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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

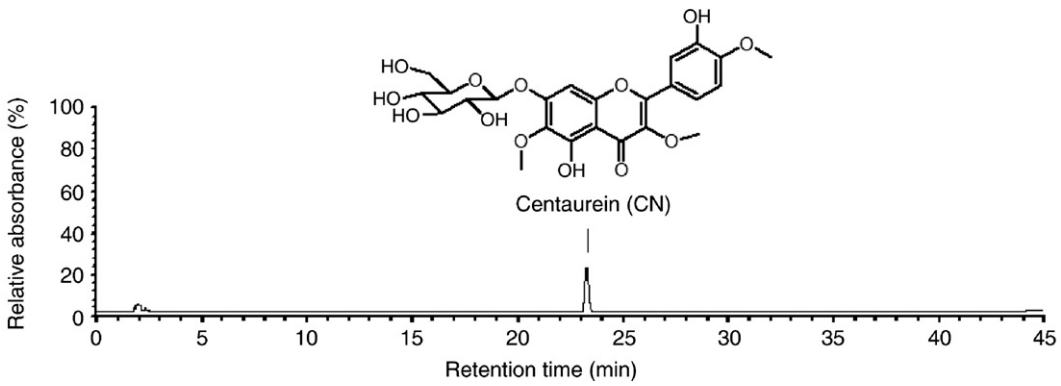

Fig. 1. Chemical structure and HPLC-determined purity of centaurein (CN). The HPLC profile was performed using an RP-18 column and detected with a UV detector at 254 nm. The structure and corresponding peak of centaurein (CN) were indicated.

also induce IFN-γ production in primary T cells as well as other immune cells, we incubated splenocytes with vehicle or centaurein and then assessed the IFN-γ production using flow cytometry. Centaurein augmented IFN-γ expression from 7% to 20% in $CD4^+$ T cells, from 21% to 41% in $CD8^+$ T cells and from 6% to 17% in NK cells (Fig. 2). These results suggest that centaurein is a universal stimulator for these IFN-γ-producing cells.

Centaurein up-regulates the transcription of T-bet and IFN-γ but not GATA-3 and IL-4 in T cells

Since centaurein induced IFN-γ expression, we wanted to test whether centaurein could regulate the transcription of T-bet as well as GATA-3 using luciferase reporter assays. Our results showed that centaurein specifically enhanced T-bet transcription by 4-fold but had no significant effect on GATA-3

