

# Phagocytosis of Apoptotic Cells Increases the Susceptibility of Macrophages to Infection with *Coxiella burnetii* Phase II through Down-Modulation of Nitric Oxide Production

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*Coxiella burnetii*, the agent of Q fever in humans and coxiellosis in other mammals, is an obligate intracellular bacterium which is sheltered and multiplies within typically large phagolysosome-like replicative vacuoles (LRVs). We have previously shown that, compared with fibroblasts, mouse resident peritoneal macrophages control the development of LRVs and bacterial multiplication within these vacuoles. Earlier experiments with the nitric oxide (NO) synthase inhibitor aminoguanidine (AG) revealed that the control is exerted by NO induced by the bacteria. We report here that phagocytosis of apoptotic-like, but not of aldehyde-killed, lymphocytes by the macrophages reduced the production of NO induced by the bacteria and increased the development of LRVs and, therefore, the total bacterial load in the cultures. Experiments with macrophages from mice deficient for inducible NO synthase (iNOS<sup>-/-</sup>) confirmed the involvement of NO in the control of infection, since neither apoptotic lymphocytes nor AG affected the development of LRVs in these phagocytes. Since macrophages are important for the clearance of apoptotic bodies and *C. burnetii* is able to induce apoptosis in human monocytes, the phenomenon shown here may be biologically relevant to the development of Q fever and coxiellosis.

The gram-negative, obligate intracellular bacterium *Coxiella burnetii*, a member of the  $\gamma$ -subdivision of *Proteobacteria*, is the agent of Q fever in humans, a zoonosis of nearly worldwide distribution (2, 3, 19, 26, 27, 31). Once internalized by permissive host cells, the bacteria are found in small phagosomes which shelter a few organisms and fuse with each other and with other endocytic or phagocytic vesicles. After two or more days, most infected cells display one or more large replicative vacuoles (LRVs), in which the bacterium multiplies (3, 4, 18, 19, 26, 33, 34).

These features describe the infection with virulent phase I or nonvirulent phase II *C. burnetii* of primary or continuous cultures of fibroblasts or epithelial, macrophage-like, or other cell types susceptible to infection (1–3, 26, 29). In contrast, other studies have suggested that human and mouse primary macrophages are less permissive to infection (14, 15, 34, 35). Although the mechanisms by which resistant host cells control *C. burnetii* infection are not entirely understood, there is evidence that tumor necrosis factor alpha and nitric oxide (NO) are involved in the control of LRV formation and of bacterial multiplication (5, 6, 14, 15, 35).

A variety of viral and nonviral pathogens have been shown to kill macrophages or other host cells by a form of programmed cell death known as apoptosis (13). *C. burnetii* is no exception, since it has also been shown to induce apoptosis in infected human monocytes (6, 7).

Apoptotic cells are recognized and phagocytosed by profes-

sional and nonprofessional phagocytes; however, the clearance is mainly made by macrophages (9, 10). It has been also shown that interaction with apoptotic cells can enhance the secretion of antiinflammatory factors, such as transforming growth factor  $\beta$  (TGF- $\beta$ ), interleukin-10 (IL-10), and prostaglandin E2 by macrophages (10, 20). However, whereas the induction of apoptosis by intracellular pathogens has been often examined, few studies have addressed the effect of apoptotic cells on the microbicidal activity of macrophages or other host cells. The realization that antiinflammatory factors such as TGF- $\beta$ , IL-10, or prostaglandin E2 also down-regulate the microbicidal activity of macrophages has led to the pioneering demonstration that uptake of apoptotic lymphocytes by macrophages increases the in vivo and in vitro susceptibility to infection of mice with the trypanosomatid parasite *Trypanosoma cruzi* (11). It was also proposed that phosphatidylserine expressed on *Leishmania* parasites has a role in the recognition and intracellular survival of the parasite in mouse macrophages (12). Indeed, it has recently been shown that the uptake of apoptotic cells by human immunodeficiency virus-infected primary macrophages increases viral replication (25).

In this report, we have investigated if the uptake of apoptotic cells enhances the infection of primary mouse macrophages by the intracellular bacterial pathogen *C. burnetii*. Our results show that cultures that have ingested apoptotic-like cells become more susceptible to infection with *C. burnetii* phase II in a process mediated by down-regulation of NO production by macrophages.

## MATERIALS AND METHODS

**Macrophage culture.** Primary macrophages were obtained from 7- to 9-week-old C57BL/6 female mice. Animals that did not express the inducible nitric oxide synthase (iNOS KO) were provided by Elaine Rodrigues (Universidade Federal

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de São Paulo). Resident peritoneal macrophages (PM $\phi$ ) were collected by wash-outs of mouse peritoneal cavities as described previously (34). Cells ( $1.5 \times 10^5$ ), attached to 13-mm-diameter glass coverslips, were placed in 2-cm<sup>2</sup> wells of 24-well tissue culture plates and kept in 0.5 ml of antibiotic-free Dulbecco modified essential medium containing 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 2 g of sodium bicarbonate/liter, 1 mM L-glutamine, and 5% (vol/vol) fetal bovine serum. Experiments were started 24 h after preparation of the PM $\phi$  cultures.

