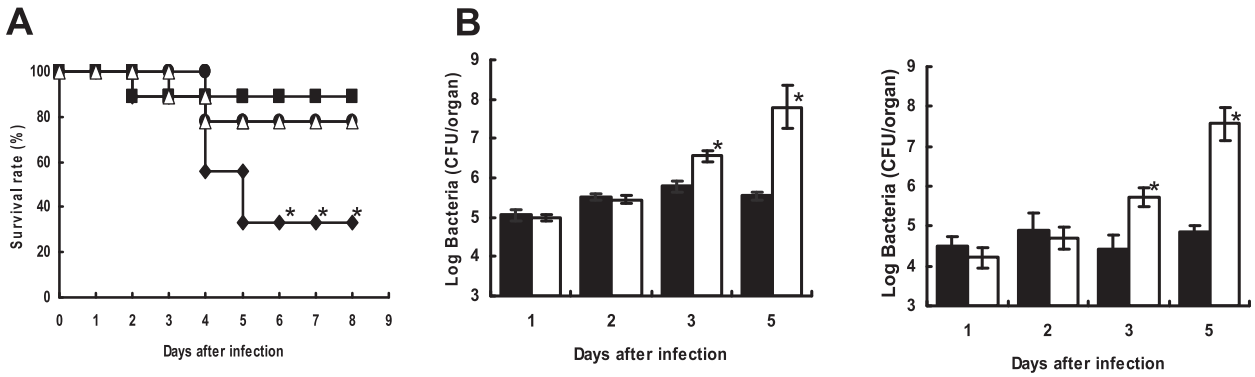

Figure 1 Ucn2 suppresses host resistance to a sublethal infection with *L. monocytogenes*. (A) Mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with 2.5μg of CRF, Ucn or Ucn2 30 min before infection. Survival rates of CRF- (circle), Ucn- (open triangle) or Ucn2 (diamond) -treated or PBS-treated control mice (square) were determined up to 10 d after infection. Each group included 9 mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P<0.05$). (B) Infection and administration of Ucn2 were carried out as described above. The numbers of viable bacteria in the spleens and livers of infected control (filled) or Ucn2-treated (open) mice were determined at various time points. Each result represents the mean and SD for a group of 6 mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P<0.01$).

250 μl of AS-30, a selective CRFR2 antagonist, in PBS 30 min prior to the injection of Ucn2 or PBS. The effect of Ucn2 treatment was canceled by the pretreatment of CRFR2 antagonist, AS-30. These results showed that the suppressive effect of Ucn2 on host resistance to *L. monocytogenes* infection was dose-dependent and mediated by CRFR2. CRFR2 reportedly distributes in the rat spleen and thymus[33]. Although CRFR2 is expressed in skin and spleen in mouse[34], cells that express CRFR2 have not been specified yet. It is possible that CRFR2 expressed on immune cells mediates the suppressive effect of Ucn2 on host resistance to L. monocytogenes infection. Ucn binds to both CRFR1 and CRFR2. However, our present results revealed that Ucn showed no significant effect on host resistance against *L. monocytogenes* infection (Table 1, Figure 1). Although it is now impossible to explain why Ucn2 but not Ucn suppresses host resistance to *L. monocytogenes* infection in spite that both Ucn and Ucn2 bind CRFR2 as a common receptor, Ucn and Ucn2 may drive different immunological pathways in listerial infection.

L. monocytogenes infection induces Th1 response in the host[27]. IFN-γ and TNF-α play protective roles and IL-10 plays a detrimental role in host resistance to *L. monocytogenes* infection[26,28-31]. IFN-γ plays a critical role in host resistance to *L. monocytogenes* infection[26,28]. IFN-γ produced by natural killer cells can activate macrophages[35]. TNF-α is also essential for primary host defense against infection with *L. monocytogenes*[36-39]. TNF-α can activate resident macrophages[37], and production of reactive oxygen and reactive nitrogen intermediates by activated macrophage is important for bactericidal activity during *L. monocytogenes* infection[40]. Therefore we assessed the production of cytokines in Ucn2-treated mice during *L. monocytogenes* infection. Mice were administered ip with 2.5μg of Ucn2 30 min before infection. The spleens were obtained from the infected mice at 1, 2, 3 or 5 d after infection, and the titers of cytokines in organ homogenates were determined (Figure 3). Titers of IFN-γ, TNF-α and IL-10 in organ homogenates and culture supernatants were determined by double sandwich ELISA as described previously[41]. The IFN-γ and TNF-α titers in the spleens from Ucn2-treated mice

Table 3. The suppressive effect of Ucn2 on host resistance to *L. monocytogenes* infection is canceled in IL-10$^{-/-}$ mice[a].

