

# Human Monocytic Cells Upregulate Superoxide-Generating Activity and mRNAs for its Components in Response to Heat-Stable and Heat-Unstable Factors Released to Medium Conditioned with *Ehrlichia Chaffeensis*-Infected THP-1 Cells

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To escape from reactive oxygen species generated in response to infection, intracellular pathogens, such as *Salmonellae* and *Anaplasma phagocytophilum*, can modulate the expression and distribution of the phagocyte NADPH oxidase components. In the present study, we analyzed the effect of *Ehrlichia chaffeensis* infection on the ability of human monocytic THP-1 cells to produce superoxide anion. Phorbol ester-stimulated superoxide generation and mRNAs for gp91<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> were significantly increased in *E. chaffeensis*-infected THP-1 cells. These increases were also achieved in THP-1 cells cultured in medium conditioned by the *E. chaffeensis*-infected cells, indicating that bystander cells can be activated for superoxide generation and implicating soluble factors in the response. Heat-stable and -unstable factors represented one-third and two-thirds of this activity, respectively. These results suggest that immature human monocytic cells increase their ability to generate superoxide anion in response to *E. chaffeensis* infection regardless they have been directly invaded or not.

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## Introduction

Monocytes/macrophages and granulocytes are essential for protecting the host from invading parasites. In spite of potent protection mechanisms, these cells occasionally permit the survival of certain parasites because of an imbalance between their protective activities and various bacterial escape mechanisms.<sup>1-3</sup> *Anaplasma phagocytophilum* (*A. phagocytophilum*), which is classified as a rickettsia, can reside in human neutrophils by escaping from the host's protection mechanisms. This rickettsia specifically inhibits the expression of CYBB (encoding gp91<sup>phox</sup>) and RAC2 (encoding rac2),<sup>4</sup> and decreases p22<sup>phox</sup>.<sup>5</sup> This eliminates the superoxide-generating phagocyte NADPH oxidase activity in human neutrophils and the myelocytic cell line, HL-60. This process mimics a chronic granulomatous disease, an inherited disease caused by the loss of one of four essential components of the phagocyte oxidase.<sup>6</sup>

Similar to *A. phagocytophilum*, *Ehrlichia chaffeensis* (*E. chaffeensis*), another rickettsia, can reside in human monocytes expressing the phagocyte NADPH oxidase system. In contrast to the effect of *E. chaffeensis* infection on cytokine release<sup>7,8</sup> and modulation of signal transduction molecules in monocytes,<sup>9-11</sup> the effect of the infection on the phagocyte NADPH oxidase system has not been well characterized. In addition, microarray analyses have not detected a significant modulation of genes for the oxidase.<sup>12</sup>

For these reasons, we analyzed the effect of *E. chaffeensis* infection on phagocyte NADPH oxidase activity and the expression of the NADPH oxidase components in human monocytic THP-1 cells. We found that the cells increased phorbol ester-stimulated superoxide generation and the expression of the oxidase components at transcriptional level in response to heat-stable and heat-unstable factors released to infected cell-conditioned medium.

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## Materials and Methods

### *E. chaffeensis* and cell culture

*E. chaffeensis* was cultivated in the THP-1 human monocytic leukemia cell line (JCRB Cell Bank, Tokyo, Japan) in RPMI 1640 medium (Asahi Technoglass Inc., Tokyo, Japan) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) as previously described.<sup>7</sup> After attaching cells to slide glasses by Auto Smear CF-12D (Sakura, Tokyo, Japan) at 1,400 rpm for 6 min, these were stained with Diff-Quik (Baxter Science Product, Obetz, OH) and the percentage of infected cells was determined by analyzing more than 300 cells. THP-1 cells with 20-50% infection were kept in Cell Banker (ZENOAQ, Kouriyama, Japan) at -80°C. For use in experiments, cells were washed twice with the medium and cultured for one or two days to achieve >90% infection. Intact cells were infected or mock-infected with *E. chaffeensis* by mixing them at a 10:1 ratio with washed >90%-infected cells or washed uninfected cells, respectively. Both infected and mock-infected cells were incubated at 37°C in an atmosphere of 95% air/5% CO<sub>2</sub>. Infection was checked daily, and, at indicated times, cells were harvested and washed once or twice with phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup> (PBS<sup>-</sup>) for use in further experiments.