Bone marrow-derived macrophages (BMM $\phi$ ) were generated from bone marrow stem cells cultured on bacteriological plastic plates for 7 days in RPMI 1640 medium containing 15 mM HEPES, 2 g of sodium bicarbonate/liter, 1 mM L-glutamine, and supplemented with 20% fetal bovine serum and 30% L929 cell-conditioned medium. Differentiated BMM $\phi$  were removed from the substrate by vigorous pipetting of ice-cold phosphate-buffered saline. Cells were seeded at  $1.5 \times 10^5$  to glass coverslips inserted in 24-well tissue culture plates and cultured in RPMI supplemented with 10% fetal bovine serum and 5% L929 cell-conditioned medium. All cultures were kept at 36°C in 5% CO<sub>2</sub> in an air atmosphere.

**Lymphocyte preparation and apoptosis induction.** Lymphocytes were purified from C57BL/6 mouse spleens. The spleen was mechanically disrupted, and the released cells were washed and treated with ACK buffer (150 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA) for 10 min at room temperature to lyse red blood cells. Splenocytes were washed and maintained in complete RPMI medium supplemented with 10% fetal bovine serum. Apoptosis of the lymphocytes was induced by heating at 56°C for 30 min (Apo-heat) or by gamma irradiation at 3,000 rads (30 Gy) (Apo-irrad) as previously described (11, 16). Lymphocytes heated to 56°C were terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling positive and displayed nuclear fragmentation. However, soon after heating these cells became permeable to propidium iodide, a characteristic of necrotic cells. These features can be prevented by pretreatment of the cells with the general caspase inhibitor z-VAD-FMC (M. F. Lopes, personal communication). It is now clear that the features classically described as specific for apoptotic cells may depend on the cell type and on the conditions that trigger apoptosis. DNA fragmentation has been described in cells subjected to treatments that are not associated with induction of apoptosis (28). For these reasons we refer to these cells as apoptotic-like. Fixed splenocytes were obtained by addition of 1% paraformaldehyde for 20 min at room temperature (followed by extensive washing). Lysed cells were obtained by freezing at -20°C and thawing at 37°C. Splenocyte suspensions were used at a multiplicity of infection of 3 cells per macrophage. Aminoguanidine (AG; Sigma) was used at a 1 mM concentration. Macrophage cultures were treated with lymphocytes or AG, 24 h prior to *C. burnetii* infection. The latter were maintained until the end of the experiment.

**Phagocytic index and nitrite determination.** To determine the phagocytic index, macrophage cultures were fixed 3 h after addition of apoptotic or fixed cells. 4',6'-diamidino-2-phenylindole (DAPI) and an in situ cell death detection kit (Roche) were used to stain the internalized fixed or apoptotic-like cells. Between 300 and 500 macrophages per treatment were scored in four different fields per coverslip to estimate the phagocytic index [percentage of macrophages that phagocytosed splenocytes  $\times$  (total number of internalized splenocytes/total number of macrophages that phagocytosed splenocytes)].

The concentration of nitrite (NO<sub>2</sub><sup>-</sup>), the stable oxidized derivative of NO, was determined in 100- $\mu$ l aliquots of cell culture supernatants transferred to 96-well plates. One hundred microliters of Griess reagent solution (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, and 2% H<sub>3</sub>PO<sub>4</sub>) was added per well, and absorbances were measured at 540 nm in a microplate enzyme-linked immunosorbent assay reader. Sodium nitrite diluted in culture medium was used as a standard.

**Bacterial preparation and infection of host cells.** The clone 4 phase II Nine Mile strain of *C. burnetii*, which is infective for cells but not for mammals, was provided by Ted Hackstadt (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Mont.) and handled in a biosafety level II facility (17, 21). Infective inocula were obtained as described previously (33) from confluent Vero cells infected with *C. burnetii* for 7 days. Prior to infection, to disrupt aggregates, suspensions containing about 10<sup>9</sup> infective bacteria per ml were mildly sonicated at 35 kHz for 15 min at room temperature. Macrophage cultures were infected with about 100 infective organisms per cell. After 24 h, infected cultures were vigorously washed with Hanks saline, and the appropriate fresh medium was added.

**Cell fixation, staining, and determination of the percentage of LRVs.** At different times after infection, cultures were fixed for 1 h in 3.5% formaldehyde containing 7.5% sucrose, stained for 15 min with 3.5  $\mu$ M DAPI, washed, and mounted in 50% glycerol (vol/vol). At least 350 cells in each of triplicate cover-

slips were scored with a 40 $\times$  objective in an inverted microscope for the presence or absence of large *C. burnetii* replicative vacuoles (LRVs).

**Confocal microscopy, image acquisition, and determination of relative *C. burnetii* load.** Images were acquired in a Bio-Rad 1024UV confocal system attached to a Zeiss Axiovert 100 microscope equipped with a 40 $\times$  water immersion objective, numerical aperture 1.2 plan-apochromatic lens and with differential interference contrast. LaserSharp 1024 version 2.1A from Bio-Rad was used for image acquisition, and MetaMorph (version 3.5; Universal Imaging Corporation) was used for image processing. Polygons were drawn onto digitized images of infected cells, and the relative fluorescence intensity of DAPI-stained bacteria within the circumscribed areas was determined. Under the measurement conditions used, the fluorescence intensity within each polygon was assumed to be proportional to the bacterial load in the region measured, as previously described (33). Between 60 and 90 vacuoles were measured in each of triplicate coverslips.