Mice	C57BL/6		IL-10$^{-/-}$	
	PBS	Ucn2	PBS	Ucn2
Spleen	5.38 ± 0.21	7.75 ± 0.88*	3.31 ± 0.18	3.08 ± 0.15
Liver	5.21 ± 0.35	7.62 ± 0.61*	3.51 ± 0.10	3.42 ± 0.21

[a]C57BL/6 mice and IL-10 deficient (IL-10$^{-/-}$) mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with 2.5 ìg of Ucn2 or PBS 30 min before infection. The numbers of viable bacteria in the spleens and livers were determined 5 d after infection. Each result represents the mean and SD for a group of six mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P < 0.01$).

Table 4. The suppressive effect of Ucn2 on host resistance to *L. monocytogenes* infection is canceled by depleting endogenous IL-10[a].

	Anti-IL-10 mAb (μg/mouse)							
	Control IgG		10		100		1000	
	PBS	Ucn2	PBS	Ucn2	PBS	Ucn2	PBS	Ucn2
Spleen	5.51 ± 0.23	7.86 ± 0.52*		5.73 ± 0.18	7.79 ± 0.62*		4.62 ± 0.21	5.18 ± 0.11 3.83 ± 0.25
Liver	4.79 ± 0.25	7.51 ± 0.41*		4.83 ± 0.23	7.45 ± 0.35*		4.01 ± 0.15	4.21 ± 0.23 3.23 ± 0.35

[a]C57BL/6 mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with 2.5 ìg of Ucn2 or PBS 30 min before infection. They were also injected ip with various doses of anti-IL-10 mAb or isotype-matched IgG 24 h before infection. The numbers of viable bacteria in the spleens and livers were determined 5 d after infection. Each result represents the mean and SD for a group of six mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P < 0.01$).

were significantly lower than those in PBS-treated mice (Figure 2A, B, P<0.01). In contrast, the IL-10 titers in the spleens from Ucn2-treated mice were significantly higher than those in the control group (Figure 2C, P<0.01). These results suggested that Ucn2 induced the up-regulation of IL-10 and the down-regulation of IFN-γ and TNF-α during *L. monocytogenes* infection in vivo. To investigate whether the suppressive effect of Ucn2 is mediated by IL-10, C57BL/6 mice and IL-10$^{-/-}$ mice were injected ip with 2.5μg of Ucn2 30 min before *L. monocytogenes* infection. The numbers of viable bacteria in the organs of mice were counted 5 d later. The Ucn2 treatment showed no effect on the bacterial numbers when administered into IL-10$^{-/-}$ mice (Table 3).

Next, we confirmed the effect of Ucn2 in mice that endogenous IL-10 had been neutralized by anti-mouse IL-10 mAb. Anti-mouse IL-10 mAb was prepared as described previously[32]. Mice were injected ip with various doses of anti-mouse IL-10 mAb or isotype-matched IgG 24 h before infection and the bacterial numbers were counted 5 d later. As expected, anti-IL-10 mAb canceled the suppressive effect of Ucn2 when mice had received 100 μg or 1000μg of anti-IL-10 mAb (Table 4).

IL-10 is known to mediate anti-inflammatory responses. IL-10 inhibits the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in cutaneous inflammation[42], and IL-10 down-regulates IL-12 production from dendritic cells[43]. Studies on experimental inflammation models have shown that IL-10 suppresses the production of TNF-α and IL-6 from macrophages, synoviocytes and T cells[44]. Moreover, IL-10 seems to mediate CD25$^+$CD4$^+$ T cell-mediated immunosuppression