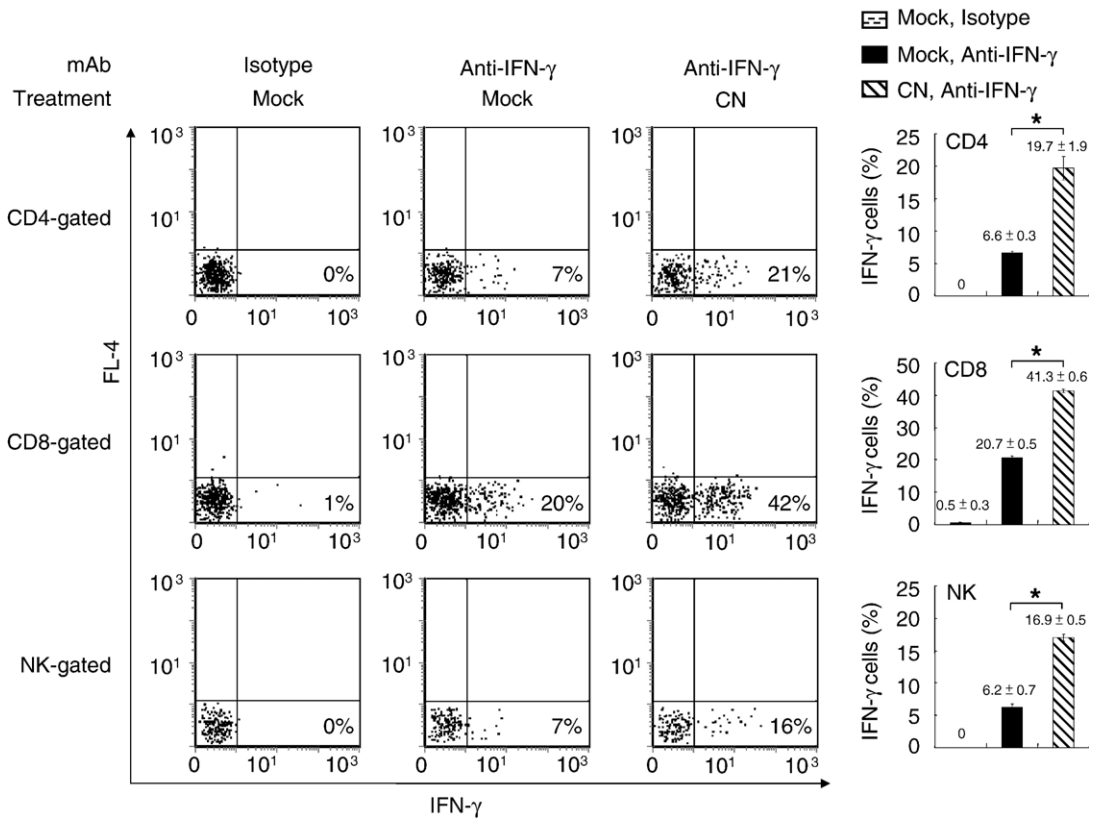

Fig. 2. Centaurein stimulates IFN-γ production in T and NK cells. Mouse splenocytes were incubated with PBS (Mock) or centaurein at 100 μg/ml (CN) for 24 h, followed by PMA/ionomycin treatment for 4 h and GolgiPlug treatment for an additional 2 h. Cells stained with isotypic mAbs or anti-IFN-γ-PE together with anti-CD4, anti-CD8 and anti-pan NK (DX5) were subjected to FACS analysis. After cells were gated, the amount of IFN-γ-producing cells was counted. The FACS profiles are representative of 3 independent experiments (left panel). The histograms (right panel) show an average of 3 independent experiments with standard error of the mean (mean ± SE). Statistical analyses were performed using Student's *t*-test. $P < 0.05$ (*) was considered to be statistically significant.

transcription in Jurkat cells (Fig. 3A). To confirm this situation in primary cells, we treated human $CD4^+$ T cells with vehicle, PHA or centaurein. RT-PCR analysis showed that centaurein increased the expression of T-bet by ~5-fold. However, centaurein had, if any, a marginal effect on that of GATA-3 (Fig. 3B). Consistently, we found that centaurein treatment also enhanced the transcription of IFN-γ but not IL-4 (Fig. 3B). These data suggest that centaurein specifically up-regulates IFN-γ expression, and T-bet is at least a nuclear factor for this IFN-γ up-regulation.

Centaurein augments the serum IFN-γ level in mice

Centaurein stimulated IFN-γ production in the main IFN-γ-producing cells (T and NK cells). Thus, we wanted to evaluate this situation in mice. C57BL/6J mice were injected intraperitoneally with 20 μg of centaurein. ELISA assays revealed that centaurein augmented the serum IFN-γ level in mice, which peaked 24 h post-injection (Fig. 4A).

IFN-γ is known to activate macrophages, resulting in *Listeria* clearance within macrophages. We next tested whether the sera from the centaurein-treated mice could activate the macrophage-mediated *Listeria* clearance. We treated mouse peritoneal exudate macrophages with the aforesaid sera, followed by incubation with GFP-expressing *Listeria*. Green fluorescence was used as an indication to monitor the quantity of GFP-expressing *Listeria* inside macrophages. FACS analysis showed that no matter what serum was incubated with the macrophages for 30 min, GFP-expressing *Listeria* was detected inside 24% of macrophages (0 h, Fig. 4B). Consistently, fluorescent images showed around 8 bacteria insides macrophages in each treatment (0 h, Fig. 4C and data not shown). These data suggest that serum treatment does not affect the initial entry of *Listeria* into macrophages. We next evaluated *Listeria* clearance inside macrophages already treated with various serum treatment for an additional 6 h. Of note, the percentage of *Listeria*-infected macrophages, pretreated with the serum of control C57BL mice, was around 46%, similar to that of *Listeria*-infected macrophages, pretreated with the serum of centaurein-treated IFN-γ knockout mice (6 h, Fig. 4B). In contrast, the percentage of *Listeria*-infected macrophages, pretreated with the serum of centaurein-treated C57BL mice, decreased to 15%. However, that of *Listeria*-infected macrophages, pretreated with the serum of centaurein-treated C57BL mice in combination with IFN-γ-neutralizing antibody, was 34% (6 h, Fig. 4B). Accordingly, fluorescent images indicated that the number of *Listeria* insides macrophages, pretreated with the control serum of C57BL mice, was 19 *Listeria*, similar to that of *Listeria* inside macrophages, pretreated with the serum of centaurein-treated IFN-γ knockout mice (6 h,