**Bacterial viability.** Infected macrophages were lysed in H<sub>2</sub>O, a step that did not reduce the bacterial infectivity; lysates were sonicated, diluted, and used to infect monolayers of Vero cells, previously irradiated (1,000 rads) to block cell multiplication. Irradiated Vero cells were transferred to glass coverslips inserted in the wells of 24-well tissue culture plates and cultured in minimal essential medium supplemented with 5% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> atmosphere. Four days after infection, Vero cells were washed with phosphate-buffered saline, fixed, and stained with DAPI as described above. An epifluorescence microscope equipped with a 40 $\times$  objective was used to score the percentage of infected cells. Dilutions chosen for counting contained in the range of 10 to 60% infected cells. About 500 cells per coverslip were scored.

**Statistical analysis.** Each experiment was performed with triplicate sets of wells. All tabulated or illustrated values are the averages of the triplicates from one representative experiment out of two or more performed. Significant differences between means were determined by either Student's *t* test or an analysis of variance. Both tests were provided by the Microsoft Excel (version 97) statistics package. Differences were taken as statistically significant when *P* was <0.05.

## RESULTS AND DISCUSSION

**Uptake of apoptotic-like splenocytes increases the formation of *C. burnetii* LRVs in macrophages.** Resident PM $\phi$  cultures were incubated with suspensions of apoptotic-like splenocytes (Apo-heat) or cells fixed with formaldehyde at a multiplicity of three particles per macrophage. Splenocytes heated to 56°C for 30 min were previously shown to display several apoptotic-like features (11, 16). Both apoptotic-like and aldehyde-fixed cells are effectively phagocytosed by macrophages. Control macrophages did not receive lymphocyte suspensions. Twenty-four hours later, cultures were washed and infected with *C. burnetii* phase II bacteria and fixed at different times after the infection. The percentage of macrophages that displayed LRVs was estimated as described in Materials and Methods. Figure 1A shows that compared to untreated or aldehyde-fixed cells, the uptake of apoptotic-like lymphocytes significantly increased the formation of LRVs, which is an essential step in the *C. burnetii* intracellular life cycle (35).

Figure 1B shows a representative field of a PM $\phi$  culture infected for 6 days with *C. burnetii* phase II: only one cell presented the typical LRV, and another two cells were also infected but controlled the LRV formation.

**Although relative bacterial loads in LRVs did not change, infective bacterial loads per well were increased in macrophage cultures that phagocytosed apoptotic cells.** Relative bacterial loads in LRVs were estimated by quantitative fluorescence in digital images (DAPI-stained cells) obtained by confocal microscopy as described in Materials and Methods. Figure 2A shows that bacterium-associated fluorescence of LRVs did not differ in control macrophage cultures when compared to cultures that internalized apoptotic-like or aldehyde-

