Deciphering the Pathways of Death of Histoplasma capsulatum-Infected Macrophages: Implications for the Immunopathogenesis of Early Infection

George S. Deepe, Jr. and William R. Buesing

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Deciphering the Pathways of Death of *Histoplasma capsulatum*-Infected Macrophages: Implications for the Immunopathogenesis of Early Infection

George S. Deepe, Jr.* † and William R. Buesing †

Apoptosis of leukocytes is known to strongly influence the immunopathogenesis of infection. In this study, we dissected the death pathways of murine macrophages (Mφs) infected with the intracellular pathogen *Histoplasma capsulatum*. Yeast cells caused apoptosis of Mφs at a wide range of multiplicity of infection, but smaller inocula resulted in delayed detection of apoptosis. Upon infection, caspases 3 and 1 were activated, and both contributed to cell death; however, only the former was involved in apoptosis. The principal driving force for apoptosis involved the extrinsic pathway via engagement of TNFRI by TNF-α. Infected Mφs produced IL-10 that dampened apoptosis. The chronology of TNF-α and IL-10 release differed in vitro. The former was detected by 2 h postinfection, and the latter was not detected until 8 h postinfection. In vivo, the lungs of TNFRI−/− mice infected for 1 d contained fewer apoptotic Mφs than wild-type mice, whereas the lungs of IL-10−/− mice exhibited more. Blockade of apoptosis by a pan-caspase inhibitor or by simvastatin sharply reduced the release of TNF-α but enhanced IL-10. However, these treatments did not modify the fungal burden in vitro over 72 h. Thus, suppressing cell death modulated cytokine release but did not alter the fungal burden. These findings provide a framework for the early pathogenesis of histoplasmosis in which yeast cell invasion of lung Mφs engenders apoptosis, triggered in part in an autocrine TNF-α–dependent manner, followed by release of IL-10 that likely prevents apoptosis of newly infected neighboring phagocytes. The Journal of Immunology, 2012, 188: 334–344.
infection and appears to be an autocrine-induced event through the release of TNF-α. Subsequently, these MΦs release IL-10, which inhibits apoptosis of neighboring phagocytes, thus promoting and delimiting the intracellular residence of this fungal pathogen.

Materials and Methods

**Mice**

C57BL/6, TNFR1−/−, and IL-10−/− male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, University of Cincinnati, which is accredited by the American Association for Accreditation of Laboratory Animal Medicine. All animal experiments were done in accordance with the Animal Welfare Act guidelines of the National Institutes of Health.

**Reagents**

Abs to TNF-α and IL-10R and isotype control were purchased from BioLegend (San Diego, CA). Campothecin was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO. It was used at a concentration of 2 μg/ml.

**Isolation of alveolar MΦs**

Mice were euthanized, and the tracheas were cannulated with a blunt-ended 20-gauge needle. The airways were lavaged five times with 1 ml chilled PBS devoid of calcium and magnesium. The lavage fluid was collected and washed three times, and cells were resuspended in DMEM containing 10% FBS and 10 μg/ml gentamicin.

**Generation of bone marrow-derived MΦs**

Bone marrow was isolated from the hind tibia and femurs of 5–6-wk-old mice by flushing with HBSS. Cells were dispensed into tissue culture flasks at a density of 2 × 10⁵ cells/ml RPMI 1640 supplemented with 10% FBS, 0.1% gentamicin sulfate, 5 × 10⁻³ M 2-ME, and 10 ng/ml mouse GM-CSF or mouse M-CSF (PeproTech, Rocky Hill, NJ). Flasks were incubated at 37°C in 5% CO₂. Bone marrow-derived MΦs (BMDMΦs) were harvested between days 5 and 7 after cultures were established. Non-adherent cells were removed, and trypsin-EDTA was added for 15 min at 37°C. Cells were gently scraped, collected, washed twice with HBSS, and dispensed into tissue culture dishes.

**Preparation of H. capsulatum and recovery of yeast cells from BMDMΦs or lungs**

*H. capsulatum* yeast cells (strain G217B) and GFP-expressing yeasts were prepared, as described previously (19, 20). To quantify the number of yeast cells from MΦs, infected cells were lysed with a hypotonic buffer containing 20 mM Tris/HCl, 10 mM NaCl, and 3 mM MgCl₂; for 5 min; yeast cells were collected and serially diluted, and aliquots were added to plates containing Mycosel agar 5 (Becton Dickinson, Walkersville, MD) supplemented with 5% sheep blood (v/v), 5% glucose (w/v), and 0.1% cys- teine hydrochloride. Yeast cells were incubated at 37°C for 30 days, and yeast growth was determined. 

**RNA isolation, cDNA synthesis, RT² Profiler PCR array, and quantitative real-time RT-PCR**

Total RNA was extracted from MΦs using an RNeasy Kit (Qiagen, Valencia, CA). cDNA was synthesized according to the manufacturer’s instructions (Qiagen). Analysis of expression of apoptosis genes was performed using the RT² Profiler PCR arrays, according to the manufacturer’s protocol (Qiagen). Gene expression was compared according to the cycle threshold value. Quantitative real-time RT-PCR for individual genes was performed using TaqMan Master Mix and primers from Applied Biosystems (Foster City, CA). Samples were analyzed on an ABI Prism 7500 (Applied Biosystems). The housekeeping gene hypoxanthine phosphoribosyltransferase was used as an internal control. The conditions for amplification were 50 °C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Assessment of cytotoxic proteins**

Protein concentrations of IL-1β, TNF-α, and IL-10 were determined by ELISA. The IL-1β and TNF-α kits were purchased from R&D Systems (Minneapolis, MN), and the IL-10 kit was purchased from eBioscience (San Diego, CA).

