Garlic (Allium sativum) Stimulates Lipopolysaccharide-induced Tumor Necrosis Factor-alpha Production from J774A.1 Murine Macrophages

Jessica Sung, Youssef Harfouche, Melissa De La Cruz, Martha P. Zamora, Yan Liu, James A. Rego and Nancy E. Buckley

1Department of Biological Sciences, California State Polytechnic University, 3801 W. Temple Ave., Pomona, CA 91768, USA
2Department of Chemistry and Biochemistry, California State Polytechnic University, 3801 W. Temple Ave., Pomona, CA 91768, USA

Garlic (Allium sativum) is known to have many beneficial attributes such as antimicrobial, antiatherosclerotic, antitumorigenetic, and immunomodulatory properties. In the present study, we investigated the effects of an aqueous garlic extract on macrophage cytokine production by challenging the macrophage J774A.1 cell line with the garlic extract in the absence or presence of lipopolysaccharide (LPS) under different conditions. The effect of allicin, the major component of crushed garlic, was also investigated. Using enzyme-linked immunosorbent assay and reverse transcriptase-quantitative polymerase chain reaction, it was found that garlic and synthetic allicin greatly stimulated tumor necrosis factor-alpha (TNF-α) production in macrophages treated with LPS. The TNF-α secretion levels peaked earlier and were sustained for a longer time in cells treated with garlic and LPS compared with cells treated with LPS alone. Garlic acted in a time-dependent manner. We suggest that garlic, at least partially via its allicin component, acts downstream from LPS to stimulate macrophage TNF-α secretion.

Keywords: garlic; allicin; macrophages; tumor necrosis factor-alpha (TNF-α); lipopolysaccharide (LPS).

INTRODUCTION

Garlic has been used as a home remedy for a myriad of diseases for thousands of years. To date, garlic and its constituents are known to have many beneficial attributes such as antimicrobial (Casella et al., 2013; Goncagul and Ayaz, 2010), antiatherosclerotic (Asdaq et al., 2009; Chan et al., 2013; Gonen et al., 2005; Malekpour-Dehkordi et al., 2013), antitumorigenetic (Iciek et al., 2009; Kaschula et al., 2011), and immunomodulatory (Iciek et al., 2009) properties. Of particular interest for our studies is the modulation of cytokine production by garlic as cytokines are important immune system mediators. Different types of garlic extracts have been shown to directly modulate cytokine production in lymphocytes (Dong et al., 2011; Liu et al., 2009; Zamani et al., 2009) and macrophages (Chang et al., 2005; Dong et al., 2011; Hodge et al., 2002; Keiss et al., 2003; Romano et al., 1997). Garlic is largely recognized as having antiinflammatory properties; however, not all reports provide evidence indicating that garlic increases antiinflammatory [i.e. interleukin-10 (IL-10)] cytokines while decreasing pro-inflammatory (i.e. tumor necrosis-alpha (TNF-α), IL-1β, and IL-6) cytokines. Thus, we set out to investigate the effect of a water garlic extract and allicin, the major component of crushed garlic (Rybak et al., 2004), on murine macrophage cytokine production induced by lipopolysaccharide (LPS). The LPS is found on the cell wall of Gram-negative bacteria (Rietschel and Brade, 1992) and has been widely used to cause an immune response in vivo and in vitro from various immune cells including the activation of macrophages (Fiorentino et al., 1991; Parameswaran and Patial, 2010; Ulmer et al., 2000). Of the cytokines investigated (IL-1β, IL-6, IL-10 and IL-12, and TNF-α), we found that a water garlic extract consistently stimulated LPS-induced TNF-α secretion from macrophages. Transcription of TNF-α was also significantly enhanced by garlic, and allicin, in LPS-treated cells. In addition, macrophages treated with the aqueous garlic extract alone secreted small, albeit significant amounts of TNF-α.