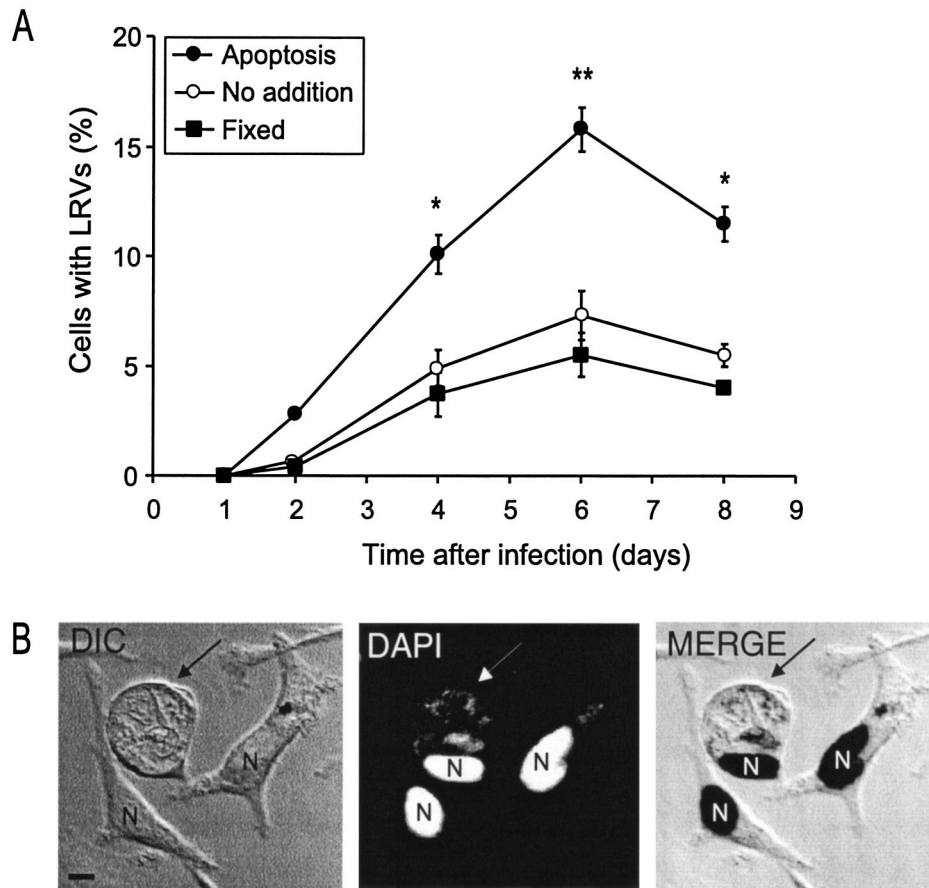


FIG. 1. Development of *C. burnetii* phase II LRVs in mouse PM $\phi$  that internalized apoptotic-like or aldehyde-fixed splenocytes. Splenocytes were added at a multiplicity of three cells per macrophage. Twenty-four hours after treatment, cultures were infected with *C. burnetii* phase II, fixed at different times, and processed as described in Materials and Methods. (A) Percentage of cells with LRVs as a function of the duration of infection. Closed circles, cultures treated with apoptotic-like heated lymphocytes (Apoptosis); open circles, no addition of splenocytes to the cultures (No addition); closed squares, cultures treated with fixed lymphocytes (Fixed). Asterisks indicate statistically significant differences between the treatments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (B) Confocal images of a macrophage culture infected for 6 days with *C. burnetii*. DIC, Nomarski differential interference contrast; DAPI, DNA fluorescence of the same field; MERGE, superimposed confocal images of DAPI fluorescence (converted to black) and Nomarski DIC (gray). The arrow points to one cell with LRV. N, cell nuclei. Bar = 10  $\mu$ m.

fixed splenocytes. However, the overall infective bacterial numbers recovered from macrophages that received apoptotic-like splenocytes were significantly higher (Fig. 2B). It was previously shown that the LRVs are the main sites of *C. burnetii* multiplication (33–35). This could explain why the uptake of apoptotic-like cells increased the formation of LRVs (Fig. 1) and the numbers of infective bacteria per culture well (Fig. 2B), although the bacterial load per LRV was similar in treated and in untreated cultures (Fig. 2A).

Interpretation of the results presented so far requires information on the relative uptake of the different splenocyte suspensions by macrophage cultures. Figure 3 shows that the phagocytic indices estimated in cultures fixed 3 h after addition of splenocytes were similar for apoptotic-like or aldehyde-fixed cells. The percentages of cells that phagocytosed heated, irradiated, or fixed splenocytes were, respectively,  $41\% \pm 10\%$ ,  $38\% \pm 3\%$ , and  $46\% \pm 13\%$  (means  $\pm$  standard deviation). The average number of splenocytes per cell in macrophages that ingested heated, irradiated, or fixed splenocytes was  $2.4 \pm$

$0.8$  ( $n = 319$ ),  $2.5 \pm 0.4$  ( $n = 436$ ), and  $2.0 \pm 0.4$  ( $n = 348$ ), respectively. Thus, statistically significant differences were not detected for either the phagocytic indices or the average numbers of particles taken up by the cells that phagocytosed.

**Uptake of apoptotic-like cells reduces *C. burnetii*-induced NO production by macrophages.** It was previously shown that infection with *C. burnetii* phase I or II induced NO production by primary macrophages (32, 35). To investigate if uptake of apoptotic-like cells affected the production of NO induced by *C. burnetii*, we measured nitrite concentrations in cultures treated or not with apoptotic-like or fixed splenocytes added to the cultures 24 h prior to *C. burnetii* bacteria. Figure 4 shows that *C. burnetii*-induced production of NO was reduced in macrophages that phagocytosed apoptotic-like but not aldehyde-fixed splenocytes. NO production by the latter did not differ from that of untreated cultures. These data are consistent with previous observations reporting that uptake of apoptotic cells down-regulates the NO production in macrophages stimulated by gamma interferon and lipopolysaccharide (11, 12).

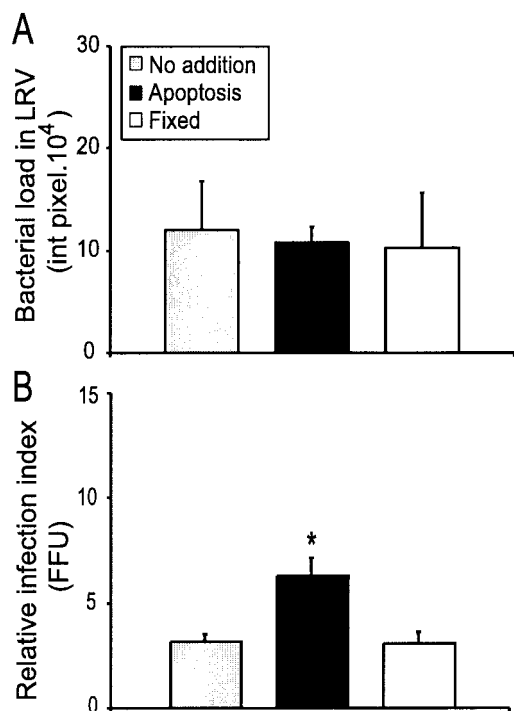


FIG. 2. Relative intracellular load of *C. burnetii* phase II in mouse PM $\phi$  cultures treated with apoptotic or aldehyde-fixed lymphocytes. Twenty-four hours prior to *C. burnetii* infection, cultures were treated with three apoptotic or fixed particles per macrophage; 6 days after infection, cultures were fixed and processed as described in Materials and Methods. (A) Relative intracellular load in the LRVs. (B) Infective bacterial load recovered from infected macrophages, with results given as the relative infection index (FFU). Gray bars, untreated cultures (No addition); black bars, treatment with apoptotic-like lymphocytes induced by heating (Apoptosis); open bars, treatment with fixed lymphocytes (Fixed). Asterisks indicate statistically significant differences between the treatments ( $P < 0.05$ ).

