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The effect of centaurein on interferon-γ expression and *Listeria* infection in mice

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Abstract

We previously found that centaurein enhanced IFN-γ transcription in T cells. Here, we demonstrate that centaurein increased the IFN-γ expression in T and NK cells and the serum IFN-γ level in mice. Centaurein elevated the transcription of T-bet but not GATA-3, which is consistent with its effect on that of IFN-γ but not IL-4. Additionally, centaurein effectively protected mice against *Listeria* infection. Moreover, centaurein per se or in combination with antibiotics could treat *Listeria* infection. Our mechanistic studies suggest that centaurein augments IFN-γ expression via a transcriptional up-regulation of T-bet and that centaurein protects against or treats *Listeria* infection via a modulation of IFN-γ expression.

Keywords: Centaurein; IFN-γ; *Listeria*; Flavonoid

Introduction

A characteristic of intracellular bacteria including *Listeria*, *Brucella*, *Legionella*, *Francisella* and *Mycobacterium* is their ability to survive and replicate within phagocytes or other cells and therefore escape from the host immune bacterial defense (Kaufmann, 1993). *Listeria* causes listeriosis in animals and humans. Antibiotics (ampicillin, vancomycin, etc.) and immunotherapeutics (IFN-γ, IL-1, IL-2, etc.) are frequently used as anti-infective agents to combat *Listeria* and other intracellular bacteria (Haak-Frendscho et al., 1989; Kurtz et al., 1989; Roll et al., 1990; Calder, 1997; Jones et al., 1997). Unfortunately, antibiotic-resistant *Listeria* species have been discovered (MacGowen et al., 1990; Facinelli et al., 1991). To overcome this antibiotic resistance, the development of immunomodulatory therapeutics for microbes such as *Listeria* is urgently needed because immunomodulatory therapeutics, in contrast with antibiotics, cannot lead to antibiotic-resistant bacteria (Buchwald and Pirofski, 2003). Phagocytes such as macrophages modulate innate immunity to *Listeria*, whereas T cells ($CD4^+$ and $CD8^+$) modulate adaptive immunity. As well, antibodies also seem to be involved in *Listeria* clearance (Edelson and Unanue, 2000).

The regulation of IFN-γ expression in T cells involves specific nuclear factors such as T-bet, NFκB, NFAT, STAT and etc. (Xu et al., 1996; Ye et al., 1996; Sica et al., 1997; Rengarajan et al., 2000). T-bet is a crucial nuclear factor known to up-regulate IFN-γ expression but down-regulate IL-4 expression by sequestering GATA-3 from the binding of GATA-3 to the IL-4 promoter (Hwang et al., 2005). Most IFN-γ production in vertebrates comes from T cells ($CD4^+$ and

Abbreviations: IFN, interferon; IL, interleukin; PBS, phosphate buffered saline; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; T-bet, T-box expressed in T cells; GATA-3, GATA binding protein 3; FACS, fluorescence-activated cell sorting; GFP, green fluorescence protein.

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$CD8^+$) and NK cells. IFN-γ is known to activate macrophages, leading to *Listeria* destruction in cell or animal models (Kaufmann et al., 1983). IFN-γ gene disruption impairs host resistance to *Listeria* infection in mice. Accordingly, defects in the IFN-γ receptor pathway are clinically associated with the susceptibility to diseases caused by intracellular pathogens such as *Mycobacteria*, *Salmonella* and some viruses (Dorman and Holland, 2000).

B. pilosa, an Asteraceae plant, has traditionally been used as a folk medicine for bacterial infections (Rabe and van Staden, 1997). However, little is known about the role of the bioactive compounds of *B. pilosa* in anti-microbial activity (Geissberger and Sequin, 1991). Previously, we had isolated centaurein, a flavonoid, from *B. pilosa* for the first time (Chiang et al., 2004) and found its ability to stimulate IFN-γ production. In the current study, we first confirmed the role of centaurein in IFN-γ production in specific immune cells and in mice. We next studied the likely mechanism by which centaurein eradicates *Listeria* infection in mice. We showed that an immune modulator such as centaurein per se or in conjunction with antibiotics in mice can protect against or treat an intracellular infectious pathogen. We also provide scientific evidence that centaurein, isolated from *B. pilosa* plant, can prevent infection of *Listeria*, an intracellular bacterium, and may explain the reason why this plant is used as an anti-microbial folk medicine.

Methods

Chemicals, cells and animals. PHA, PMA and ionomycin were purchased from Sigma. Jurkat cells (a T cell line) were obtained from American Type Culture Collection. *Listeria monocytogenes* (BCRC 15386) was obtained from Bioresource Collection and Research Center (Taiwan). Human cord blood cells were obtained from Taipei Medical University Hospital. C57BL/6J mice (National Laboratory Animal Center, Taiwan) and IFN-γ-knockout mice on a C57BL/6J background (Jackson Laboratory) were maintained and handled according to the guidelines of Academia Sinica Institutional Animal Care and Utilization Committee. Female or male mice with similar body weight, 6- to 8-week-old, were used in all our experiments.

Centaurein. Centaurein ($C_{24}H_{26}O_{13}$, MW = 522.5 Da) with more than 98% purity was prepared from *B. pilosa* and structurally determined as previously published (Chiang et al., 2004).