MATERIALS AND METHODS

Preparation of water garlic extract. Garlic was purchased at the local grocery store. The cloves of garlic from two garlic bulbs were peeled, and any discolored parts or blemishes were cut off. Garlic was weighed (56.77 g) and added to a Waring blender containing 113.54 mL of pyrogen-free water (HOSPIRA, Inc., Lake Forest, IL). The garlic to water ratio was modeled after the aqueous garlic extraction method reported by Gamboa-León et al. (2007). The garlic was then blended for 1 min, and the homogenate was passed through grade 50 cheesecloth (Lymex, Chicopee, MA). In a tissue culture hood, the flow-through was then filter-
sterilized using a 150-mL Nalgene 0.2-μm pore-sized filter unit (Nalge Nunc International, Rochester, NY) connected to a vacuum pump. The flow-through from the Nalgene filter was aliquoted into microcentrifuge tubes and stored at −80°C until used. To determine endotoxin contamination, the garlic extract was sent for endotoxin testing using the kinetic chromogenic limulus amebocyte lysate assay (Charles River Laboratories, Charleston, SC).

**Determination of allicin content in garlic.** Allicin in the garlic extracts was quantified using a reversed-phase HPLC method that employed the Agilent-1100 HPLC system (Agilent, CA) with a Zorbax Eclipse XDB-C18 column. The garlic extracts were eluted through the column by the isocratic solvent of water/methanol (50/50) at a flow rate of 0.75 mL/min, and the absorbance of the eluate was monitored at 254 nm.

**Allicin synthesis.** Allicin was synthetized by the peroxoyacid oxidation of diallyl disulphide (Alfa Aesar, Ward Hill, MA) according to the procedure of Small et al. (1947). Pre-purification of diallyl disulphide by fractional vacuum distillation to remove diallyl sulphide and diallyl trisulphide was critical for obtaining pure allicin after oxidation. Because of the thermal instability of allicin, the crude product was purified by silica gel chromatography rather than distillation.

**J774A.1 macrophage culture and treatment.** The murine macrophage cell line J774A.1 was purchased from ATCC (Manassas, VA) and maintained in complete media: Dulbecco’s modified Eagle’s medium (ATCC), 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and penicillin (100 units/mL)/streptomycin (100 μg/mL) (Invitrogen). For experiments, J774A.1 macrophages were plated at a concentration of 1.25 × 10⁶ cells/mL, 200 μL/well in complete media in 96 well tissue culture plates. Cells between passages 6–15 were used for all experiments.

Before treatment, the conditioned media was removed from the cells 24 h after plating them. Fresh media was then added to the cells, and they were treated with the garlic or synthetic allicin. The garlic extract was added to the cells at varying dilutions in pyrogen-free water (1:40, 1:100, 1:200, 1:500, 1:1000, and 1:2000). Alternatively, allicin (1–10 μg/mL) also diluted in pyrogen-free water was added to the cells. Garlic or allicin was added in the presence or absence of 0.1 μg/mL LPS (LPS from *Escherichia coli* 055:B5, Sigma, St. Louis, MO). The cells were then incubated at 37°C and 5% CO₂ for 24 h. After this incubation period, cell supernatants were collected and stored at −80°C until assaying for cytokine secretion. Analysis of cytokine levels revealed an effective dilution of garlic (1:500) that modulated cytokine production.

To determine the minimum LPS concentration that would cause the maximum TNF-α stimulation in the presence of garlic, J774A.1 macrophages were plated in 96 well plates as described earlier and treated with the garlic extract (G1:500) and different concentrations of LPS (0.001 to 10 μg/mL). To further characterize the interaction between LPS and the effective garlic extract dilution (G1:500), J774A.1 macrophages were plated in 96 well plates as described earlier. The cells were then treated with G1:500 for different periods before, after, or at the same time as LPS (0.1 μg/mL). The cells were incubated at 37°C for 24 h (or for the time specified in the figure legend). At the end of this incubation period, the supernatants were collected from the wells and stored at −80°C until assaying for cytokine secretion. In some experiments, G1:500 was removed prior to the addition of the LPS. In these experiments, the conditioned media in the wells was replaced with fresh media prior to adding the LPS. The cells were incubated at 37°C for 24 h after the addition of LPS. At the end of the 24 h, the supernatants were collected from the wells and stored at −80°C until assaying for cytokine secretion. Alternatively, in other experiments, LPS was added to the cells for different periods and removed prior to the addition of G1:500. In these experiments, the conditioned media in the wells was replaced with fresh media prior to adding G1:500. These cells were then incubated at 37°C for 24 h after the addition of G1:500. At the end of the 24 h, the supernatants were collected from the wells and stored at −80°C until assaying for cytokine secretion.