### Trans-well assay

THP-1 cells (1×10<sup>6</sup>/2 ml) maximally infected with *E. chaffeensis* were seeded onto 6-well culture plates. Fresh THP-1 cells (2.5×10<sup>5</sup>/2 ml) were added to trans-well inserts with 0.2µm-pore membranes (Nalge Nunc International, Roskilde, Denmark). Once a day for four days, the inserted trans-wells were transferred to new wells containing maximally infected THP-1 cells.

### Conditioned media

Conditioned media were prepared from dishes with *E. chaffeensis*-infected and mock-infected cells at day 2.5 of infection by a series of centrifugations (500 ×g for 3 min followed by 155,000 ×g for 45 min), followed by sterilization by filtration through 0.2 µm-pore membranes.

### Heat inactivation

Portions of the conditioned media were heat-treated in a water bath by gradually increasing the temperature to 100°C and then maintaining the temperature at 100°C for 15 min. The media were diluted with the same volume of fresh culture medium and sterilized by filtration through 0.2 µm-pore membranes.

### Assay of phorbol ester-stimulated superoxide-generating activity by enhancer-amplified luminol chemiluminescence

Superoxide-generating activity of THP-1 cells was assayed as reported by de Mendez et al.<sup>13</sup> with a small modification. THP-1 cells

washed with PBS<sup>-</sup> were suspended in PBS<sup>-</sup> containing 2.5 mM glucose, 0.25 mM CaCl<sub>2</sub>, and 0.5 mM MgCl<sub>2</sub> (PBS<sup>+</sup>) at a concentration of 0.5-2.0 ×10<sup>6</sup>/ml. One hundred µl of the cell suspension was diluted with a 100-µl PBS<sup>+</sup> containing 0.5 mM luminol (Sigma) and 150-fold diluted Diogenes (National Diagnostics, Atlanta, GA) and incubated for 0.5 to 1 h at 37°C for the detection of chemiluminescence in a Berthold LB9505C (Berthold, Japan K.K., Tokyo, Japan). Cells were stimulated with 40 ng phorbol myristate acetate (PMA, Sigma) in the presence or absence of 500 U/ml superoxide dismutase (SOD, Wako Chemicals, Tokyo, Japan). Superoxide-generating activity was expressed as PMA-stimulated and SOD-sensitive counts per minute (cpm) per cell observed 15 min after stimulation. The SOD-insensitive fraction of chemiluminescence was approximately 1% of the total.