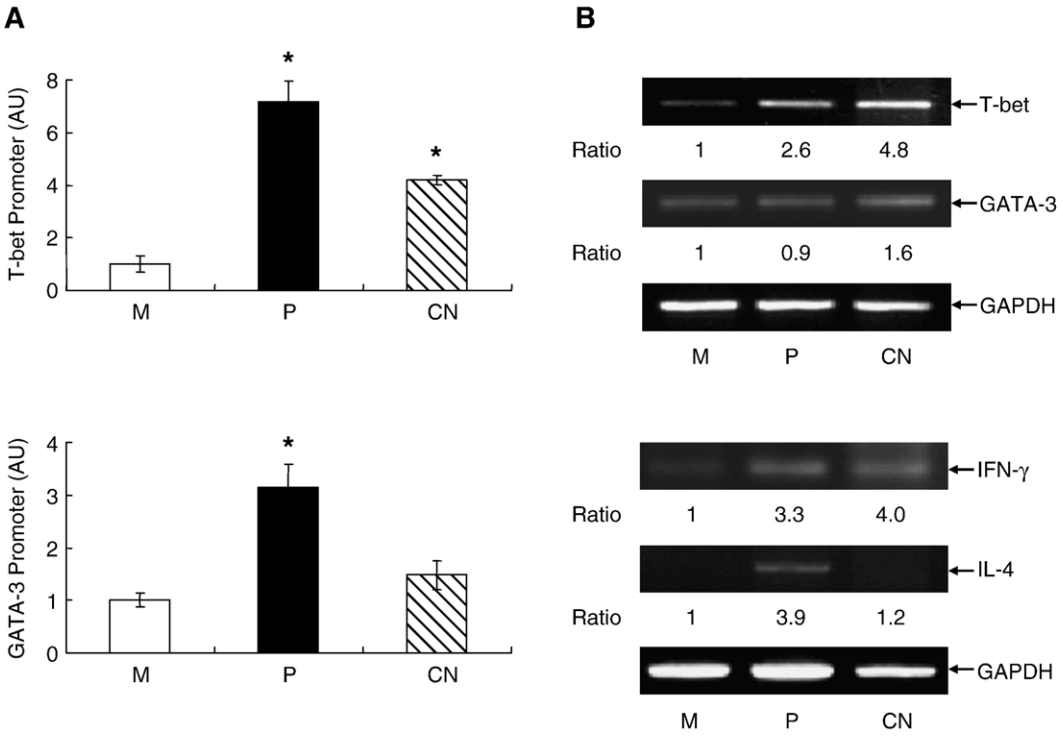

Fig. 3. Centaurein increases the gene expression of T-bet and IFN-γ but not GATA-3 and IL-4 in T cells. (A) Jurkat cells electroporated with pT-bet-Luc (top panel) or pGATA-3-Luc (bottom panel) plus pRL-TK were stimulated with vehicle (M), PHA (P, 1 μg/ml) or centaurein (CN, 100 μg/ml). The induction fold (AU) was obtained from the ratio of luciferase activity to that of *Renilla* luciferase in each group. The histograms represent an average of 3 independent experiments with standard error of the mean (mean±SE). $P<0.05$ (*) is considered to be statistically significant based on Student's *t*-test. (B) Human umbilical T helper cells were treated with vehicle (M), PHA (P, 1 μg/ml) or centaurein (CN, 100 μg/ml) for 24 h. Total RNAs from various treatments underwent RT-PCR analysis. PCR products were analyzed using DNA gel electrophoresis and ethidium bromide. Arbitrary units (AU) were obtained from the ratio of the signal of IFN-γ, IL-4, T-bet or GATA-3 bands to that of the corresponding GAPDH bands. The data are representative of 3 independent experiments.