**Cell proliferation XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] assay.** A cell proliferation assay was carried out to determine the effect of garlic or allicin treatment on macrophage cell viability. Thus, macrophages were plated (2.5 × 10⁵ cells/mL, 100 μL/well) in a 96 well plate and incubated at 37°C. After incubation for 24 h, the complete media was removed, and new complete media was added (100 μL/well). The cells were then treated with different dilutions of the garlic extract or synthetic allicin in the absence or presence of LPS (0.1 μg/mL). The effect of the garlic extract on cell viability was assayed using the Cell Proliferation Kit II (XTT) from Roche (Indianapolis, IN) as described by the manufacturer.

**Cytokine assays.** Cell supernatants were assayed for the presence of cytokines (TNF-α, IL-1β, IL-6, IL-10, and IL-12) using cytokine specific ELISAs (BD OptEIA, BD Biosciences, San Diego, CA) as indicated by the manufacturer.

**mRNA quantification.** To determine the effect of garlic and allicin on TNF-α and toll-like receptor 4 (TLR4) mRNA levels, J774A.1 macrophages were plated at a concentration of 1.25 × 10⁵ cells/mL, 5 mL/well in complete media in six well tissue culture plates. The conditioned media was removed from the cells 24 h after plating them, and fresh media was added. The cells were then treated for 2 h with the garlic or synthetic allicin in the absence or presence of LPS (0.1 μg/mL). Preparation of RNA, cDNA, and qPCR was carried out with reagents from Bio-Rad (Hercules, CA). Total RNA extraction and cDNA synthesis were carried out as indicated by the manufacturer using the Aurum Total RNA Mini kit and the iScript reverse transcriptase, respectively. Real-time PCR was performed in the CFX96 Touch Real-Time PCR Instrument using Sso Advanced Universal SYBR Green and primers (PrimePCR SYBR Assay) for TNF-α, Actin-β, or TLR4.
The reaction was run at 95 °C for 2 min for 1 cycle, 95 °C for 5 s for 40 cycles, 60 °C for 30 s for 40 cycles, and 65–95 °C (0.5 °C increments) for 5 s/step for 1 cycle. The data, expressed as $2^{-\Delta\Delta C_{q}}$, were obtained by normalizing against the actin gene and then comparing the treated cells against the untreated cells.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism Version 6.0. An unpaired one-way analysis of variance (ANOVA) was performed with a Tukey’s multiple comparison test to compare all groups against each other. $p < 0.05$ was considered statistically significant.

**RESULTS**

The aqueous garlic extract (G) was tested at different dilutions to investigate whether it had any effects on cytokine levels. Of the cytokines tested (TNF-α, IL-1β, IL-6, IL-10, and IL-12), G at dilutions of 1:200 to 1:1000 consistently increased TNF-α secretion from the macrophages (Table 1) compared with pyrogen-free water (PFW). Furthermore, G was very effective at stimulating LPS (0.1 μg/mL)-induced TNF-α (Table 1) from macrophages. Thus, we focused on the production of TNF-α. To rule out that the stimulation of the garlic extract on TNF-α secretion was due to endotoxin contamination, the garlic extract was sent out for testing. The levels were found to be 0.0025 endotoxin units/mg in 5 mg/mL of garlic extract. These endotoxin levels were negligible, and thus, we concluded that component(s) within the garlic extract was responsible for stimulating TNF-α production from the macrophages. We next looked at cell proliferation using the XTT assay to determine whether garlic had a mitogenic effect. We found that garlic did not stimulate cell proliferation, indicating that the increase in TNF-α was not because of an increase in cell number. However, garlic at a dilution of 1:40, in the absence of LPS, decreased metabolic activity by 10% as compared with untreated cells (data not shown). This suggests that at this dilution, garlic had a slightly toxic effect on the cells. This finding is similar to that by Shin et al. (2013) who also reported that at high raw garlic extract concentrations, RAW cell viability was reduced (Shin et al., 2013).