**Assessment of apoptosis, necrosis, and caspase activity, cytotoxicity, and cell viability in vitro**

To assess apoptosis of MΦs, we used the ELISA-formatted Cell Death kit (Roche Applied Sciences, Indianapolis, IN) and the ELISA-based ssDNA Apoptosis ELISA kit (Millipore, Billerica, MA). In these studies, we calculated the enrichment factor using the following formula: absorbance of cells incubated with yeast cells/absorbance of cells incubated in medium alone. For graphic purposes, the enrichment factor for cells incubated in medium alone was assigned a value of 1. Necrosis was analyzed by recovering supernatants from cells exposed or not exposed to yeast cells. A colorimetric kit (Thermo Scientific, Waltham, MA) was used to assay for caspase 3. The activity of caspase 3 was standardized to the quantity of whole-cell protein using Janus green staining. Apoptosis in vivo was assessed as reported (13). Caspase 1 activity was assessed using a colorimetric assay from Millipore. Uniform amounts of protein were analyzed among groups. Release of lactate dehydrogenase was used to assess cytotoxicity using the Cyto-Tox assay from Promega (Madison, WI), and cell viability was assayed by PrestoBlue (Invitrogen, San Diego, CA).

**Use of caspase inhibitors and simvastatin in vitro**

The pan caspase inhibitors Boc-Asp (OMe) fluoromethyl ketone (Boc-D-FMK; Sigma-Aldrich) and quinolinyl-vanyl-O-methylaspartyl- [2,6-difluorophenoxyl]-methyl ketone (QVD-OPh) (R&D Systems), the caspase 3 inhibitor acetylated-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), or the control peptide N-benzoyloxycarbonyl-Phe-Asp-fluoromethylketone (z-FA-FMK; Sigma-Aldrich) was dissolved in DMSO and incubated in vitro at a concentration of 20 μM. Simvastatin (Sigma-Aldrich) was dissolved in DMSO and used in experiments at a concentration of 10 μM. Caspase 1 inhibitors acetylated Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) and Z-Trp-Glu-His-Asp fluoromethylketone (Z-WEHD-FMK) were purchased from EMD Chemicals (Darmstadt, Germany) and R&D Systems, respectively, and dissolved in DMSO. Final concentration was 20 μM. Cells were exposed to the compounds for 24 h before exposure to the fungus. In preliminary studies, we found that the above concentrations were optimal without causing toxicity. When experiments extended beyond 24 h, the cells were re-exposed every 24 h to the compounds to adjust for their reversibility.

**In vivo treatment with simvastatin**

Simvastatin was given i.p. on the day before and the day of intranasal infection with *H. capsulatum*. As a control, PBS containing the same concentration of DMSO was used.

**Isolation of lung leukocytes**

Lungs were homogenized with the gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) in 5 ml HBSS with 2 mg/ml collagenase D (Roche) and 40 U/ml DNase I (Roche) for 30 min at 37°C. Following treatment, the homogenate was percolated through a 60-μm nylon mesh (Spectrum Laboratories, Rancho Domincigue, CA) and washed three times with HBSS. Leukocytes were isolated by separation on Lympholyte M (Cedarlane Laboratories, Burlington, ON, Canada).

**Flow cytometry**

Apoptosis was assessed, as previously described, using the In Situ Cell Death Detection Kit (Roche) (13). All surface staining was performed before analysis of apoptosis. To determine the phenotype of apoptotic cells, lung leukocytes were adjusted to a concentration of 2 × 10⁷ cells/200 μl staining buffer (PBS containing 2% BSA and 0.02% sodium azide) and incubated with 0.5 μg one or more of the following: CD3-allophycocyanin, Ly6G-PerCP, Mac-3–PE, CD11c-PerCP, CD124 (IL-4Rα)–PE, or CD11b-allophycocyanin. These Abs were purchased from BD Pharmingen (San Diego, CA). Other Abs include biotinylated Mac-3 purchased from BioLegend (San Diego, CA), F4/80-PerCP from BioLegend (San Diego, CA), and CD206-PE from AbD Serotec (Oxford, U.K.). Streptavidin-PE-Cy7, Cy-chrome, VEGF, and CD38-FITC were purchased from BD Biosciences (San Jose, CA). Viability was assessed by PrestoBlue (Invitrogen, San Diego, CA).

**Statistics**

ANOVA was used to compare groups. Correction for multiple comparisons was accomplished using the Tukey test.