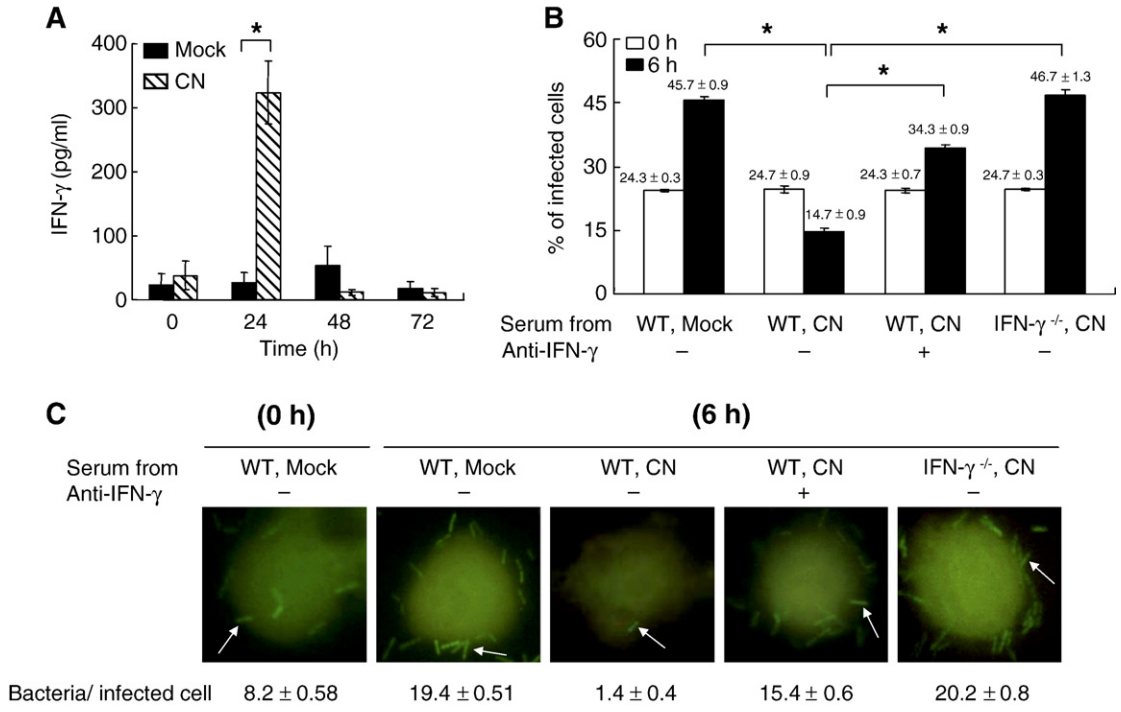

Fig. 4. Centaurein elevates the serum levels of IFN-γ in mice and decreases *Listeria* level inside macrophages. (A) Blood samples from 3 mice per group were collected from C57BL/6J mice injected with PBS (Mock) or centaurein (CN, 20 μg/mouse) at 0, 24, 48 and 72 h post-injection. The serum concentration of IFN-γ was measured using an ELISA kit. The bar graphs represent an average of 3 independent experiments with standard error of the mean (mean±SE). $P<0.05$ (*) is considered to be statistically significant based on Student's *t*-test. (B) Mouse resident peritoneal macrophages (2×10^5) were incubated with the serum of C57BL/6J mice with a 24-h injection of PBS (WT, Mock) or centaurein (WT, CN), a mixture of anti-IFN-γ antibody and the serum of C57BL/6J mice with a 24-h injection of centaurein (WT, CN+anti-IFN-γ) and the serum from IFN-γ knockout mice with a 24-h injection of centaurein (IFN-$\gamma^{-/-}$, CN). After a 16-h incubation with the sera, the cells were infected with GFP-expressing *Listeria* for 30 min. Following extensive washing, the cells either started to undergo FACS analysis (0 h) or an additional 6-h culture (6 h), followed by FACS analysis. The histograms show an average of 3 independent experiments with an error bar (mean±SE), indicating the percentage of macrophages with GFP-expressing *Listeria*. (C) The *Listeria* inside macrophages was visualized using a fluorescent microscope. The average bacteria number per infected macrophage is expressed as mean±SE. White arrows show the place where *Listeria* is located.