For subsequent studies, we used the highest dilution of garlic that caused the maximum stimulation of LPS-induced TNF-α secretion, which was seen using the 1:500 dilution (G1:500). We next varied the concentration of LPS to determine the LPS concentration to use in subsequent experiments. As seen in Fig. 1A, garlic stimulated LPS-induced TNF-α macrophage secretion at all LPS concentrations studied. Furthermore, although TNF-α secretion increased in a concentration-dependent manner

### Table 1. Garlic stimulates TNF-α secretion from J774A.1 macrophages in the absence and presence of lipopolysaccharide (LPS)

| Treatment          | TNF-α (pg/mL) avg ± SD |
|--------------------|------------------------|
| Pyrogen-free water | 21.72 ± 8.86           |
| G1:200             | 357.67 ± 47.35*        |
| G1:500             | 86.81 ± 16.23*         |
| G1:1000            | 31.63 ± 8.98           |
| LPS 0.1 μg/mL      | 1471.75 ± 453.90       |
| G1:200 + LPS       | 8401.25 ± 112.40f      |
| G1:500 + LPS       | 8315.00 ± 271.77f      |
| G1:1000 + LPS      | 3502.81 ± 427.94d      |

Cells were plated at 1.25 × 10⁵ cells/mL, 200 μL/well in 96 well plates, and incubated at 37 °C. The media was replaced with fresh media 24 h after plating. The garlic extract (G) was then added to the cells at the indicated dilutions in the absence or presence of 0.1 μg/mL LPS, and the cells were incubated for 24 h. After this incubation period, cell supernatants were collected and assayed for TNF-α using ELISA.

$p < 0.0001$

$p = 0.0434$ compared with the control pyrogen-free water.

$p < 0.0001$

$p = 0.0002$ compared with LPS 0.1 μg/mL. This is a representative experiment from at least five independent experiments.

© 2014 The Authors. *Phytotherapy Research* published by John Wiley & Sons, Ltd.
when the cells were treated with only LPS, in the presence of garlic (G1:500) and LPS, TNF-α secretion reached a plateau at 0.1 μg/mL LPS. Analysis of the TNF-α transcription levels after a 2-h G1:500 and 0.1 μg/mL LPS cell treatment also revealed a significant increase in TNF-α production (Table 2). Thus, we decided to continue to use 0.1 μg/mL LPS in our subsequent studies.

To further characterize the interaction between the garlic extract and LPS, the cells were treated with G1:500 for different periods before, after, or at the same time as LPS (0.1 μg/mL). G1:500 stimulated LPS-induced TNF-α at all time points studied, although the maximum stimulation by G1:500 occurred when the extract was added 15 min before to 1 h after LPS cell treatment (Fig. 1B). These results suggest the presence of a garlic component that has a maximum stimulatory effect when it is added around the same time as LPS. We next investigated whether preincubating the cells with garlic had an effect on LPS-induced TNF-α secretion. Thus, the cells were pretreated with G1:500 for different times; however, this time, garlic was removed prior to LPS addition. We found that only cells treated with G1:500 in conjunction with LPS showed TNF-α secretion (Fig. 2A), suggesting that either a labile garlic compound was responsible for the stimulatory effect on LPS-induced TNF-α secretion or that garlic acted downstream from LPS. Thus, we investigated whether preincubating the cells with LPS had an effect on G1:500 stimulation of TNF-α. Hence, the cells were pretreated with LPS for different times, and then, the LPS was removed. The cells then received either the PFW control or G1:500. Cells receiving PFW after LPS removal showed that only cells pretreated with LPS for 0.5 to 1 h induced TNF-α secretion (Fig. 2B, set of white bars). However, these TNF-α levels were smaller than the levels observed when LPS remained with the cells for 24 h (Fig. 2B, last white bar). Interestingly, we found that addition of G1:500 after LPS removal further enhanced cellular TNF-α production when compared with the cells pretreated with LPS followed by PFW (Fig. 2B, set of black bars compared with white bars). This effect was seen at all LPS pretreatment times studied but was greatest after cells were preincubated with LPS for 0.5 h (Fig. 2B, set of black bars). These are representative experiments from at least three independent experiments. *p < 0.001 compared with LPS alone.