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**References**

(DeNovo Software, Los Angeles, CA).
Results

Apoptosis of MΦs during early infection with H. capsulatum

Previously, we reported that phagocytes constituted a very small proportion (<5%) of the apoptotic leukocytes at days 7–21 of murine histoplasmosis (13). This finding seemed perplexing because phagocytes, and in particular MΦs, are crucial effector cells in the host response to this fungus. We reasoned that day 7 may be too late to detect a high proportion of apoptotic MΦs; rather, programmed cell death of these cells may transpire very early in the course of infection and is followed by their ingestion. Hence, the lungs of mice infected for 1 or 3 d were analyzed for the presence of apoptotic leukocytes. In uninfected mice, the mean percentage (± SEM) of apoptotic cells (n = 8) was 5.4 ± 0.4%, which was similar to what we reported (13). On day 1, the proportion of apoptotic cells was 9.8 ± 0.8% (n = 8). Of these, MΦs (Mac-3+, autofluorescent, CD11b+, I-A<sup>+</sup> low/intermediate, CD11c<sup>+</sup>) constituted 42.3 ± 4.1% of the apoptotic cells. By day 3, the proportion of apoptotic cells was 12.9 ± 2.1% (n = 8), and MΦs made up 24.7 ± 1.8% of these cells. These values were derived by calculating the total number of each population and dividing by the total number of apoptotic cells. These results indicated that, in acute pulmonary histoplasmosis, MΦs constituted a more important population of apoptotic leukocytes than was appreciated in our prior study.

We queried whether apoptosis of MΦs was restricted to a distinct subpopulation of these cells. To approach this possibility, we examined apoptosis of classically and alternatively activated cells. The classically activated cells were defined as F4/80<sup>+</sup> or Mac-3<sup>+</sup>, CD206<sup>low/intermediate</sup>, and IL-4R<sub>a</sub>low, and alternatively activated cells were classified as F4/80<sup>+</sup> or Mac-3<sup>+</sup>, IL-4R<sub>a</sub>high, CD206<sup>high</sup>, IL-4R<sub>a</sub>high, and CD206<sub>high</sub> (22–25). Based on this categorization, we found that >95% of the apoptotic MΦs were detected in the CD206<sub>low/intermediate</sub>, IL-4R<sub>a</sub>low subpopulation at both days 1 and 3 (n = 8) (Supplemental Fig. 1). This distribution did not merely reflect the proportions of classically and alternatively activated cells, because 21.6 ± 0.7% and 26.3 ± 1.6% were CD206<sub>high</sub>, IL-4R<sub>a</sub>high at days 1 and 3 of infection, respectively (Supplemental Fig. 1).

Apoptosis of MΦs is dependent on multiplicity of infection and time of exposure

GM-CSF–differentiated BMDM<sub>M</sub>s were incubated with increasing numbers of H. capsulatum yeast cells for 24, 48, and 72 h, and apoptosis was assessed using ELISA. At 24 h, apoptosis was detected only in cells with a high multiplicity of infection (MOI): 5 yeast cells:1 MΦ and 20 yeast cells:1 MΦ (Fig. IA). By 48 h, apoptosis of MΦs was observed only when incubated with 1 yeast cell:1 MΦ and 5 yeast cells:1 MΦ. At 72 h, apoptosis was no longer detected in MΦs exposed to a high MOI but to lower numbers of yeast cells. M-CSF also differentiates murine bone marrow monocytes into MΦs, but it induces a distinct differentiation pathway (22, 26, 27). Hence, we asked whether these cells underwent apoptosis in response to H. capsulatum. Incubation with a 5:1 or 20:1 ratio induced apoptosis after 24 h (Fig. IB). To put these data into perspective, we assessed the mean percentage of MΦs infected with H. capsulatum using GFP-expressing yeast cells (20). The mean percentage (± SEM) of MΦs infected was 21.5 ± 2.2% when exposed to 0.1 yeast cell:1 MΦ; 32.3 ± 4.3% when exposed to 0.5 yeast cell:1 MΦ; 36.8 ± 4.9% when exposed to 1 yeast cell:1 MΦ; and >98% when exposed to 5 or 20 yeast cells:1 MΦ.

We used another ELISA for adherent cells that identifies denatured DNA in response to formamide. Using this assay, the results were virtually identical to the aforementioned results (Fig. 1C). Thus, we verified by two different assays that H. capsulatum provoked apoptosis in MΦs.

High MOI stimulates necrosis

We noted that cells exposed to a high MOI manifested a sharp decrement at 48 h in the enrichment factor, with a value that was far below that observed with cells alone. This finding raised the possibility that cells may be undergoing necrosis subsequent to apoptosis. Indeed, at 20 yeast cells:1 MΦ, necrosis was observed as early as 24 h, and at 48 h, it was only found when the yeast cell/MΦ ratio was 5:1 (Fig. 1D). By 72 h, the wells incubated with 20 yeast cells:1 MΦ were largely obliterated (Fig. 2). This destruction of the monolayer of heavily infected cells was affirmed by a cytotoxicity assay. The percentage of cell death varied between 85 and 90% (n = 4) compared with uninfected cells. At this time, necrosis was only detected in cells infected with 1 yeast cell/1 MΦ. The most heavily infected MΦs manifested a heterogeneous picture of apoptosis and necrosis.