Fig. 4C). The number of *Listeria* insides macrophages, pretreated with the serum of centaurein-treated C57BL mice, was 1. However, the number of *Listeria* insides macrophages, pretreated with IFN-γ-neutralizing antibody and the serum of the centaurein-treated mice, was 15 (6 h, Fig. 4C). Taken together, our data showed that sera with elevated IFN-γ levels from the centaurein-treated mice could clear *Listeria* in macrophages more efficiently than control sera.

Centaurein protects mice against Listeria infection through IFN-γ

Since centaurein up-regulated IFN-γ production and thus, macrophage activation, we next examined whether centaurein could protect mice against *Listeria* infection. Our results showed that centaurein protected mice against *Listeria* infection in a dose-dependent fashion (Fig. 5A). We hypothesized that centaurein protected mice against *Listeria* infection via IFN-γ production. To test this hypothesis, we assessed whether IFN-γ-knockout mice challenged with *Listeria* could be rescued by centaurein. Our results demonstrated that centaurein lost its ability to protect IFN-γ-knockout mice against *Listeria* infection. In contrast, ampicillin at 1000 μg/ml could protect against *Listeria* infection (Fig. 5B). Thus, these results showed that centaurein cannot protect mice without IFN-γ against *Listeria* infection.

Centaurein treats Listeria infection in mice alone or in combination with antibiotics

Since centaurein protected mice against *Listeria* infection, we next investigated whether centaurein could be used to treat mice already infected with *Listeria*. Our data showed that centaurein treatment (20 μg/mouse) rescued 30% of the mice infected with a lethal dose of *Listeria* (2×10^6 CFU) (Fig. 6). In contrast, ampicillin (5 μg ampicillin, 2 times per day for 3 days) rescued 50% of the mice that received the lethal dose of *Listeria*. Interestingly, a combination of ampicillin (5 μg/mouse, 2 times/day for 3 days) and centaurein (20 μg/mouse) rescued 70% of the mice that received the lethal dose of *Listeria*. Importantly, this combination, better than ampicillin or centaurein alone, had an additive effect on the mice already infected with *Listeria* (Fig. 6). A high dosage of ampicillin (30 μg/mouse) fully treated *Listeria* infection (Fig. 6) as published elsewhere (van Ogtrop et al., 1992). Overall, results suggest that centaurein protects against and treats *Listeria* infection in mice via up-regulation of IFN-γ and macrophage activation.

Discussion

The annual incidence of listeriosis in humans is rare, ~0.4 to 7.4 per million people (Calder, 1997). But among those who develop listeriosis, the death rate is high (~25 to 30%) (Rouquette and Berche, 1996). Antibiotic drugs are currently used for *Listeria* infection. However, antibiotic-resistant *Listeria* strains have been increasingly reported (MacGowen et al., 1990; Facinelli et al., 1991). Therefore, it is necessary to develop new therapeutics for treating *Listeria* infection.

Several herbal medicines have been reported to have a protective effect against *Listeria* infection in mice. For example, the crude extract of a Chinese medicine, Bu-Zhong-Yi-Qi-Tang, up-regulates IFN-γ production and, therefore, eradicates *Listeria* infection in mice (Yamaoka et al., 1998; Yamaoka et al., 2000; Yamaoka et al., 2001). Some polysaccharides isolated from the *Echinacea purpurea* plant protect mice against *Listeria* infection (Steinmuller et al., 1993). Unfortunately, the detailed pharmacological mechanisms and/or the bioactive compounds in the above systems remain to be elucidated.