### Quantification of mRNA by LightCycler PCR

Total RNA was extracted from *E. chaffeensis*-infected and mock-infected cells (1-5 ×10<sup>6</sup>) using TRIZOL LS Reagent (Life Technologies, Gaithersburg, MD). Two micrograms of total RNA was reverse-transcribed in a 20-µl reaction mixture containing 2.5 µM random 9-mer primers, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dNTP, 1 U/µl RNase inhibitor, and 0.25 U/µl AMV reverse transcriptaseXL (TaKaRa Biomedical, Kyoto, Japan) in 10 mM Tris-HCl buffer, pH 8.3. Next, the first strand cDNA was amplified in a sealed capillary glass using the LightCycler-FastStart DNA Master SYBR Green I Kit for real-time PCR (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's instructions. Each reaction mixture contained 1×SYBR Green I Master Mix supplemented with 2.5 mM MgCl<sub>2</sub>, 0.5 µM sense- and anti-sense primers, and target cDNA derived from 75 ng total RNA in a total volume of 10 µl. Real-time PCR was monitored by a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). Primer sequences used in this study were as follows: 5'-CAT GAA GTG TGA CGT GGA CAT CC-3' and 5'-GCT GAT CCA CAT CTG CTG GAA GG-3' for β-actin; 5'-AAA GGA ATG CCC AAT CCC TC-3' and 5'-TCT TCA CTG GCA GTG CCA AA-3' for gp91<sup>phox</sup>; 5'-CTT CGT ACC CAG CCA GCA CTA TGT-3' and 5'-GGT GGG GGA TGA TCC TGT TC-3' for p47<sup>phox</sup>; and 5'-GTT TGC CTG TGA GGT GTT ATA T-3' and 5'-CCA GAG AAA CTG TCT TGA TCC A-3' for p67<sup>phox</sup>. All primers were supplied by Hokkaido System Science (Sapporo, Japan). PCR was performed using an initial denaturation for 10 sec at 95°C, followed by 36 cycles of annealing for 10 sec at 62°C, elongation for 15 sec at 72°C, 1 sec at various temperatures to check fluorescence intensities, and denaturation for 0 sec at 95°C. Fluorescence intensities of the PCR products were checked at the highest temperature that did not allow specific melting: 87°C for β-actin, 83°C for gp91<sup>phox</sup>, 82°C for p47<sup>phox</sup>, and 85°C for p67<sup>phox</sup>. The sizes of the PCR products determined by agarose gel electrophoresis matched their predicted sizes: 229 bp for β-actin, 429 bp for gp91<sup>phox</sup>, 173 bp for p47<sup>phox</sup>, and 294 bp for p67<sup>phox</sup>. Standard lines were generated using serial dilutions of purified PCR products (typically, 3×10<sup>-17</sup> to 3×10<sup>-22</sup> mol), and the log of the fluorescence intensity was plotted

as a function of the cycle number. Linear regression analysis was performed to determine the best fit for the standard line. In addition, the minimal detectable amount of DNA was determined by preliminary experiments. Because infection with *E. chaffeensis* did not change the amount of  $\beta$ -actin mRNA that could be detected in a fixed amount of total RNA, it was used as an internal standard for calculating the relative amount of mRNA.

#### Flow cytometry for p47<sup>phox</sup>

*E. chaffeensis*-infected and mock-infected THP-1 cells ( $10^6$ ) were fixed in 10 ml of 3% paraformaldehyde (Wako Chemicals, Tokyo, Japan) for 30 min at room temperature. Next, the sample was centrifuged and permeabilized for 10 min at room temperature in PBS<sup>-</sup> containing 0.2% Triton X-100 (ICN Biochemicals, Aurora, OH) and 2% bovine serum albumin (BSA, Sigma). Permeabilized cells were treated for 20 min at room temperature with 3 mg/ml human globulin (Cohn Fraction III, Sigma) to prevent nonspecific antibody binding. Cells were mixed overnight at 4°C with a primary mouse monoclonal antibody (IgG<sub>1</sub>, Transduction Laboratories, Lexington, KY) to amino acids 129-153 of human p47<sup>phox</sup> or its corresponding control IgG<sub>1</sub>. Antibody was removed by washing the cells twice with PBS<sup>-</sup> containing 0.2% Triton X-100 and 2% BSA. Washed cells were incubated at room temperature for 1 h with fluorescein isothiocyanate-conjugated secondary antibodies. The cells were washed twice with PBS<sup>-</sup> and suspended in 0.5 ml of PBS<sup>-</sup> containing 0.5 mM EDTA, 10 mM NaN<sub>3</sub>, and 0.2% BSA. Fluorescence was monitored on a FACScan (Nippon Becton-Dickinson K.K., Tokyo, Japan).