**Increased LRV formation in macrophages that have internalized apoptotic-like cells is NO dependent.** The results described above demonstrate that uptake of apoptotic-like cells increased the development of LRVs in macrophages while it diminished *C. burnetii*-induced NO production. We have previously found that the development of LRVs is increased by AG, an inhibitor of iNOS, added to the macrophage cultures together with the bacteria (35). To further examine the role of NO in the increased formation of LRVs induced by the uptake of apoptotic cells, we investigated the effects of apoptotic cells and of AG when given singly or in combination to peritoneal or bone marrow-derived macrophages 1 day prior to infection with *C. burnetii* phase II bacteria. We have previously shown that PM $\phi$  were more able to restrict LRV formation than BMM $\phi$  (35). This difference is also evident in the results shown in Table 1. However, the effects of the different treatments were similar in both macrophage populations.

As previously described, AG or apoptotic-like cells (Apo-heat)—but not fixed cells—given alone increased similarly the percentage of cells that developed LRVs in peritoneal as well as in BMM $\phi$  (Table 1). However, the effects of AG and of apoptotic-like cells on the percentage of LRVs in peritoneal or BMM $\phi$  were not additive, i.e., joint treatment with apoptotic-

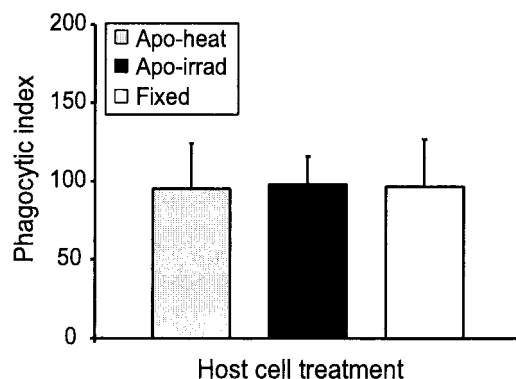


FIG. 3. Uptake of splenocytes by PM $\phi$ . Cultures received splenocytes that were either heat treated (Apo-heat), irradiated with 3,000 rads (Apo-irrad), or fixed at a multiplicity of three cells per macrophage and were fixed after 3 h. Phagocytic indices were determined as described in Materials and Methods. Gray bars, Apo-heat; black bars, Apo-irrad; open bars, fixed. Statistically significant differences were not found between the treatments ( $P > 0.05$ ).

like cells and AG did not significantly increase the formation of LRVs compared to Apo-heat or AG alone (Table 1). These results suggest that NO may play a role in LRV formation in cultures treated by apoptotic cells. Table 1 also shows the effects of the uptake of other particles in the control of LRV formation by PM $\phi$  or BMM $\phi$ . For both PM $\phi$  and BMM $\phi$ , statistically significant differences between untreated versus treated cultures were found in the Apo-heat, Apo-irrad, AG, and Apo-heat-plus-AG groups (Table 1).

To further examine the role of NO in the increased LRV formation after uptake of apoptotic cells, we performed experiments with macrophages from mice deficient in the iNOS gene (iNOS<sup>-/-</sup>). As described in Fig. 1 and Table 1, wild-type macrophages increased the frequency of LRVs when treated

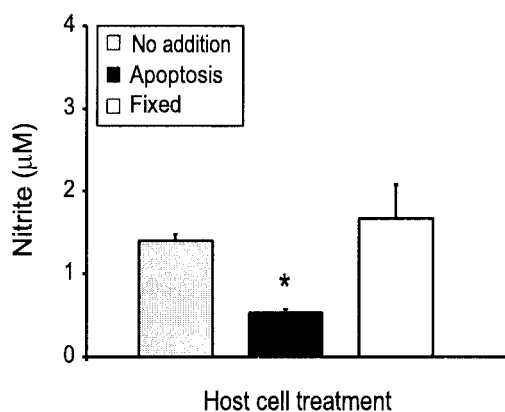


FIG. 4. Effect of splenocyte treatments on NO production induced by *C. burnetii* infection. Twenty-four hours prior to *C. burnetii* infection, cultures received apoptotic-like (heat-treated) or aldehyde-fixed splenocytes at a multiplicity of three cells per macrophage. Culture supernatants were collected for nitrite determination 1 day after infection as described in Materials and Methods. Gray bars, untreated cultures (No addition); black bars, cultures treated with apoptotic-like heated lymphocytes (Apoptosis); open bars, cultures treated with fixed lymphocytes (Fixed). Asterisks indicate statistically significant differences between the treatments ( $P < 0.05$ ).