Because we could not detect apoptosis in cells exposed to lower MOI until later in infection, we queried whether infection with lower numbers of yeast cells inhibited apoptosis, at least transiently. We infected MΦs with 0.1, 0.5, or 1 yeast cell/1 MΦ and exposed them to camptothecin. This agent induced apoptosis in uninfected cells as robustly as it did in infected cells. For example, the mean enrichment factor ± SEM (n = 3) for uninfected cells exposed to camptothecin was 1.93 ± 0.34, and it was 1.87 ± 0.29, 1.79 ± 0.42, and 2.05 ± 0.21 for 1, 0.5, and 0.1 yeast cells/1 MΦ, respectively. These results indicated that yeast cells did not manipulate the host to block apoptosis.

Alveolar MΦs undergo apoptosis in response to H. capsulatum yeast cells

The lungs are the portal of entry for H. capsulatum, and yeast cells are likely to be in alveolar MΦs prior to the marshaling of an inflammatory response; therefore, we asked whether H. capsulatum yeast cells induced apoptosis in this MΦ population. Phagocytes were exposed to an increasing number of yeast cells, and apoptosis was analyzed after 24 h. The profile of apoptotic responses for this cell population was nearly identical to that of BMDM<sub>M</sub>s (Fig. 1E). Thus, H. capsulatum provoked the apoptotic pathway in both phagocyte populations.

Modulation of programmed cell death-signaling molecules by H. capsulatum

To understand, in greater depth, the molecular character of apoptosis induced by H. capsulatum, we analyzed the transcripts of numerous genes known to contribute to programmed cell death. MΦs were incubated with 5- or 20-fold more yeast cells than MΦs for 24 h, and transcripts were assessed using the RT<sup>2</sup> apoptosis array. We focused our analysis on MΦ genes whose expression was strikingly increased in both experimental groups. Interestingly, a number of these genes were associated with the TNF family. These included TNF-α, TRAF 1–3, CD40, and CD70. A few genes that contribute to the intrinsic pathway were upregulated in both experimental groups, including BCL2/adenovirus E1B interacting protein 3 and BH3 interacting domain death agonist. Expression of several caspases was also found to be elevated in infected MΦs (Supplemental Table I). Expression of several caspases, including caspase 3 and 1, was upregulated. In addition, we discovered that the anti-inflammatory, antiapoptotic cytokine, IL-10, was significantly enhanced in MΦs exposed to yeast cells. We validated the transcriptional analysis of TNF-α and IL-10 by assaying protein content in supernatants from MΦs infected with
yeast cells. The concentrations of both cytokines were markedly increased compared with uninfected cells (Fig. 3). This finding was quite unexpected, because IL-10 and TNF often deliver antipodal signals in the apoptosis cascade (28–30).

IL-10 induces the expression of suppressor of cytokine synthesis (SOCS)3, which depresses cytokine signaling (31). If IL-10 were actively engaging its own receptor and transmitting signals, it is likely that SOCS3 would be upregulated. Expression of this regulatory genetic element was increased in infected cells compared with uninfected controls (Fig. 3). Its expression was largely, but not exclusively, dependent on IL-10, because the response by BMDMFs from IL-10−/− mice was diminished but not abolished.

The chronology of the generation of TNF-α and IL-10 was examined to determine whether their secretion was synchronized over 24 h. Cells were infected, and TNF-α and IL-10 generation was assessed at serial intervals. TNF-α was released as early as 2 h postinfection and continued to increase thereafter. In contrast, IL-10 was not detected until 8 h and declined by 24 h (Fig. 3C, 3D). Thus, the generation of these two cytokines was not contemporaneous.

Apoptosis of infected MΦs is associated with activation of the caspase pathway

Much of the apoptotic pathway is dependent on the activation of caspases (6, 7, 32, 33). By contrast, apoptosis of murine MΦs associated with a high MOI (>25 bacilli/MΦ) of virulent Mycobacterium tuberculosis is cathepsin dependent (34). To ascertain the role of initiator and effector caspases in our experimental work, infected cells were incubated with the pan-caspase inhibitors QVD-OPh or Boc-D-FMK or, as a control, z-FA-FMK. Both pan-caspase inhibitors prevented the induction of apoptosis at the high MOI at 24 h or at the lower MOI at 72 h (Fig. 4A, 4B). Statins, which are 3-hydroxymethylglutaryl coenzyme A reductase inhibitors, were reported to reduce apoptosis of cells, including MΦs, in selected model systems (35–37). As illustrated in Fig. 4, this statin diminished apoptosis induced by H. capsulatum yeast cells. We subsequently endeavored to determine whether the in vitro findings could be reproduced in vivo. Mice were treated

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**FIGURE 1.** Apoptosis of MΦs infected with H. capsulatum. A, Studies done using the Cell Death ELISA kit. B, Response by BMDMΦs differentiated with M-CSF. C, Apoptotic response of BMDMΦs using a kit from Millipore. D, Necrosis. E, Apoptotic response by alveolar MΦs. The enrichment factor was calculated using the formula: absorbance of cells incubated with yeast cells/absorbance of cells incubated in medium alone. For graphic purposes, the enrichment factor for cells incubated in medium alone was assigned a value of 1. The horizontal dashed line is the enrichment factor of the uninfected cells. The data represent the mean ± SEM of 6–12 experiments. The enrichment factor for H. capsulatum incubated in the absence of MΦs was <0.01 for each ratio of yeast/MΦs. *p < 0.05, **p < 0.01. Hc, H. capsulatum.