Table 2. Effect of garlic and alllicin on TNF-α and toll-like receptor 4 (TLR4) mRNA levels

| Treatment                        | TNF-α (fold expression) | TLR4 (fold expression) |
|----------------------------------|--------------------------|-------------------------|
| G1:500                           | 0.82 ± 0.52              | 0.68 ± 0.33             |
| A 5 μg/mL                        | 0.84 ± 0.4               | 0.64 ± 0.30             |
| Lipopolysaccharide (LPS)         | 17.58 ± 12.81            | 0.42 ± 0.30             |
| 0.1 μg/mL                        |                          |                         |
| LPS 0.1 μg/mL + G1:500           | 61.30 ± 17.05*           | 0.50 ± 0.30             |
| LPS 0.1 μg/mL + A 5 μg/mL        | 43.86 ± 20.71            | 0.54 ± 0.30             |

Cells were plated at a concentration of 1.25 × 10^5 cells/mL, 5 mL/well in complete media in six well tissue culture plates. The conditioned media was removed from the cells 24 h after plating them, and fresh media was added. The cells were then treated for 2 h with garlic (G) or synthetic alllicin (A) at the indicated concentrations in the absence or presence of LPS (0.1 μg/mL). Total RNA was extracted, reverse transcribed, and the cDNA amplified to determine TNF-α and TLR4 mRNA levels. The data were normalized against the actin gene and are expressed as fold expression compared with untreated cells. *p < 0.02 compared with LPS 0.1 μg/mL.

to 1 h (Fig. 2B, set of black bars). These results suggest that garlic acts downstream from LPS to enhance LPS-induced TNF-α secretion or that a labile garlic component is responsible for garlic’s effect. To confirm that the garlic extract acts downstream of the LPS-signaling cascade, we investigated the effect of garlic on TLR4 mRNA expression. The TLR4 is known to be the receptor for LPS (Parameswaran and Patial, 2010). As is shown in Table 2, garlic did not significantly alter TLR4 expression in the absence or presence of LPS.

We next investigated the length of time that garlic could stimulate LPS-induced TNF-α secretion. To this end, cells were treated with G1:500 + LPS or LPS alone, and cell supernatants were collected at different time points after LPS addition. We found, as before, that in the presence of garlic, LPS-induced TNF-α secretion was significantly greater in cells treated with LPS alone (Fig. 3). Furthermore, in cells treated with

Figure 2. Effect of cell pretreatment with garlic or LPS on TNF-α secretion from J774A.1 macrophages. Cells were plated as described in Fig. 1. (A) The pyrogen-free water (PFW) or the garlic extract (G1:500) was then added to the cells. G1:500 was removed 3 h (−3 h), 1 h (−1 h), 0.5 h (−0.5 h), 15 min (−0.25 h), or 5 min (−0.083 h), prior to LPS addition, by replacing the media. Cells were treated with LPS (0.1 μg/mL) and further incubated at 37 °C for 24 h after which cell supernatants were collected and assayed for TNF-α levels via ELISA. G1:500 + LPS treatment (black bar) was significantly different (p < 0.001) from all other treatments. (B) PFW or LPS (0.1 μg/mL) was added to the cells. The LPS was removed 3 h (−3 h), 2 h (−2 h), 1 h (−1 h), 0.5 h (−0.5 h), 15 min (−0.25 h), or 5 min (−0.083 h), prior to garlic extract (G1:500) addition, by replacing the media. Cells were then treated with G1:500 and further incubated at 37 °C for 24 h after which cell supernatants were collected and assayed for TNF-α levels via ELISA. With respect to each time point, the black bars (G1:500 treated cells) are statistically significantly different from corresponding white bars (p < 0.001). Within the black bar group or the white bar group, similar letters correspond to equivalent values within that group. These are representative experiments from at least three independent experiments. *p < 0.001 compared with LPS alone.
Tumor necrosis factor-α is a pleiotropic cytokine that, depending on the cellular context, can regulate a number of cellular functions, including inflammation, proliferation, differentiation, and cell death or survival (Parameswaran and Patial, 2010; Varfolomeev and Ashkenazi, 2004). The major producers of TNF-α are macrophages, important players in the innate immune response (Parameswaran and Patial, 2010). In the present study, we demonstrate that an aqueous garlic extract and synthetic alliin, in the absence or presence of LPS, stimulate TNF-α secretion from the murine macrophage cell line J774A.1.