#### Statistical analysis

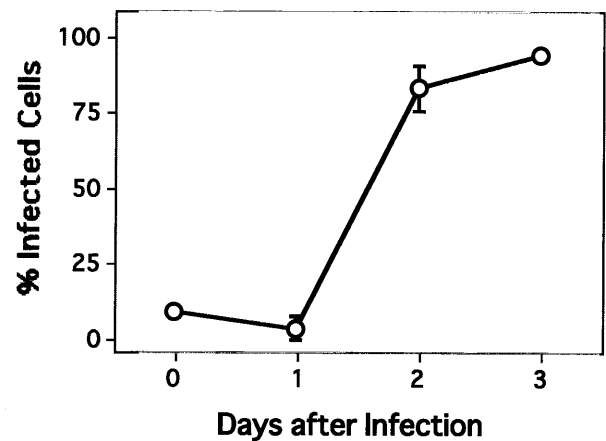
Unless otherwise stated, data represent the mean  $\pm$  standard deviation (SD) from three measurements. In all cases, similar results were reproduced in one or two additional independent experiments. The significance of differences was assessed using Student's *t*-test, and *p*-values <0.05 were considered significant.

## Results

### *E. chaffeensis* infection increases superoxide-generating activity, mRNAs for the phagocyte NADPH oxidase components, and p47<sup>phox</sup> protein in THP-1 cells.

Figure 1 shows the time course of *E. chaffeensis* infection of human monocytic THP-1 cells. There was no significant increase in the infection after one day. After two days, however, there was an abrupt increase in the level of infection ( $p=0.0001$ ), and after three days, approximately one-fourth of the infected cells had burst (data not shown).

As shown in Figure 2 A at day 0, THP-1 cells that were not exposed to *E. chaffeensis* had significant but low PMA-stimulated and SOD-sensitive superoxide generation ( $p=0.0005$ ). The ability to produce superoxide anion increased logarithmically with the number days



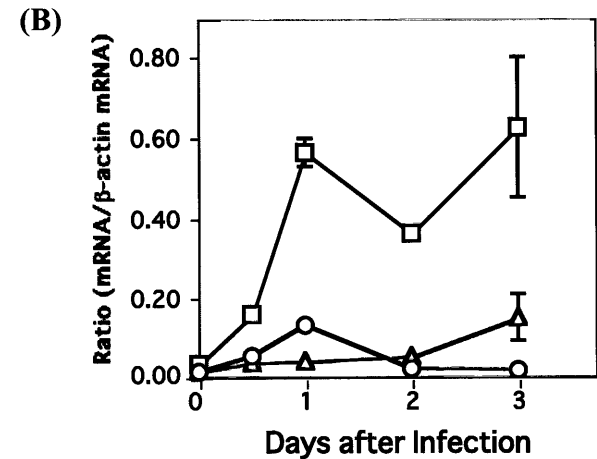
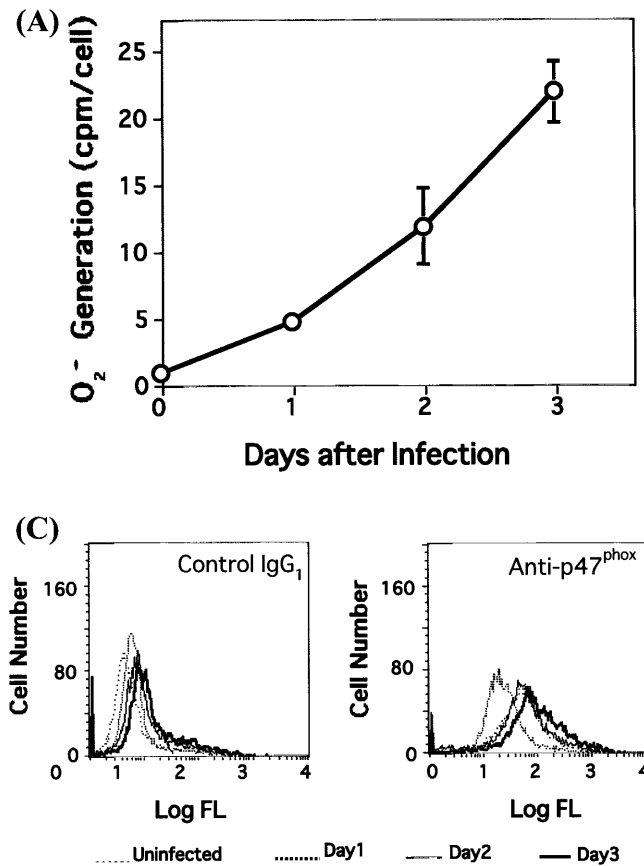
**Figure 1.** Time course of *E. chaffeensis* infection in THP-1 cells. The percent of cells infected with *E. chaffeensis* was determined at the indicated time points. For each time point, more than 300 cells were counted. The results represent the mean  $\pm$  SD ( $n=3$ ). The value for day 2 was significantly different from values for days 0 and 1 ( $p=0.0001$  in both pairs).