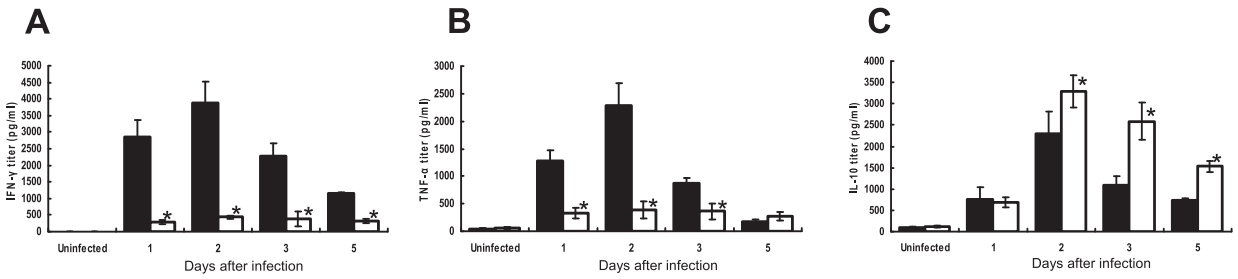

Figure 2 IFN-γ and TNF-α production is down-regulated and IL-10 production is up-regulated in the organs of Ucn2-treated mice during *L. monocytogenes* infection. Mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with 2.5 μg of Ucn2 30 min before infection. Titers of IFN-γ (A), TNF-α (B) and IL-10 (C) in the spleens of Ucn2-treated (open) or PBS-treated (filled) mice were determined at various time points. Each result represents the mean and SD for a group of 6 mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P<0.01$).

in autoimmune or inflammatory disease[45,46]. IL-10 plays a detrimental role in host resistance to *L. monocytogenes* infection[29-31,47]. In this study, IL-10 production induced by *L. monocytogenes* infection was up-regulated by Ucn2 treatment in vivo (Figure 2C). Neither CRF nor Ucn showed a significant effect on IL-10 production (data not shown). Macrophages can produce IL-10 in response to bacterial infections[48,49]. These results suggested that IL-10 played a critical role in the suppression of host resistance to *L. monocytogenes* infection by Ucn2.

Signal transducers and activators of transcription (STAT) is signal transducer of various cytokines. IFN-γ activates STAT1[50] and IL-10 activates STAT3[51]. Therefore we investigated the effect of Ucn2 on the expression and activation of STAT1 and STAT3 during a sublethal infection with *L. monocytogenes*. We prepared mRNAs and proteins from the spleens of Ucn2-treated mice 24 h and 48 h after infection. Real-time quantitative RT-PCR was carried out as previously described[52]. Our results showed that STAT1 expression was up-regulated in the spleens from PBS-treated mice 24 h after infection, whereas STAT1 expression was not up-regulated in the spleens from Ucn2-treated mice (Figure 3A). In contrast, STAT3 expression was up-regulated in the spleens from Ucn2-treated mice 24 h after infection, whereas the spleens from PBS-treated mice showed no change in the expression of STAT3 (Figure 3B). Next, we confirmed the expressions of non-phosphorylated STATs and p-STATs by western blotting. Primary antibodies for STAT1, pSTAT1, STAT3 and pSTAT3 were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The expressions of STAT1 and p-STAT1 were up-regulated in the spleens from PBS-treated mice 48 h after infection, whereas the up-regulation of neither STAT1 nor p-STAT1 expression was observed in the spleens from Ucn2-treated mice (Figure 3C). Conversely, the expressions of STAT3 and p-STAT3 were up-regulated in the spleens from Ucn2-treated mice compared with PBS-treated mice 48 h after infection (Figure 3C). These results suggested that Ucn2 up-regulated STAT3 and down-regulated STAT1 during *L. monocytogenes* infection. Although turnover of Ucn2 is very short, the up-regulation of IL-10 in the early phase of infection might cause the sustained suppression of host resistance against *L. monocytogenes* infection.

To specify the source of IL-10 during infection with *L. monocyotogenes*, we investigated the effect of Ucn2 on cytokine responses by stimulating with HKLM for 48 h in murine macrophages. Murine macrophage cell line