TABLE 1. Effect of uptake of different agents on infection of BMM $\phi$  or PM $\phi$  with *C. burnetii* phase II

Host cell treatment <sup>a</sup>	PM $\phi$ <sup>b</sup>	BMM $\phi$ <sup>b</sup>
No addition	8.0 $\pm$ 0.4	11.1 $\pm$ 1.2
Apo-heat	14.7 $\pm$ 0.2**	33.4 $\pm$ 7.4*
Apo-irrad	14.5 $\pm$ 1.7*	20.6 $\pm$ 2.5**
Fixed	9.2 $\pm$ 1.3	15.1 $\pm$ 5.8
Lysed	8.5 $\pm$ 0.2	13.4 $\pm$ 0.7
Live splenocytes	9.3 $\pm$ 0.6	9.0 $\pm$ 0.5
AG	13.8 $\pm$ 0.2**	22.8 $\pm$ 0.9*
Apo-heat + AG	15.2 $\pm$ 0.9*	28.0 $\pm$ 5.6*

<sup>a</sup> Macrophages were treated 24 h prior to infection with three particles per cell or 1 mM AG.

<sup>b</sup> Percentage of cells (mean  $\pm$  standard error) containing LRVs 6 days after *C. burnetii* infection. Significantly different result compared to control cells are indicated with asterisks (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

with AG or heat-treated but not fixed splenocytes. In contrast, macrophages from *iNOS*<sup>-/-</sup> mice did not statistically differ between the treatments (Fig. 5). It is of particular interest that, in *iNOS*<sup>-/-</sup> macrophages, the frequency of LRVs was increased in all groups, i.e., in infected but otherwise untreated controls and cultures treated with AG as well as in those that phagocytosed apoptotic-like and fixed splenocytes (Fig. 5).

**The increased bacterial load recovered from macrophage cultures that internalized apoptotic-like cells was NO dependent.** We have shown that uptake of apoptotic cells increased the frequency of LRVs in macrophages from wild-type but not from *iNOS*<sup>-/-</sup> mice (Fig. 5). If LRVs are the main sites of *C. burnetii* multiplication, we should be able to recover higher

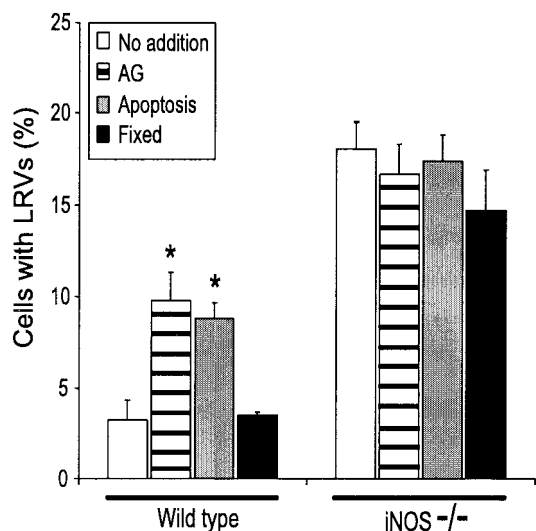


FIG. 5. Effects of treatment with AG or apoptotic or fixed cells on the formation of LRVs in PM $\phi$  from wild-type or *iNOS*<sup>-/-</sup> mice (*iNOS*<sup>-/-</sup>). Twenty-four hours prior to *C. burnetii* phase II infection, cultures were treated with three apoptotic or fixed particles per macrophage or with 1 mM AG. Six days after infection, cultures were fixed and processed as described in Materials and Methods. Open bars, untreated cultures (No addition); hatched bars, cultures treated with AG; gray bars, cultures treated with apoptotic-like heated lymphocytes (Apoptosis); black bars, cultures treated with fixed lymphocytes (Fixed). Asterisks indicate statistically significant differences between the treatments ( $P < 0.05$ ).

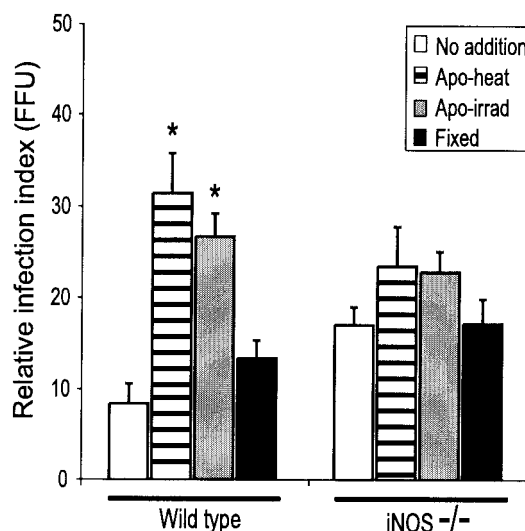


FIG. 6. Effect of treatment with apoptotic or fixed cells on the infective bacterial load (FFU) recovered from PM $\phi$  from wild-type or *iNOS*<sup>-/-</sup> mice. Twenty-four hours prior to *C. burnetii* phase II infection, cultures were treated with three apoptotic or fixed splenocytes per macrophage. Six days after infection, cultures were fixed and processed as described in Materials and Methods. Open bars, untreated cultures (No addition); hatched bars, cultures treated with apoptotic-like heated lymphocytes (Apo-heat); gray bars, cultures treated with apoptotic-like irradiated lymphocytes (Apo-irrad); black bars, cultures treated with fixed lymphocytes (Fixed). Asterisks indicate statistically significant differences between the treatments ( $P < 0.05$ ).