Plasmids. pGATA-3-Luc and pT-bet-Luc containing a GATA-3 promoter and a T-bet promoter linked to a luciferase gene, respectively, were prepared as previously described (Chang et al., 2005). An internal control vector, pRL-TK, was purchased from Promega. Plasmid plmo-GFP, composed of a *Listeria*-specific lmo2219 promoter and a GFP reporter gene, was previously described (Wilson et al., 2001).

Electroporation and luciferase assay. Jurkat cells were electroporated with pT-bet-Luc or pGATA-3-Luc reporter constructs together with the pRL-TK plasmid. After a 2-h recovery, the cells were incubated with vehicle, PHA at 1 μg/ml or centaurein at 100 μg/ml for 24 h. Following cell lysis, 10 μg of the cell lysate underwent dual luciferase reporter assays (Promega). The ratio of firefly luciferase activity to *Renilla* luciferase activity in each lysate was determined as previously published (Yang et al., 2001).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Human umbilical $CD4^+$ T cells, purified with MACS (Miltenyi Biotech), were treated with 1 μg/ml PHA, centaurein (100 μg/ml) or DMSO (vehicle control) for 24 h. Total RNAs were isolated with Trizol solution (Life Technologies) and converted into cDNAs with use of a first-strand cDNA synthesis kit (Amersham Biosciences). Total cDNAs were used as templates for PCR performed in the thermocycler at 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min for 27 cycles with following specific primer sets: IFN-γ, ACGAGATGACTTCGAAAAGCTG and TTTAGCTGCTGGCGACAGTTC; T-bet, CTAAAGCTCACAAACAACAAGG and AGAAGCGGCTGGGAACAGGAT; GATA-3, GTCCTGTGCGAACTGTCAGA and TAAACGAGCTGTTCTTGGGG; IL-4, GCGATATCACCTTACAGGAG and TCAGCTCGAACACTTTGAATAT; and GAPDH, ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA.

IFN-γ detection in splenocytes and mice. Mouse splenocytes were treated with PBS (vehicle control) or centaurein (100 μg/ml) for 24 h, followed by PMA/ionomycin treatment for 4 h plus GolgiPlug treatment (BD Biosciences) for an additional 2 h. The cells were stained with CD4 (BioLegend), CD8 (Caltag) or NK (BioLegend) antibodies. Following intracellular staining with anti-IFN-γ antibody (BioLegend), the cells underwent fluorescence-activated cell sorting (FACS) analysis. To measure the serum IFN-γ level, C57BL/6J mice were intraperitoneally injected with centaurein at 20 μg. The serum IFN-γ concentration was determined using an ELISA kit (eBioscience).

Listeria detection in macrophages. For serum concentration, the sera (1.5 ml) from C57BL/6J mice or IFN-γ-knockout mice, already treated with PBS or centaurein for 24 h, were concentrated 3-fold using SpeedVac® concentrators. For macrophage preparation, resident exudate macrophages from C57BL/6J mice were harvested by peritoneal lavage with 5 ml of ice-cold PBS, followed by centrifugation (Andrade et al., 2005).

Peritoneal macrophages (2×10^5 cells) were incubated with 0.5 ml of the concentrated sera or a volume-matched mixture of anti-IFN-γ antibody (1 μg) and the serum of C57BL/6J mice with a 24-h injection of centaurein for 16 h. The cells were incubated with GFP-expressing *Listeria* (5×10^6 CFU), which was already transformed with plmo-GFP plasmid, for 30 min. After extensive washing, the cells were analyzed with FACS and fluorescent microscopy (0 h) or subjected to an additional 6-h incubation with gentamicin (40 μg/ml) and analyzed with FACS and fluorescent microscopy.

Listeria challenge. For prevention study, wild-type or IFN-$\gamma^{-/-}$ C57BL/6J mice were pretreated with vehicle, centaurein (a single dose at 10 or 20 μg/mouse) or ampicillin (1000 μg/mouse, 2 times per day for 3 days). After 24 h, mice were intraperitoneally injected with *Listeria* (1×10^6 CFU). For treatment study, 6- to 8-week-old C57BL/6J mice were intraperitoneally injected with *Listeria* (2×10^6 CFU). After 12 h, mice were treated with vehicle, centaurein (a single dose at 20 μg/mouse), ampicillin (5 or 30 μg/mouse, 2 times per day for 3 days) or a combination of centaurein (a single dose at 20 μg/mouse) and ampicillin (5 μg/mouse, 2 times per day for 3 days). The animals were then observed every day for determination of mortality.

Statistical analysis. Data from three independent experiments or more are pooled and expressed as an average of all the experiments with standard error of the mean (mean ± SE). For the survival data, the log-rank test was used to determine if a group was statistically significant from the control group. For the other experiments, the Student's *t*-test was performed to determine whether there was a significant difference between treatment groups and mock control groups. $P<0.05$ (*) was considered to be statistically significant.

Results

Centaurein elevates IFN-γ expression in T cells and NK cells

Centaurein, isolated from *B. pilosa* (Fig. 1), was used to study its role in T cell function. Previous data showed that centaurein up-regulated IFN-γ transcription in Jurkat cells (unpublished data). To understand whether centaurein could