of infection with *E. chaffeensis*, and the maximal level was observed after three days ( $p=0.0089$ ). Further observation was not possible because almost all cells were dead four days after infection.

To determine the mechanism of the increased ability to produce superoxide anion, we examined the expression of mRNA for phagocyte NADPH oxidase by real-time PCR. As shown in Figure 2 B, mRNAs for gp91<sup>phox</sup> and p47<sup>phox</sup> significantly increased one day after infection ( $p=0.0001$  in both cases), whereas mRNA for p67<sup>phox</sup> increased after three days ( $p=0.0134$ ). The maximal increases in mRNAs expression for gp91<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> were 8.6-, 15.4-, and 8.3-fold, respectively, compared to uninfected cells. The enhanced expression of p47<sup>phox</sup> mRNA coincided with a significant increase in p47<sup>phox</sup> protein (Figure 2 C). These results suggest that the enhanced ability to generate superoxide anion primarily depends on the increased transcription of genes for the phagocyte NADPH oxidase. We were unable to detect gp91<sup>phox</sup> and p67<sup>phox</sup> protein by FACS, even in the infected THP-1 cells, suggesting that these genes were still in an early stage of activation.

### Soluble factors from *E. chaffeensis*-infected cells enhance the ability of THP-1 cells to produce superoxide anion.

The direct invasion of *E. chaffeensis* to host THP-1 cells did not correlate with the increased ability to produce superoxide anion because mRNAs for gp91<sup>phox</sup> and p47<sup>phox</sup> increased one day after infection (Figure 2 B) when less than 5% of the cells were infected (Figure 1). Therefore, we suspected that factors produced by infected cells and/or *E. chaffeensis* itself could affect non-infected cells. To avoid the effect of direct invasion of *E. chaffeensis*, we continuously exposed THP-1 cells to the culture medium conditioned by *E. chaffeensis*-infected THP-1 cells by using a trans-well culture system. In this system, uninfected cells were kept in an upper trans-well, which allowed only soluble factors to enter from the infected cells



**Figure 2.** Time course of changes in *E. chaffeensis*-infected THP-1 cells. (A) PMA-stimulated superoxide generation following infection. All values significantly differed from each other ( $p=0.0001-0.0005$ ). Values represent the means  $\pm$  SD ( $n=3$ ). (B) Expression of mRNAs for phagocyte NADPH oxidase components following infection. The mRNA levels were normalized to the level of  $\beta$ -actin mRNA. Values represent the means  $\pm$  SD. The values for days 0.5 and 2 represent single determinations, while all others represent three determinations. The relative levels for gp91<sup>phox</sup> (○) and p47<sup>phox</sup> (□) were high on day one, while the level of mRNA for p67<sup>phox</sup> (△) was high on day 3 ( $p=0.001-0.013$ ). (C) Expression of p47<sup>phox</sup>. Infected cells were stained for flow cytometry with control-IgG<sub>1</sub> (left) or anti-p47<sup>phox</sup> (right) before (---), or one (---), two (—), or three days (—) after infection.