amounts of infective bacteria (focus-forming units [FFU]) from cultures that exhibit larger numbers of LRVs. Thus, we performed an experiment similar to those shown in Fig. 5, but instead of estimating the proportion of LRVs in cultures we determined the relative loads of infective bacteria recovered from the cultures as FFU (6, 22, 35). Figure 6 shows that cultures treated with apoptotic splenocytes yielded more infective *C. burnetii* bacteria than untreated cultures or those treated with fixed splenocytes. In contrast, similar amounts of infective bacteria were recovered from *iNOS*<sup>-/-</sup> macrophages treated with either apoptotic or fixed splenocytes (Fig. 6). The results shown in Fig. 5 and 6 strongly suggest that the uptake of apoptotic-like splenocytes but not that of control particles increased the susceptibility of primary macrophages to infection with *C. burnetii* phase II. Furthermore, the effect of apoptotic lymphocytes, similar to that of the NO synthase inhibitor AG, was mediated by down-regulation of the production of NO by the phagocytes. Consistent with these data, it was recently and independently shown that NO controls *C. burnetii* phase II multiplication and the formation of LRVs (22, 35).

Due to the importance of the LRVs to *C. burnetii* pathogenesis, the control of their formation could be important in establishing the difference between resistance and susceptibility to infection. However, mechanisms other than those involving NO may also be involved in the control (35). Interestingly, the amount of intracellular bacteria does not appear to affect the formation of LRVs, since the infection of host cells with lower or higher multiplicities of infection (30, 100, or 300 bacteria/cell) did not affect the proportion of LRVs in murine macrophages (data not shown). Accordingly, Howe and his col-

leagues have recently demonstrated that only 10 infective *C. burnetii* bacteria are sufficient to induce LRV formation in Vero cells (23).

Future studies will be required to assess the pathways and mechanisms by which the uptake of apoptotic cells down-modulates the production of NO. In this context, several studies have revealed that phagocytosis of apoptotic cells inhibits the production of IL-1 $\beta$ , IL-8, IL-12, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha (8, 12, 24, 30). In contrast, the production of other mediators such as TGF- $\beta$ , prostaglandin E2, and platelet-activating factor increased (8, 11, 12, 24). It is possible that some of these cytokines, singly or in combination, may account for the modulation of NO production and therefore affect the intracellular growth of *C. burnetii*.