**FIGURE 2.** Photomicrographs of MΦs and H. capsulatum. Phase-contrast photomicrographs of uninfected and infected MΦs at 24 and 72 h postexposure to yeast cells. Original magnification ×200. Hc, H. capsulatum.
with 40 or 200 μg of simvastatin or vehicle the day before and the day of infection; apoptosis of lung MΦs was determined 24 h later. The mean number of apoptotic MΦs (±SEM) in mice (n = 5) that received vehicle (2.67 ± 0.37 × 10^5) was significantly higher (p < 0.05) than that in mice that received either 40 μg (1.69 ± 0.09 × 10^5) or 200 μg (1.06 ± 0.22 × 10^5) of simvastatin. The number of apoptotic cells in uninfected mice (n = 6) given vehicle (1.75 ± 0.18 × 10^5) did not differ (p > 0.05) from those given 40 μg (1.58 ± 0.23 × 10^5) or 200 μg (1.86 ± 0.17 × 10^5) of simvastatin. The data indicated that the effect of simvastatin was not limited to an in vitro phenomenon. However, the alteration in apoptosis exerted by simvastatin required a stimulus, in this case *H. capsulatum*, because we failed to observe a change in uninfected mice treated with a single dose.

Caspase 3 is a principal effector caspase that drives the cleavage of selected proteins, including actin, MAPK, lamins, poly ADP-ribose polymerase, and inhibitor of caspase-activated DNase (6, 7, 38). Caspase 1 is a critical mediator of IL-1β generation and another form of cell death, termed pyroptosis (4). Because both were upregulated in our screening assay, we queried whether the activity of either caspase was altered following in vitro infection. Caspase 3 activation was noted in cells with the high MOI at 24 h and with the lower MOI at 72 h (Fig. 5A). As with caspase 3 activity, caspase 1 activity was elevated in MΦs after 24 h (Fig. 5B).

Subsequently, we determined whether the caspase 3 inhibitor Ac-DEVD-CHO or the caspase 1 inhibitors, Ac-YVAD-CHO or Z-WEHD-FMK, modified the apoptotic response by BMDMΦs infected with yeast cells. The caspase 3 inhibitor, but not the
FIGURE 5. Caspase activity and inhibition of apoptosis. A, BMDMs were incubated with H. capsulatum for 24 or 72 h, and caspase 3 activity was assessed. B, Caspase 1 activity was assessed at 24 h postinfection. C, Apoptosis was assessed in cells exposed to the caspase 3 inhibitor Ac-DEVD-CHO or the caspase 1 inhibitors Ac-YVAD-CHO or Z-WEHD-FMK 24 h postinfection. The horizontal dashed line is the enrichment factor of the uninfected cells. D, Caspase 3 activity was assessed after exposure to z-FA-FMK, QVD-OPh, or simvastatin (Sim). E, Cell death analysis in the presence or absence of the caspase 3 inhibitors. F, IL-1β generation in infected cells in the presence or absence of caspase 1 inhibitors. *p < 0.05; **p < 0.01. Hc, H. capsulatum; ND, not detected.

caspase 1 inhibitors, blocked apoptosis in cells infected with a high MOI (Fig. 5C). Moreover, caspase 3 activity was significantly depressed (p < 0.01) by QVD-OPh or simvastatin (Fig. 5D). Collectively, these data denoted that activation of caspase 3 is central in apoptosis of H. capsulatum-infected MΦs. Inhibition of caspase 1 activity did not alter apoptosis, and it reduced (p < 0.05) cell death only at a ratio of 5 yeast cells:1 MΦ by the Cytotox assay (Fig. 5E) and PrestoBlue assay (data not shown). In addition, IL-1β generation was diminished (p < 0.01) by inhibitors of caspase 1 (Fig. 5F).

TNF-α and apoptosis

The above data affirmed that some caspases are necessary for the induction of apoptosis in H. capsulatum-infected MΦs. Because both TNF-α and IL-10 were strongly induced by infection, we ascertained whether neutralization of one of these cytokines would alter apoptosis. A concentration of 1000 ng/ml of anti–TNF-α, but not 1000 ng/ml of anti–IL-10R, blocked apoptosis (Fig. 6A, 6B). However, antagonism of IL-10R caused a moderate elevation in apoptosis, suggesting that IL-10 signaling was dampening apoptosis response. Because TNF-α generation induced apoptosis of infected MΦs, we sought to determine whether apoptosis was mediated by TNFR1 signaling based on prior data that apoptosis of lung leukocytes is reduced in infected TNFR1−/− mice (13). BMDMs from TNFR1−/− mice did not undergo apoptosis (Fig. 6C). This failure was not attributable to an inability to produce TNF-α, because both (wild-type) WT and TNFR1−/− MΦs produced equivalent amounts of cytokine after 24 h of incubation (Fig. 6D). Inhibition of apoptosis alters IL-10 and TNF-α by MΦs

We asked whether inhibition of apoptosis by QVD-OPh or by simvastatin modified secretion of either TNF-α or IL-10. Infected MΦs were incubated in the presence or absence of the apoptosis inhibitor; after 24 h, supernatants were assayed for each cytokine. Suppression of apoptosis blunted TNF-α release and caused a pronounced increase in IL-10 production (Fig. 7A, 7B). Thus, induction of apoptosis is important in the control of the pro- and anti-inflammatory profile of H. capsulatum-infected MΦs.