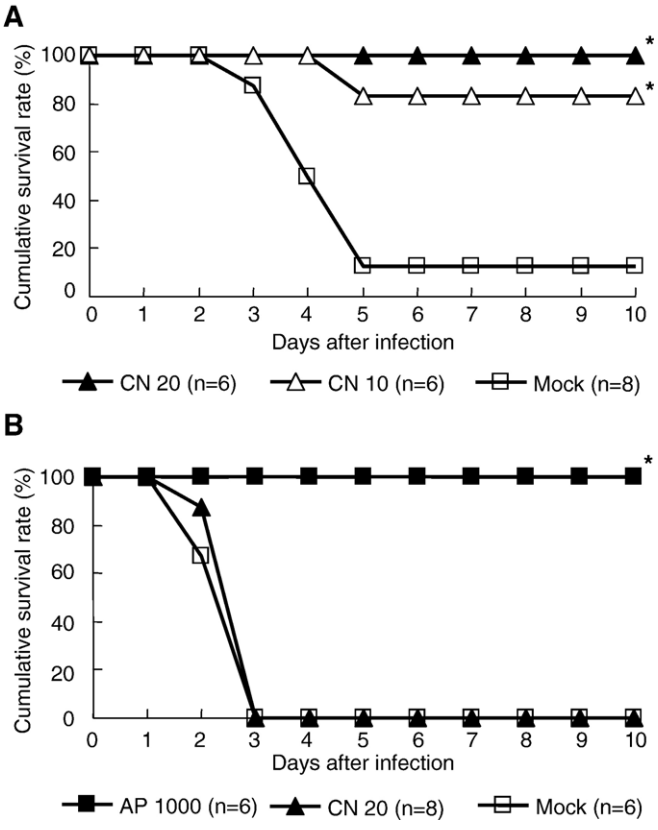

Fig. 5. IFN-γ gene disruption abolishes the centaurein-mediated *Listeria* prevention in mice. (A) 6- to 8-week-old C57BL/6J mice received a single intraperitoneal injection of PBS (Mock, □) and centaurein at 10 μg (CN 10, △) or 20 μg (CN 20, ▲) per mouse. After 24 h, the mice were intraperitoneally challenged with *Listeria* (1×10^6 CFU). The cumulative survival rates of mice were determined. (B) 6- to 8-week-old IFN-$\gamma^{-/-}$ C57BL/6J mice received a single intraperitoneal injection of PBS (Mock, □), centaurein at 20 μg (CN 20, ▲) or ampicillin at 1000 μg for 3 days, twice a day (AP 1000, ■). After 24 h, the mice were intraperitoneally challenged with *Listeria* (1×10^6 CFU). The cumulative survival rates of mice were determined. Mouse numbers per group are indicated in parenthesis. $P<0.05$ (*) is significantly different from controls based on log-rank test.

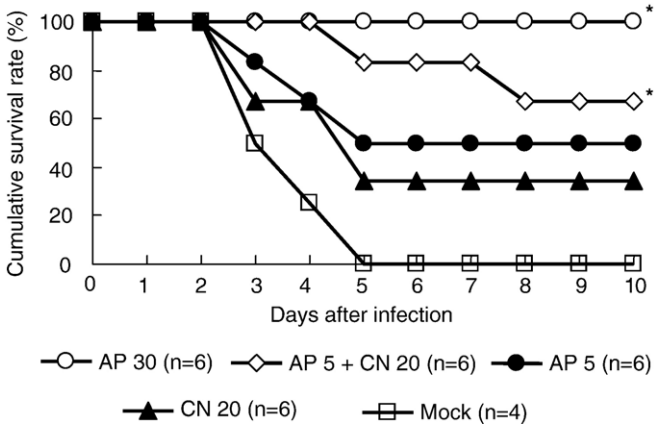

Fig. 6. Centaurein per se or in combination with ampicillin can treat *Listeria* infection in mice. 6- to 8-week-old C57BL/6J mice were intraperitoneally challenged with *Listeria* on day 0. After 12 h, the mice received PBS (Mock, □), ampicillin at 5 (AP 5, ●) or 30 (AP 30, ○) μg, centaurein at 20 μg (CN 20, ▲), or ampicillin at 5 μg plus centaurein at 20 μg (AP 5+CN 20, ◊). Ampicillin was given twice a day for 3 days, whereas centaurein was given in a single dose. The cumulative survival rates of mice were determined. Mouse numbers per group are indicated in parenthesis. $P<0.05$ (*) is significantly different from controls based on log-rank test.