Our findings are in contrast to those of other investigators who used organic garlic extracts or organosulphur garlic derivatives and diverse concentrations of LPS. At micromolar concentrations, ajoene, a sulphur-containing compound found in ethanol garlic extracts, was found to inhibit LPS (100 ng)-induced TNF-α production from thioglycolate-induced mouse peritoneal macrophages (Romano et al., 1997). Similarly, the garlic oil derivatives diallyl sulphide (DAS), diallyl disulphide (DADS), and allyl methyl sulphide (AMS) were shown to modulate LPS (330 ng/mL)-induced TNF-α production from the murine macrophage cell line RAW264.7. The AMS at 5 and 10 μM inhibited TNF-α production. The DADS at 0.1 μM slightly enhanced TNF-α production but was inhibitory at 0.5 and 2 μM. The AMS inhibited TNF-α at 2, 10, and 20 μM concentrations (Chang et al., 2005). In addition, garlic powder extract (0.1 mg/mL) dissolved in dimethyl sulfoxide inhibited LPS (10 ng/mL)-induced TNF-α production from human whole blood (Keiss et al., 2003). This same group also found that DADS and alliin at 100 μM reduced LPS-induced TNF-α levels (Keiss et al., 2003). More recently, using RAW264.7 cells, Lee et al. (2012) showed that ajoene extracted from garlic inhibited the expression of TNF-α mRNA (Lee et al., 2012), and You et al. (2013) reported that diallyl trisulphide (DATS, 100 μM) inhibited several cytokines including TNF-α (You et al., 2013). Using RAW264.7 macrophages, Shin et al. (2013) found that raw garlic extracts decreased LPS (1 μg/mL)-induced TNF-α secretion but that alliin (1 μg/mL) had no effect on the production of this cytokine by LPS (Shin et al., 2013).

In our experiments, we have been unable to observe an effect of garlic on the secretion of other cytokines whether pro-inflammatory (i.e. IL-1β, IL-6, and IL-12) or antiinflammatory (i.e. IL-10). Others have conflicting reports on the effect of garlic or its derivatives on these cytokines. It was found that DADS stimulated the pro-inflammatory cytokines IL-1β and IL-6, and that AMS stimulated the antiinflammatory cytokine IL-10 (Chang et al., 2005; Hodge et al., 2002; Liu et al., 2009). It was also reported that DADS inhibited IL-10 (Chang et al., 2005). An aqueous garlic extract was also shown to inhibit IL-12 from monocytes (Hodge et al., 2002), whereas another group reported garlic lectins as stimulating IL-12 from monocytes (Dong et al., 2011). Others have reported garlic or its derivatives as inhibiting the pro-inflammatory cytokines IL-1β (Keiss et al., 2003) and IL-6 (Hodge et al., 2002).

We suggest that our results are different from other studies carried out in macrophages because of the nature and concentration of garlic compounds found in organic extracts compared with our aqueous garlic.

**DISCUSSION**

G1:500 + LPS, TNF-α levels began and peaked earlier than in cells treated with LPS alone. Thirdly, TNF-α secretion remained elevated longer in cells treated with G1:500 + LPS as compared with cells treated with LPS alone (Fig. 3). Thus, garlic enhances and prolongs the cellular response to LPS in terms of TNF-α production.

To determine if the stimulating component in the garlic was alliin, we quantified the alliin content in our garlic extract using HPLC. More than five peaks, because of different components in the garlic extract, were observed on the chromatogram. We identified the peak for alliin by its migration time (1.286 min) and by running it alongside the synthetic alliin. Upon integrating all peaks on the chromatogram, the peak area of alliin accounts for ~24% of overall peak areas, indicating that alliin is one of the major components of our aqueous garlic extract. We calculated the alliin content in garlic to be 60 ± 14 μM. Using synthetic alliin, we found that alliin [2.5 (15 μM), 5 (31 μM), and 10 μg/mL (62 μM)] enhanced LPS-induced TNF-α secretion (Fig. 4). Furthermore, alliin (5 μg/mL) increased LPS-induced TNF-α mRNA levels as well (Table 2). These findings suggest that alliin, at least in part, is responsible for garlic’s stimulation of TNF-α production from the macrophages.