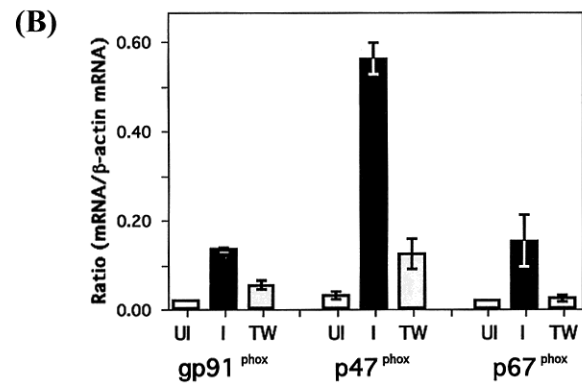
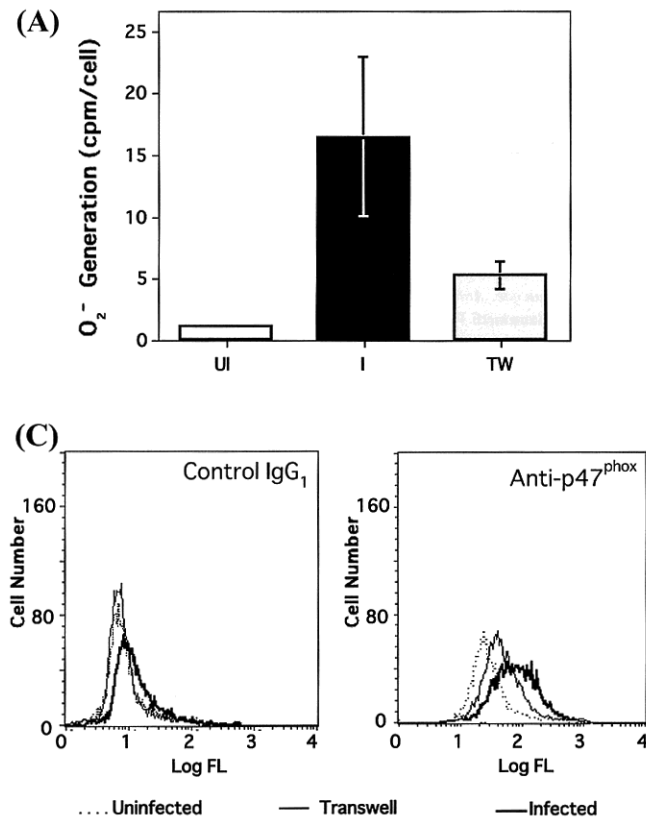
below.

We found that PMA-stimulated superoxide production in the trans-well cells increased significantly ( $p=0.0028$ ), reaching approximately one-third of the activity of directly infected cells (Figure 3 A). *E. chaffeensis* particles were not detected in the upper well THP-1 cells (data not shown), indicating that the results were not due to an accidental contamination with the bacterial particles. These results suggest that soluble factors released from infected cells and/or *E. chaffeensis* can lead to an increase in the ability to produce superoxide anion. Real-time PCR revealed significant increases in the mRNAs for p47<sup>phox</sup> ( $p=0.0037$ ) and gp91<sup>phox</sup> ( $p=0.0103$ ) in the upper wells one day after exposure to the medium of fully infected lower well cells. (Figure 3 B). In addition, we detected the expression of p47<sup>phox</sup> protein in the upper-well cells four days after the exposure (Figure 3 C, right, a solid histogram), though the intensity of the expression was about one-third of that in the cells directly infected with the bacterium for three days (Figure 3 C, right, a bold solid histogram). Therefore, the cognate interaction of THP-1 cells with the *E. chaffeensis* is not essential for the increased expression of the phagocyte oxidase components and the associated increase in the ability to produce superoxide anion. Rather, our results suggest that these effects are mediated by soluble factors released from *E. chaffeensis*-infected THP-1 cells or from the bacterium itself.

*Both heat-stable and -unstable factors of medium conditioned by E. chaffeensis-infected cells can increase the ability of THP-1 cells to produce superoxide anion.*

We further investigated the properties of the factors by directly exposing fresh cells to medium conditioned by *E. chaffeensis*-infected and uninfected cells. In addition, we examined the effect of heat treatment, which denatures protein components, to help determine the nature of the soluble factors. We calculated the heat-unstable fractions and net increases dependent only on *E. chaffeensis* infection based on the ability of the four types of media (heat-treated and -untreated conditioned media from infected and non-infected cells) to increase the ability of THP-1 cells to produce superoxide anion. As shown in Table 1, the heat-stable and calculated heat-unstable fractions of the infection-dependent superoxide production were  $3.5 \pm 0.7$  cpm/cell and  $6.8 \pm 1.7$  cpm/cell, respectively. Therefore the heat-unstable fraction accounted for approximately two-thirds of the total increase activity in the conditioned medium, while the heat-stable portion accounted for approximately one-third of the activity.