Centaurein was previously isolated from a plant (*B. pilosa*) with a folk tradition of anti-bacterial use (Chiang et al., 2004). Here, we, for the first time, found that NK and T cells increase IFN-γ production in response to centaurein. This IFN-γ increase was also observed in mice. The fact that centaurein up-regulates T-bet expression suggests a molecular mechanism by which centaurein mediates IFN-γ expression via an IFN-γ regulator, T-bet. In the present paper, we confirm that centaurein can protect against or treat *Listeria* infection in mice via up-regulation of IFN-γ and macrophage activation.

IFN-γ can activate the macrophage-mediated killing of intracellular pathogens. Both IFN-γ and macrophage activations are pivotal for *Listeria* eradication in cell or animal models (Hubel et al., 2002). IFN-γ, alone or in conjunction with antimicrobial agents, is also reported to clinically treat patients infected with an intracellular microbe, *Mycobacteria* (Hubel et al., 2002). Of note, IFN-γ showed a promising effect on the adjunctive treatment of multidrug-resistant *Mycobacteria* in patients (Hubel et al., 2002). We report that centaurein alone or in combination with antibiotics protects against and treats *Listeria* infection via up-regulation of IFN-γ. In addition, the anti-bacterial susceptibility test showed that the minimal inhibitory concentration of centaurein for *Listeria* is over 200 μg/ml (data not shown), indicating that centaurein itself did not show any significant bacteriocidal or bacteriostatic activity against *Listeria* because of its high minimal inhibitory concentration. On the contrary, centaurein can prevent and treat *Listeria* infection indirectly via boosting immune responses (IFN-γ production and macrophage activation).

Our results are encouraging for the use of centaurein protecting against and treating antibiotic-resistant intracellular

bacteria via enhanced IFN-γ production. Similar approaches can be used to develop immune modulators and prophylactics/therapeutics for infectious pathogens. However, it is noteworthy that the beneficial therapeutic effect of centaurein in our studies was only based on healthy young mice and/or IFN-γ knockout mice with serious innate immunodeficiency. Additional experiments in evaluating the efficacy of centaurein in partially immunocompromised mice, such as dexamethasone-treated mice, needs be considered. The use of healthy mice and mice with innate or acquired immunodeficiency to evaluate the therapeutic effect of centaurein on *Listeria* infection helps draw the conclusion on the efficacy of centaurein in *Listeria* elimination and may be more like humans susceptible to *Listeria* infection.

T-bet is required for IFN-γ production in T cells and NK cells (Szabo et al., 2002; Townsend et al., 2004). However, T-bet was reported not to be required for host resistance to *Listeria* infection (Way and Wilson, 2004). During *Listeria* infection, IFN-γ production significantly decreased in $CD4^+$ T cells but not $CD8^+$ T and NK cells, leading to a comparable level of IFN-γ production in control mice and T-bet-knockout mice. Therefore, T-bet-knockout mice maintained their resistance to *Listeria*. Our results showed that centaurein increases IFN-γ production in cells and mice. Such an increase accompanies T-bet up-regulation. Therefore, our results strongly suggest that centaurein elevates IFN-γ production via control of T-bet. IFN-γ was reported to induce T-bet expression (Lighvani et al., 2001), raising the possibility that the up-regulation of T-bet is an indirect consequence of centaurein inducing IFN-γ. However, this should not be the case because centaurein still up-regulates T-bet transcription in Jurkat cells, in which IFN-γ production is defective. The regulation of IFN-γ expression involves a complicated mechanism mediated by various nuclear factors. Other nuclear factors, in addition to T-bet, might also take part in centaurein-mediated IFN-γ transcription. Accordingly, our unpublished data suggest that centaurein may activate NFκB and NFAT enhancer activities (data not shown). Both enhancer sites exist in IFN-γ promoter and in T-bet promoter.

The concentrations of centaurein used in the paper are 50 to 100 μg/ml in cells and 10 to 20 μg/mouse in mice. The above doses, probably not excellent for a drug, seem reasonable for a lead compound or food supplement. Besides, we also found that centaureidin, an aglycone of centaurein, could enhance IFN-γ production 30 times more than centaurein (unpublished data). Therefore, there is great potential for use of centaurein or its derivatives to treat infectious pathogens.

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