**Figure 3.** Garlic stimulates LPS-induced TNF-α secretion from J774A.1 macrophages more efficiently than LPS alone. Cells were plated as described in Fig. 1. Cells were treated with PFW or the garlic extract (G) and LPS (0.1 μg/mL). Cell supernatants were then collected at different times (from 3 to 48 h after LPS treatment) and assayed for TNF-α concentrations of allicin. Cell supernatants were collected 24 h after LPS addition and assayed for TNF-α production.

**Figure 4.** Allicin stimulates LPS-induced TNF-α secretion from J774A.1 macrophages. Cells were plated as described in Fig. 1. Cells were treated with LPS (0.1 μg/mL) and the indicated concentrations of allicin. Cell supernatants were collected h after LPS addition and assayed for TNF-α levels via ELISA. Statistical significance: *p < 0.02, **p < 0.006 compared with LPS-only treatment. This is a representative experiment of at least two independent experiments.

© 2014 The Authors. Phytotherapy Research published by John Wiley & Sons, Ltd.
Garlic has been recognized to have immunostimulatory effects. Although garlic has been largely recognized as having anti-inflammatory response, the induction of TNF-α by garlic does not contradict the notion that this herb helps immunity. The increase in TNF-α is necessary when an organism is injured or infected. We suggest that garlic renders macrophages more efficient at producing this cytokine in response to infections as evidenced by a more rapid increase in TNF-α and prolongation of this response. As is true to a healthy pro-inflammatory response, the pro-inflammatory cytokines cannot remain elevated or they can be fatal to the organism. Thus, we find that although garlic does increase LPS-induced TNF-α, this elevation is temporary as evidenced by the subsequent decrease in the levels of this cytokine. Therefore, we believe that our findings support the notion that garlic is beneficial to the immune system.

Acknowledgements
This research was supported by the Agricultural Research Initiative (ARI) grant number 10-4-184-12.

Conflict of Interest
The authors have declared having no conflict of interest.

REFERENCES

Asadq SM, Inamdar MN, Asad M. 2009. Effect of conventional antihypertensive drugs on hypolipidemic action of garlic in rats. Indian J Exp Biol 47: 176–181.

Brodnitz MH, Pascale JV, van Derslice L. 1971. Flavor components of garlic extract. J Agric Food Chem 19: 273–275.

Casella S, Leonardi M, Melai B, Fratini F, Pistelli L. 2013. The role of diallyl sulfides and dipropyl sulfides in the in vitro antimicrobial activity of the essential oil of garlic, Allium sativum L., and leek, Allium porrum L. [Comparative study]. Phytother Res 27: 380–383.

Chang HP, Huang SY, Chen YH. 2005. Modulation of cytokine secretion by garlic oil derivatives is associated with suppressed nitric oxide production in stimulated macrophages. J Agr Food Chem 53: 2530–2534.

Dong Q, Sugita T, Toyohira Y, Yoshida Y, Yanagihara N, Karasaki Y. 2011. Stimulation of IFN-γ production by garlic lectin in mouse spleen cells: involvement of IL-12 via activation of p38 MAPK and ERK in macrophages. Phytomedicine 18: 309–316.

Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. 1991. IL-10 inhibits cytokine production by activated macrophages. J Immunol 147: 3815–3822.

Gamboa-Leon MR, Aranda-Gonzalez I, Mut-Martin M, Garcia-Miss MR, Dumontel E. 2007. In vivo and in vitro control of Leishmania mexicana due to garlic-induced NO production. Scand J Immunol 66: 508–514.

Goncagul G, Ayaz E. 2010. Antimicrobial effect of garlic (Allium sativum). Recent Pat Antiinfect Drug Discov 5: 91–93.

Gonen A, Harats D, Rabinkov A, et al. 2005. The antiatherogenic effect of allicin: possible mode of action. Pathobiology 72: 325–334.