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#### REFERENCES

- Baca, O. G., E. T. Akporiaye, A. S. Aragon, I. L. Martinez, M. V. Robles, and N. L. Warner. 1981. Fate of phase I and phase II *Coxiella burnetii* in several macrophage-like tumor cell lines. *Infect. Immun.* **33**:258–266.
- Baca, O. G., and D. Paretsky. 1983. Q fever and *Coxiella burnetii*: a model for host-parasite interactions. *Microbiol. Rev.* **46**:127–149.
- Baca, O. G., Y. P. Li, and H. Kumar. 1994. Survival of the Q fever agent *Coxiella burnetii* in the phagolysosome. *Trends Microbiol.* **2**:476–480.
- Beron, W., M. Gutierrez, M. Rabinovitch, and M. I. Colombo. *Coxiella burnetii* phase II localizes in a Rab-7-labeled compartment with autophagic characteristics. *Infect. Immun.* **70**:5816–5821.
- Capo, C., F. P. Lindberg, S. Meconi, Y. Zaffran, G. Tardei, E. J. Brown, D. Raoult, and J. L. Mege. 1999. Subversion of monocyte functions by *Coxiella burnetii*: impairment of the cross-talk between  $\alpha_v\beta_3$  integrin and CR3. *J. Immunol.* **163**:6078–6085.
- Dellacasagrande, J., C. Capo, D. Raoult, and J. L. Mege. 1999. IFN- $\gamma$ -mediated control of *Coxiella burnetii* survival in monocytes: the role of cell apoptosis and TNF. *J. Immunol.* **162**:2259–2265.
- Dellacasagrande, J., E. Ghigo, D. Raoult, and J. L. Mege. 2002. IFN- $\gamma$ -induced apoptosis and microbicidal activity in monocytes harboring the intracellular bacterium *Coxiella burnetii* require membrane TNF and homotypic cell adherence. *J. Immunol.* **169**:6309–6315.
- Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott, and P. M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- $\beta$ , PGE2, and PAF. *J. Clin. Investig.* **101**:890–898.
- Fadok, V. A., D. L. Bratton, and P. M. Henson. 2001. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J. Clin. Investig.* **108**:957–962.
- Fadok, V. A., and G. Chimini. 2001. The phagocytosis of apoptotic cells. *Semin. Immunol.* **13**:365–372.
- Freire-de-Lima, C. G., D. O. Nascimento, M. B. Soares, P. T. Bozza, H. C. Castro-Faria-Neto, F. G. de Mello, G. A. DosReis, and M. F. Lopes. 2000. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* **403**:199–203.
- Freitas Balanco, J. M., M. E. Moreira, A. Bonomo, P. T. Bozza, G. Amante-Mendes, C. Pirmez, and M. A. Barcinski. 2001. Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. *Curr. Biol.* **11**:1870–1873.
- Gao, L. Y., and Y. A. Kwaik. 2000. The modulation of host cell apoptosis by intracellular bacterial pathogens. *Trends Microbiol.* **8**:306–313.
- Ghigo, E., C. Capo, D. Raoult, and J. L. Mege. 2001. Interleukin-10 stimulates *Coxiella burnetii* replication in human monocytes through tumor necrosis factor down-modulation: role in microbicidal defect of Q fever. *Infect. Immun.* **69**:2345–2352.
- Ghigo, E., C. Capo, C. H. Tung, D. Raoult, J. P. Gorvel, and J. L. Mege. 2002. *Coxiella burnetii* survival in THP-1 monocytes involves the impairment of phagosome maturation: IFN- $\gamma$  mediates its restoration and bacterial killing. *J. Immunol.* **169**:4488–4495.
- Griffith, T. S., X. Yu, J. M. Herndon, D. R. Green, and T. A. Ferguson. 1996. CD95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance. *Immunity* **5**:7–16.
- Hackstadt, T. 1996. Biosafety concerns and *Coxiella burnetii*. *Trends Microbiol.* **4**:341–342.
- Heinzen, R. A., M. A. Scidmore, D. D. Rockey, and T. Hackstadt. 1996. Differential interaction with endocytic and exocytic pathways distinguishing parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. *Infect. Immun.* **64**:796–809.
- Heinzen, R. A., T. Hackstadt, and J. E. Samuel. 1999. Developmental biology of *Coxiella burnetii*. *Trends Microbiol.* **7**:149–154.
- Henson, P. M., D. L. Bratton, and V. A. Fadok. 2001. Apoptotic cell removal. *Curr. Biol.* **11**:R795–R805.
- Hoover, T. A., D. W. Culp, M. H. Vodkin, J. C. Williams, and H. A. Thompson. 2002. Chromosomal DNA deletions explain phenotypic characteristics of two antigenic variants, phase II and RSA 514 (Crazy), of the *Coxiella burnetii* Nine Mile strain. *Infect. Immun.* **70**:6726–6733.
- Howe, D., L. F. Barrows, N. M. Lindstrom, and R. A. Heinzen. 2002. Nitric oxide inhibits *Coxiella burnetii* replication and parasitophorous vacuole maturation. *Infect. Immun.* **70**:5140–5147.
- Howe, D., J. Melnickova, I. Barak, and R. A. Heinzen. 2003. Maturation of the *Coxiella burnetii* parasitophorous vacuole requires bacterial protein synthesis but not replication. *Cell. Microbiol.* **5**:469–480.
- Huynh, M. L., V. A. Fadok, and P. M. Henson. 2002. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF- $\beta$ 1 secretion and the resolution of inflammation. *J. Clin. Investig.* **109**:41–50.
- Lima, R. G., J. V. Weyenbergh, E. M. B. Saraiva, M. Barral-Netto, B. Galvao-Castro, and D. C. Bou-Habib. 2002. The replication of HIV type 1 in macrophages is enhanced after phagocytosis of apoptotic cells. *J. Infect. Dis.* **185**:1561–1566.
- Maurin, M., and D. Raoult. 1999. Q Fever. *Clin. Microbiol. Rev.* **14**:518–553.
- Norlander, L. 2000. Q fever epidemiology and pathogenesis. *Microbes Infect.* **2**:417–424.
- Proskuryakov, S. Y., V. L. Gabai, and A. G. Konoplyannikov. 2002. Necrosis is an active and controlled form of programmed cell death. *Biochemistry* **67**:387–408.
- Tujulin, E., B. Lilliehook, A. Macellaro, A. Sjøstedt, and L. Norlander. 1999. Early cytokine induction in mouse P388D1 macrophages infected by *Coxiella burnetii*. *Vet. Immunol. Immunopathol.* **68**:159–168.
- Voll, R. E., M. Herrmann, E. A. Roth, C. Stach, J. R. Kalden, and I. Girkontaite. 1997. Immunosuppressive effects of apoptotic cells. *Nature* **390**:350–351.
- Williams, J. C., and H. A. Thompson (ed.). 1991. Q fever: the biology of *Coxiella burnetii*. CRC Press, Boca Raton, Fla.
- Yoshiie, K., S. Matayoshi, T. Fujimura, N. Maeno, and H. Oda. 1999. Induced production of nitric oxide and sensitivity of alveolar macrophages derived from mice with different sensitivity to *Coxiella burnetii*. *Acta Virol.* **43**:273–278.
- Zamboni, D. S., R. A. Mortara, and M. Rabinovitch. 2001. Infection of Vero cells with *Coxiella burnetii* phase II: relative intracellular bacterial load and distribution estimated by confocal laser scanning microscopy and morphometry. *J. Microbiol. Methods* **43**:223–232.
- Zamboni, D. S., E. Freymuller, R. A. Mortara, and M. Rabinovitch. 2002. Mouse resident peritoneal macrophages partially control in vitro infection with *Coxiella burnetii* phase II. *Microbes Infect.* **4**:591–598.
- Zamboni, D. S., and M. Rabinovitch. 2003. Nitric oxide partially controls *Coxiella burnetii* phase II infection in mouse primary macrophages. *Infect. Immun.* **71**:1225–1233.