The above results suggested that TNF-α and IL-10 are critically important in the initial apoptotic response to H. capsulatum. We extended these studies in vivo. WT, TNFR1−/−, and IL-10−/− mice were infected with H. capsulatum, and apoptosis of lung MΦs was evaluated at day 1 postinfection. The number of apoptotic MΦs in WT mice exceeded that in TNFR1−/− mice but was less than that in IL-10−/− mice (Table I). The alteration in the number of apoptotic MΦs was associated with modest changes in the number of CFU recovered from the lungs of mice (Table I).

Inhibition of apoptosis and MΦ handling of H. capsulatum

We postulated that inhibition of apoptosis would provide a more permissive environment for the intracellular growth of this fungus. Prior to studying the intracellular behavior of H. capsulatum, it was necessary to ascertain whether any of the modulators of apoptosis would directly affect growth of the fungus. Yeast cells (5 × 10⁵) were exposed for 24 or 48 h to z-FA-FMK, QVD-OPh, or simvastatin. The growth of the yeast cells was not impacted by the presence of these compounds (data not shown). BMDMs or
alveolar MΦs were pretreated with z-FA-FMK, QVD-OPh, or simvastatin for 24 h and subsequently incubated with yeast cells for 2, 24, or 48 h. The number of CFU was determined at each of these time points. Exposure to an apoptosis inhibitor did not significantly alter \((p > 0.05)\) the number of CFU recovered at 2, 24, or 48 h (Fig. 8). Contrary to expectations, inhibition of apoptosis for up to 48 h did not manifestly promote intracellular growth.

**Discussion**

The pathogenesis of early histoplasmosis is a highly complex process that entails the struggle of the fungus to combat the innate immune system. The outcome of exposure to *H. capsulatum* from the environment largely relies on a highly synchronized immune response to invasion. In contradistinction, survival of the fungus during the acute phase of infection requires that it manipulates innate immunity to obviate at least rapid elimination. We previously reported that apoptosis was a pivotal feature of a successful host response, and inhibition of this process by several approaches was associated with a progressive infection (13). This finding was certainly different from that found in other infectious disease model systems, in which apoptosis impedes the host from mounting an effective response (2, 16, 39, 40). A curiosity of our prior work was that MΦs were not a significant constituent of the apoptotic response, despite their central influence in defenses to this fungus. We reasoned that our failure to detect larger numbers of apoptosis-positive MΦs was likely due to the rapid turnover of these cells in the lung environment. In the current study, we aimed to address this issue by extending our analysis to include late time points and by investigating the role of caspase-8 and caspase-3 in MΦ apoptosis.

**Figure 6.** TNF-α and IL-10 modulation of apoptosis. A and B, Apoptosis of BMDMΦs in the presence of isotype control mAb, anti-TNF-α, or anti-IL-10R. Cells were incubated with the mAb, infected for 24 h, and assessed for apoptosis. C, Apoptotic response by BMDMΦs from TNFR1−/− and WT mice. D, BMDMΦs from WT and TNFR1−/− mice produce TNF-α. The horizontal dashed line is the enrichment factor of the uninfected cells. The data represent the mean ± SEM of five experiments. In A and B, statistical analysis was performed by comparing Ab-treated cells with isotype controls. *p < 0.05, **p < 0.01. Hc, *H. capsulatum.*

**Figure 7.** TNF-α and IL-10 production in the presence of inhibitors of apoptosis. BMDMΦs were incubated with z-FA-FMK, QVD-OPh, or simvastatin (Sim) and infected with *H. capsulatum* for 24 h. Supernatants were removed and assayed for cytokines by ELISA. Data represent the mean ± SEM of six or seven experiments. TNF-α and IL-10 levels were not detected in uninfected MΦs treated with z-FA-FMK, QVD-OPh, or simvastatin. Hc, *H. capsulatum.*
of apoptotic MΦs was a consequence of timing. In the original publication, in vivo analysis of apoptosis was initiated on day 7 and continued until day 14. Because the early pathogenesis necessitates the interaction of resident MΦs with the fungus, we explored the apoptotic response in the very early period of infection, days 1–3, prior to the massive influx of inflammatory cells. Analysis of the infected lungs at days 1 and 3 clearly demonstrated that MΦs make up a much more significant population of apoptotic cells than that seen on day 7 (13). Moreover, we demonstrated that the distribution of apoptotic MΦs was heavily weighted to a phenotype that was not indicative of an alternatively activated MΦ. This finding reflects the strong bias toward a Th1 phenotype in murine histoplasmosis, but also suggests that the paucity of apoptosis in alternatively activated cells may promote survival of *H. capsulatum* in a permissive cell (41, 42).