Because we suspected that at least a part of the heat-unstable activity is due to cytokines released from *E. chaffeensis*-infected cells, we examined the ability of various cytokine-blocking antibodies to inhibit the increase in ability to produce superoxide anion. However, when added 30 min after infection with *E. chaffeensis*, antibodies



**Figure 3.** Effect of conditioned medium from *E. chaffeensis*-infected THP-1 cells. Uninfected (UI) THP-1 cells or cells infected with *E. chaffeensis* (I), or exposed in trans-wells to medium conditioned by infected cells (TW). **(A)** PMA-stimulated superoxide generation. Values represent the mean ± SD (n=3). TW values were higher than UI values (p=0.0028). **(B)** Expression of mRNAs for the phagocyte NADPH oxidase components. Messenger RNAs for gp91<sup>phox</sup> and p47<sup>phox</sup> and mRNA for p67<sup>phox</sup> were assayed days 1 and 3, respectively. Values represent the means ± SD (n=3). TW values were significantly higher than UI values in expressions of gp91<sup>phox</sup> (p=0.0037) and p47<sup>phox</sup> (p=0.01), but not in the expression of p67<sup>phox</sup> (p=0.434). **(C)** Expression of p47<sup>phox</sup>. Uninfected (---) cells, *E. chaffeensis*-infected cells (—), and cells treated in trans-wells with conditioned medium (—) were stained for flow cytometry with control-IgG<sub>1</sub> (left) or anti-p47<sup>phox</sup> (right).

**Table 1** Heat stability of conditioned medium from uninfected- or *E. chaffeensis*-infected THP-1 cells for increase in superoxide generation<sup>a</sup>

Medium conditioned with	Treatment			p-value <sup>e</sup>
	Heat (-)	Heat (+)	Heat-unstable	
Uninfected cells	1.2±0.4	1.7±0.2	-0.4±0.4	
Infected cells	11.6±1.5	5.2±0.7	6.3±1.6	0.0026
Net increase	10.3±1.5	3.5±0.7	6.8±1.7 <sup>d</sup>	0.0021
p-value <sup>b</sup>	0.0003	0.0011		

<sup>a</sup>Portions of conditioned media were heat-treated (Heat(+)) or -untreated (Heat(-)). THP-1 cells were cultured in these media for 2.5 days and their superoxide-generating activities were assayed. All values with SD were expressed as cpm/cell of PMA-stimulated and SOD-sensitive superoxide generation.

<sup>b</sup>For testing no increase in the net increase (infected cells – uninfected cells).

<sup>c</sup>For testing no heat-unstable fraction (Heat (-) – Heat (+)).

<sup>d</sup>Heat-unstable fraction in net increase. This value was comparable to the net increase calculated in this heat-unstable column (5.9±1.9 cpm/cell).

against TNFα, TGFβ, GM-CSF, IL-1α, IL-1β, IL-6, and IL-10 were unable to inhibit the increased ability of infected THP-1 cells to produce superoxide anion (data not shown). Therefore, it appears that, if a cytokine is involved, it is not one of the ones that we examined. Finally, PCR for mycoplasma-specific 16S rRNA genes was negative (data not shown), excluding any contribution of lipopeptides from mycoplasma to the observed increased ability to produce superoxide anion.