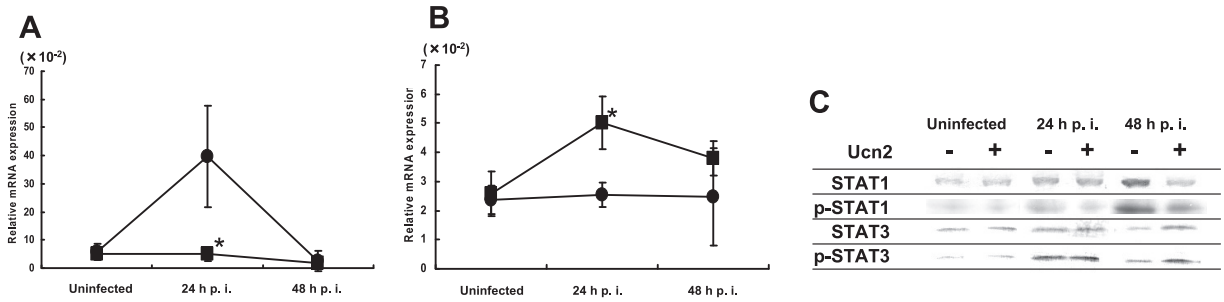

Figure 3 Expression of STAT1 and STAT3 in the spleens from Ucn2-treated mice during *L. monocytogenes* infection. Mice were infected iv with 5×10^5 CFU of *L. monocytogenes*. They were administered ip with 2.5 μg of Ucn2 30 min before infection. (A, B) The spleens from Ucn2-treated (square) or PBS-treated (circle) mice were obtained 24 h and 48 h after infection. The spleens from uninfected mice that were treated or untreated with Ucn2 were obtained as the controls. The relative expression of STAT1 (A) and STAT3 (B) in the splenic mRNA was determined by real-time quantitative RT-PCR. Each result represents the mean and SD for a group of six mice. An asterisk indicates that the value is significantly different from the PBS-treated control group ($P<0.01$). (C) The spleens from Ucn2-treated or PBS-treated mice were obtained 24 h and 48 h after infection and those from uninfected mice treated or untreated with Ucn2 were obtained as the controls. Expression of STAT1, p-STAT1 (p-STAT1), STAT3 and p-STAT3 were detected by western blotting.

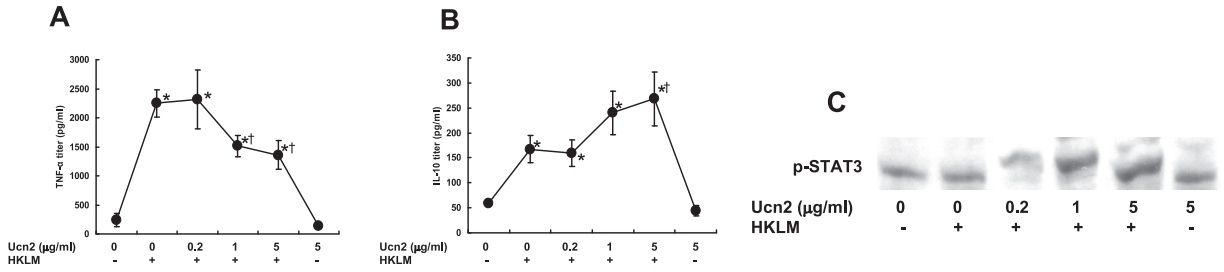

Figure 4 TNF-α production is down-regulated and IL-10 production is up-regulated by Ucn2-treatment in response to HKLM in vitro. RAW264.7 cells were stimulated with HKLM in the absence or presence of Ucn2. Titers of TNF-α (A) and IL-10 (B) in the culture supernatants were determined by ELISA. Each result represents the mean and SD for a group of 6 samples. An asterisk indicates that value is significantly different from Ucn2- and HKLM-untreated group ($P<0.01$). A cross indicates that values are significantly different from Ucn2-untreated and HKLM-stimulated group ($P<0.05$). (C) Proteins from cultured cells were prepared and detection of p-STAT3 was carried out by western blotting.

RAW264.7 was purchased from Dainippon pharmaceutical Co. Ltd., Osaka, Japan and were cultured in Dulbecco's modified Eagle medium (Nissui) supplemented with 10% of fetal bovine serum (JRH Biosciences, Lenexa, KS), 3% of L-glutamine (Wako). Cells at 2×10^6/ml were stimulated with heat-killed *L. monocytogenes* (HKLM) at 2×10^7/ml in the presence or absence of Ucn2 for 48 h. Cytokine titers in the culture supernatants were determined by ELISA (Figure 4A, B). Treatment of Ucn2 significantly decreased the TNF-α titers (Figure 4A) and increased the IL-10 titers (Figure 4B) in a dose-dependent manner. IFN-γ was not detected in any group of culture (data not shown). To investigate the activation of STAT3 by the increased IL-10, p-STAT3 was detected in Ucn2-treated RAW264.7 cells by western blotting. Ucn2 treatment up-regulated the expression of p-STAT3 dose-dependently (Figure 4C). These results suggested that Ucn2 treatment induced up-regulation of IL-10 and down-regulation of TNF-α in vitro.

In conclusion, our present results demonstrated

that CRF family peptide Ucn2 converts a sublethal infection with *L. monocytogenes* to the lethal infection in mice through up-regulation of IL-10. Our study supported that the existence of close interaction between endocrine system and immune system during bacterial infection.

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