These results prompted us to examine the functional consequences of apoptosis of MΦs. We should denote that the results were largely obtained with BMDMΦs, although data with alveolar MΦs were congruent with that of the former. BMDMΦs were analyzed to reduce the use of the large number of mice that would have been required to obtain a sufficient number of alveolar MΦs to conduct these experiments. The kinetics of apoptosis was dependent on the MOI. Heavier infection produced apoptosis and necrosis by 24 h, whereas smaller inocula required 48–72 h to manifest apoptosis. This chronology provides an insight into the possible order of pro- and anti-inflammatory signaling by surrounding or emigrating leukocytes. Ingestion of apoptotic bodies induces the generation of anti-inflammatory signals, whereas engulfment of necrotic cells prompts the release of proinflammatory signals (1, 11, 43). The simultaneous occurrence of apoptosis and necrosis from heavily infected MΦs most likely results in a stochastic delivery of pro- and anti-inflammatory signals by phagocytes that have ingested these particles. The balance of the opposing signals will greatly depend on the magnitude of apoptosis and necrosis. In contrast, in cells that are not as heavily infected, the pattern is that of apoptosis first, followed by necrosis. Therefore, it is likely that, in those regions of tissue or in lighter infections, there is a transient anti-inflammatory environment until necrotic cells are phagocytosed, prompting the release of proinflammatory mediators. In addition, apoptotic infected MΦs are crucial for activation of CD8+ T cells through cross-presentation by dendritic cells (14). This is not true for necrotic cells. Therefore, apoptotic cells are of considerable importance in triggering CD8+ T cell activation. Although this T cell population is subordinate to CD4+ T cells in the clearance of *H. capsulatum*, its importance to host defenses is not negligible and is heightened when CD4+ T cells are deficient or dysfunctional (14).

Of note, other investigators reported that *H. capsulatum* inhibits apoptosis of peritoneal MΦs after injecting live yeasts into the peritoneal cavity (15). This assertion was based on the finding that peritoneal MΦs from PBS-injected animals manifested a higher proportion of apoptotic cells than those recovered from animals infected i.p. with the fungus. There are several reasons why these data differ from ours: a different route of inoculation and studies of apoptosis in the peritoneal cavity versus the lung, a different strain of *H. capsulatum*, and differences in the baseline apoptosis of cells from uninfected animals (13). The proportion of apoptotic MΦs from PBS-injected animals approximated 60%, with only 25% viability. This value was far greater than we found in uninfected lungs.

An unbiased screen of the more heavily infected MΦs was conducted to determine the pathway or pathways engaged in apoptosis. We anticipated that the intracellular residence of *H. capsulatum* would most likely lead to activation of the intrinsic pathway. Unexpectedly, there was little evidence that the intrinsic pathway was engaged; only a few genes therein manifested a significant increase in transcription. Rather, the majority of up-

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**FIGURE 8.** Recovery of *H. capsulatum* from BMDMΦs. BMDMΦs infected with *H. capsulatum* and exposed to medium only, z-FA-FMK, QVD-OPh, or simvastatin (Sim) were lysed at 24 and 48 h postinfection and plated. Data represent the mean CFU ± SEM of five experiments.

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**Table I.** Number of apoptotic lung MΦs in mice infected with *H. capsulatum* for 24 h

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Uninfected</th>
<th>Infected*</th>
<th>CFU (Mean ± SEM [×10⁵])</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.20 ± 0.04</td>
<td>14.53 ± 2.06</td>
<td>1.18 ± 0.06</td>
</tr>
<tr>
<td>TNFR1−/−</td>
<td>0.18 ± 0.05</td>
<td>6.54 ± 0.51*</td>
<td>1.33 ± 0.05**</td>
</tr>
<tr>
<td>IL-10−/−</td>
<td>0.23 ± 0.06</td>
<td>27.07 ± 2.09*</td>
<td>0.83 ± 0.06**</td>
</tr>
</tbody>
</table>

*Mice (n = 5–6) were challenged with 2 × 10⁸ yeast cells, and 24-h lung leukocytes were collected. MΦs were identified as described in Results.

*p < 0.01 compared with WT, **p < 0.05 compared with WT.
regulated genes consisted of those that participate in the extrinsic pathway mediated by TNF-α, but not Fas. Concomitantly, transcription of initiator and effector caspases was increased, as was caspase 1. The increase in expression of the genes encoding the caspases was confirmed in activity assays; both caspase 3 and caspase 1 displayed increased activity upon infection. However, only inhibition of the former blocked cell death.

Activation of caspase 1 is critical for elaboration of IL-1, and it produces cell death (termed pyroptosis) distinct from that of caspase 3 (4). Inhibition of caspase 1 activity modestly lessened the cytotoxic effect of H. capsulatum, but only at an MOI of 5 yeast cells/l MΦ. This finding is likely a consequence of the vigor of caspase 1 activity in MΦs exposed to five yeast cells compared with an MOI of 20:1. Thus, the cell death in the higher MOI is largely, if not exclusively, attributable to apoptosis. Activation of caspase 1 was essential for generation of IL-1β in H. capsulatum-infected MΦs. IL-1β is required for host control of infection (44); therefore, activation of caspase 1 and the inflammasome appears to be an essential constituent in the evolution of the protective immune response to this fungus.