Hodge G, Hodge S, Han P. 2002. Allium sativum (garlic) suppresses leukocyte inflammatory cytokine production in vitro: potential therapeutic use in the treatment of inflammatory bowel disease. Cytometry 48: 2009–2215.

Iciek M, Kwiecien I, Wlodiek L. 2009. Biological properties of garlic and garlic-derived organosulfur compounds. Environ Mol Mutagen 50: 247–265.

Kang N, Moon E, Cho C, Pyo S. 2001. Immunomodulating effect of synthetic ajoene analogues on cancer cell-lines. Anticancer Agents Med Chem 11: 260–266.

Keiss HP, Dirsch VM, Hartung T, et al. 2003. Garlic (Allium sativum L.) modulates cytokine expression in lipopolysaccharide-activated human blood thereby inhibiting NF-κB activity. J Nutr 133: 2171–2175.
Lee DY, Li H, Lim HJ, Lee HJ, Jeon R, Ryu JH. 2012. Anti-inflammatory activity of sulfur-containing compounds from garlic. *J Med Food* **15**:992–999.

Liu CT, Su HM, Lii CK, Sheen LY. 2009. Effects of supplementation with garlic oil on activity of Th1 and Th2 lymphocytes from rats. *Planta Med* **75**: 205–210.

Malekpour-Dehkordi Z, Javadi E, Doosti M, et al. 2013. S-Allylcysteine, a garlic compound, increases ABCA1 expression in human THP-1 macrophages. [Research support, Non-U.S. Gov't]. *Phytother Res* **27**: 357–361.

Parameswaran N, Patial, S. 2010. Tumor necrosis factor-alpha signaling in macrophages. *Crit Rev Eukaryot Gene Expr* **20**: 87–103.

Rabinkov A, Wilchek M, Mirelman D. 1995. Alliinase (alliin lyase) from garlic (*Allium sativum*) is glycosylated at ASN146 and forms a complex with a garlic mannose-specific lectin. *Glycoconj J* **12**: 690–698.

Rietschel ET, Brade H. 1992. Bacterial endotoxins. *Sci Am* **267**: 53–61.

Romano EL, Montano RF, Brito B, *et al*. 1997. Effects of ajoene on lymphocyte and macrophage membrane-dependent functions. *Immunopharmacol Immunotoxicol* **19**: 15–36.

Rybak ME, Calvey EM, Harnly JM. 2004. Quantitative determination of allicin in garlic: supercritical fluid extraction and standard addition of alliin. *J Agric Food Chem* **52**: 682–687.

Shin JH, Ryu JH, Kang MJ, Hwang CR, Han J, Kang D. 2013. Short-term heating reduces the anti-inflammatory effects of fresh raw garlic extracts on the LPS-induced production of NO and pro-inflammatory cytokines by downregulating allicin activity in RAW 264.7 macrophages. [Research support, Non-U.S. Gov't]. *Food Chem Toxicol* **58**: 545–551.

Small LD, Bailey JH, Cavallito C.J. 1947. Alkyl thiolsulfonates. *J Am Chem Soc* **69**: 1710–1713.

Ulmer AJ, Flad H, Rietschel T, Mattern T. 2000. Induction of proliferation and cytokine production in human T lymphocytes by lipopolysaccharide (LPS). *Toxicology* **152**: 37–45.

Varfolomeev E, Ashkenazi A. 2004. Tumor necrosis factor: an apoptosis JuNKiE? *Cell* **116**: 491–497.

Wang H, Li X, Liu S, Jin S. 2010. Quantitative determination of allicin in *Allium sativum* L. bulbs by UPLC. *Chromatographia* **71**: 159–161.

You S, Nakanishi E, Kuwata H, *et al*. 2013. Inhibitory effects and molecular mechanisms of garlic organosulfur compounds on the production of inflammatory mediators. [Research support, Non-U.S. Gov't]. *Mol Nutr Food Res* **57**: 2049–2060.

Zamani A, Vahidinia A, Sabouri Ghannad M. 2009. The effect of garlic consumption on Th1/Th2 cytokines in phytohemagglutinin (PHA) activated rat spleen lymphocytes. *Phytother Res* **23**: 579–581.