### Discussion

In the current report, we show for the first time that infection of THP-1 cells with *E. chaffeensis* increases their ability to produce superoxide anion and enhances the expression of mRNAs for components of the phagocyte NADPH oxidase (gp91<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup>) and at least one of their protein products, p47<sup>phox</sup>. These results contrast with the finding that the ability to produce superoxide anion is strongly down-regulated in human neutrophils by *A. phagocytophilum* infection as a result of specific inhibition of the CYBB- and RAC2 expression<sup>4</sup> and the reduction of p22<sup>phox</sup>.<sup>5</sup>

*E. chaffeensis* resides *in vivo* in monocytes that can generate superoxide anion. One possible role for the up-regulation of NADPH oxidase components and the result increase in superoxide production is that the superoxide anion plays a direct role in the killing of bacteria. However, even three days after infection, the ability of THP-1 cells to produce superoxide anion is only approximately 1% of the level in human peripheral blood monocytes. Therefore, increased superoxide production may play a limited role in bacterial killing at early stages of infection. Indeed, superoxide anions and the reactive oxygen derivatives that they generate may not be directly involved in the protection against *E. chaffeensis*<sup>14</sup> but rather protect the cells against concomitant infection by other bacteria.

The other possible role for superoxide production is in the induction of specific biological responses to infection by *E. chaffeensis*. For example, superoxide anion increases the effect of nitric oxide, which is generated by an inducible nitric oxide synthase and may be important

in the defense against *E. chaffeensis*.<sup>15</sup> Reactive oxygen and nitrogen species generated in response to bacterial lipopolysaccharide (LPS) act as a second messenger to modulate the intracellular redox potential. In THP-1 cells<sup>16</sup> and pulmonary tissues,<sup>17</sup> LPS activates a mitogen-activated protein kinase pathway and NF- $\kappa$ B, resulting in the induction of inflammation-related genes. However, exposure of THP-1 cells to *E. chaffeensis* does not activate NF- $\kappa$ B *in vitro* in the absence of specific antibody against the bacterium.<sup>8</sup>

In these studies, we found that conditioned medium from *E. chaffeensis*-infected cells can enhance the ability of THP-1 cells to produce superoxide anion. This suggests that monocytic cells, even if immature, can be activated by neighboring *E. chaffeensis*-infected monocytes to generate higher levels of superoxide anion in response to certain phagocytosing stimuli. Moreover, this implies that there are soluble factors that are released by infected cells that can increase the ability of non-infected cells to produce superoxide anion. Indeed, we found that heat-stable and -unstable factors are released by the infected THP-1 cells. These may be either produced by the infected host cells or derived from the bacterium. The nature and origin of the heat-stable factor is not yet clear, although it is likely different from LPS because *E. chaffeensis* lacks the genes for key enzymes in LPS synthesis.<sup>18</sup> Other types of bacterial heat-stable factors, such as lipopeptides and peptide glycans, are candidates, but the purification of the heat-stable factor(s) will be necessary for its identification. Finally, although the heat-stable activity accounts for a substantial portion of the total, most of the activity was heat-sensitive. This activity is similar to the activity of IL-10-inducing activity of *E. chaffeensis* because approximately 80% of it is heat-sensitive, while IL-1 $\beta$ - and IL-8-inducing activities are completely heat-resistant.<sup>7</sup>

Whether the mitogen-activated protein kinase pathway participates in the superoxide-generating response of THP-1 cells to *E. chaffeensis* remains to be determined.

Lin and Rikihisa recently reported that the *E. chaffeensis* infection of human monocytes down-regulates PU.1,<sup>11</sup> a major transcriptional activator for gp91<sup>phox</sup>.<sup>19,20</sup> PU.1 was also reported to be a transcriptional activator of p47<sup>phox</sup>.<sup>21</sup> Based on this information, both gp91<sup>phox</sup> and p47<sup>phox</sup> were expected to be down-regulated in our *E. chaffeensis*-infected THP-1 cells. However, our results show that the transcription of both genes is up-regulated. This discrepancy may imply some stage-specific difference in the role of PU.1 or may reflect differences in the way that the cells were infected; Lin and Rikihisa infected THP-1 cells with purified *E. chaffeensis* while we mixed non-infected cells with infected cells.

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