A surprising finding was that suppression of apoptosis did not necessarily lead to increased recovery of yeast cells from MΦs, at least over the 48-h observation period. This time span is sufficient for the yeast cells to double their numbers twice or three times. The results indicated that inhibition of apoptosis does not necessarily accelerate intracellular growth but provides a niche for the organism to replicate. In contrast to the lack of change in CFU following inhibition of apoptosis, the release of TNF-α was vitally dependent on the ability of cells to undergo apoptosis. When apoptosis was blocked with either QVD-OPh or simvastatin, secretion of TNF-α was dramatically lower, and IL-10 was sharply higher. These findings point to a role for caspase activation in the generation of these two cytokines. Moreover, the findings indicated that the process of apoptosis may have more to do with cytokine regulation than regulating intracellular growth.

MΦs infected with a high MOI of M. tuberculosis bacilli undergo apoptosis through a caspase-independent, cathepsin-dependent pathway (34). In contrast, H. capsulatum is strictly dependent on caspase activity. In fact, we surveyed several cathepsins to determine whether they were activated in response to infection, and we failed to detect upregulation in four major cathepsins. Thus, despite the common feature of intracellular residence, it is clear that these organisms trigger different pathways of apoptosis.

In these studies, we used the lipid-lowering agent simvastatin to assess whether it could modify apoptosis of Histoplasma-infected MΦs. The study of this compound arose out of work that examined its effect on expression of the transcription factor Krüppel-like factor 2, which contributes to cytokine and chemokine signaling of leukocytes, as well as directs thymic trafficking of T cells (45-49). Among its many properties, Krüppel-like factor 2 is antiapoptotic, and simvastatin is known to increase its expression (45, 50, 51). Therefore, we examined whether simvastatin could modify apoptosis and found that it did so in conjunction with modifying caspase 3 activity. Simvastatin was reported to decrease apoptosis and to enhance apoptosis (36, 52-55). The effects of simvastatin on apoptosis are caspase 3 dependent and independent (52, 53). Hence, the action of simvastatin on apoptosis varies widely, and there does not appear to be uniform or common motifs to explain its effect on the apoptotic pathway. However, it is apparent that this agent modified apoptosis of H. capsulatum-infected MΦs both in vitro and in vivo, thus indicating our in vitro findings were not an anomaly. Because we have shown that apoptosis is crucial in host defenses, it is possible that this agent may exacerbate infection, although one must consider that simvastatin has a multitude of effects on the genetic machinery of cells and, therefore, modulation of apoptosis in vivo may not be its only activity.

Apoptosis at 24 h was accompanied by upregulation of both TNF-α and IL-10. This finding was unexpected because these two cytokines exert distinctly polar effects on MΦs, although other investigators reported that both are produced by MΦs in response to mycobacterial infection (28, 29, 56). To confirm our array data, we assessed secretion of both of the proteins encoded by the genes. The kinetics of production were distinct between these two cytokines. TNF-α was released earlier than IL-10 and continued to increase as time progressed. In contrast, IL-10 peaked at 8 h and declined by 24 h. Both of these cytokines participated in the apoptotic response. Neutralization of TNF-α prevented apoptosis, whereas blockade of IL-10 signaling actually enhanced the apoptotic response. Moreover, the ability of TNF-α was critically dependent on TNFR1 signaling. Our previous data highlighted the pivotal influence of TNFR1 in apoptosis of lung leukocytes in the lungs of mice infected with H. capsulatum (13). The current finding indicates that TNFR1 expression on a specific cell population, MΦs, is important in the regulation of apoptosis. We validated these in vitro experiments by demonstrating that, in vivo, apoptosis was reduced in lung MΦs in TNFR1−/− mice and increased in IL-10−/− mice at 24 h postinfection. Moreover, the differences in the degree of apoptosis in vivo were inversely correlated with changes in the recovery of CFU. Less apoptosis was associated with greater fungal recovery and vice versa. The differences at 24 h were modest, largely because the generation time of H. capsulatum ranges from 18 to 24 h (57). In such a brief span, it would be unusual to find dramatic changes for this slow-growing fungus. The results contrast with the recovery of fungi when apoptosis was blocked in vitro; no change in CFU was observed between treated and control MΦs. The observed differences likely reflect the complexities of the in vivo interactions, in which secreted soluble mediators and freshly recruited inflammatory cells also influence the fungal burden. The complex nature of the apoptotic response is further highlighted by the fact that both infected and uninfected MΦs undergo apoptosis in vivo, indicating that it is not solely the fungus that drives apoptosis (20).

Extrapolating our findings to the in vivo situation, it is possible that initial invasion of MΦs by H. capsulatum leads to the release of TNF-α, which initiates the apoptotic pathway through engagement of TNFR1 in an autocrine manner. As yeast cells are released by apoptotic cells, they enter into adjacent phagocytes that have been exposed to IL-10. This cytokine prevents apoptosis through several mechanisms, thus providing a niche for the fungus to grow. One includes the activation of SOCS3. Expression of this gene leads to inhibition of TNF-α and NO release in newly infected cells, thus prolonging survival of yeast cells (58, 59). Moreover, SOCS3 depresses IFN-γ signaling, thus blunting another antifungal host-defense mechanism (60). The net result is that infection of MΦs produces a trans effect, in which the yeast cells initially trigger apoptosis to find niches that are more likely to accommodate their survival. In this circumstance, the newly released yeast cells enter phagocytes that are less likely to undergo apoptosis and to become activated.

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References


