Immunization with Apoptotic Phagocytes Containing *Histoplasma capsulatum* Activates Functional CD8⁺ T Cells To Protect against Histoplasmosis

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We have previously revealed the protective role of CD8⁺ T cells in host defense against *Histoplasma capsulatum* in animals with CD4⁺ T cell deficiency and demonstrated that sensitized CD8⁺ T cells are restimulated in *vitro* by dendritic cells that have ingested apoptotic macrophage-associated *Histoplasma* antigen. Here we show that immunization with apoptotic phagocytes containing heat-killed *Histoplasma* efficiently activated functional CD8⁺ T cells whose contribution was equal to that of CD4⁺ T cells in protection against *Histoplasma* challenge. Inhibition of macrophage apoptosis due to inducible nitric oxide synthase (iNOS) deficiency or by caspase inhibitor treatment dampened the CD8⁺ T cell but not the CD4⁺ T cell response to pulmonary *Histoplasma* infection. In mice subcutaneously immunized with viable *Histoplasma* yeasts whose CD8⁺ T cells are protective against *Histoplasma* challenge, there was heavy granulocyte and macrophage infiltration and the infiltrating cells became apoptotic. In mice subcutaneously immunized with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled apoptotic macrophages containing heat-killed *Histoplasma*, the CFSE-labeled macrophage material was found to localize within dendritic cells in the draining lymph node. Moreover, depleting dendritic cells in immunized CD11c-DTR mice significantly reduced CD8⁺ T cell activation. Taken together, our results revealed that phagocyte apoptosis in the *Histoplasma*-infected host is associated with CD8⁺ T cell activation and that immunization with apoptotic phagocytes containing heat-killed *Histoplasma* efficiently evokes a protective CD8⁺ T cell response. These results suggest that employing apoptotic phagocytes as antigen donor cells is a viable approach for the development of efficacious vaccines to elicit strong CD8⁺ T cell as well as CD4⁺ T cell responses to *Histoplasma* infection.

*Histoplasma capsulatum* is an opportunistic fungal pathogen that threatens the life of immune-compromised individuals, especially those infected with HIV (8). Upon entry into the respiratory system, the fungus transforms into yeast cells and is phagocytosed by the macrophage. The yeast cells of *Histoplasma* reside and replicate primarily in the phagosomes of macrophages (16). Gamma interferon (IFN-γ) produced by CD4⁺ and CD8⁺ T cells can activate macrophages to produce reactive oxygen species and nitric oxide for fungal clearance (3, 14, 16, 31). A previous depletion study established the vital role of CD4⁺ T cells in clearing *Histoplasma* in intranasal infection of wild-type mice (3). Depletion of CD8⁺ T cells or a deficiency in major histocompatibility complex class I (MHC-I), on the other hand, has little effect on fungal clearance (3, 5). However, the protective role of CD8⁺ T cells is prominent in infection of mice with MHC-II deficiency (14), demonstrating that CD8⁺ T cells can be protective against histoplasmosis in the absence of functional CD4⁺ T cells. In HIV-infected individuals whose CD4⁺ T cell responses gradually decline, the CD8⁺ T cells become the major effectors defending against opportunistic infections. Thus, developing vaccines that aim to induce functional CD8⁺ T cell immune responses would be valuable.

Vaccines designed for nonviral pathogens often focus on eliciting CD4⁺ T cell and B cell immune responses (15, 24). The CD8⁺ T cell response receives relatively little attention. Recently, increasing evidence demonstrated that exogenous or nonviral antigens can be presented on MHC-I molecules to prime CD8⁺ T cell responses, processes referred to as “cross-presentation” and “cross-priming” (12). Exogenous antigens coupled with heat shock protein (25), exosomes (29), immune complexes (20), and latex beads (22) all can be cross-presented to prime CD8⁺ T cells. Immunizing mice with antigen-containing dead cells or with dead cell-pulsed dendritic cells is a well-recognized strategy in the development of cancer vaccines to elicit strong CD8⁺ T cell responses (6, 9). In their studies of infectious diseases, Albert et al. were the first to report that apoptotic monocytes deliver influenza antigens to dendritic cells and trigger CD8⁺ T cell immune responses (1). Nonviral intracellular pathogens such as *Salmonella* and *Mycobacterium* induce macrophage apoptosis, and the apoptotic cell blebs shuttle the bacterial antigens to uninfected bystander antigen-presenting cells to cross-prime CD8⁺ T cells (21, 33). We previously showed that sensitized CD8⁺ T cells were restimulated in *vitro* by dendritic cells that acquired *Histoplasma* antigens through phagocytosis of heat-killed *Histoplasma*-containing apoptotic macrophages (14). In addition, Winau et al. showed that subcutaneously immunizing mice with apoptotic

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vesicles prepared from *Mycobacterium bovis* BCG-ovalbumin-infected macrophages not only induces adoptively transferred OT-1 CD8+ T cell division and IFN-γ production in vivo but also protects mice from tuberculosis (28). The results of these studies together raise the possibility that a similar strategy aiming to cross-prime strong CD8+ T cell responses could be applied to develop fungal vaccines.

Here we show that immunization with apoptotic phagocytes (macrophages or neutrophils) containing heat-killed *Histoplasma* efficiently activated functional CD8+ and CD4+ T cells. Inhibiting apoptosis during the early phase of infection weakened the CD8+ T cell but not the CD4+ T cell response to pulmonary *Histoplasma* infection. We also demonstrate that, in mice subcutaneously immunized with viable *Histoplasma* yeasts in which CD8+ T cells are protective, there was heavy granulocyte and macrophage infiltration and the infiltrating cells became apoptotic. While the carboxyfluorescein diacetate succinimidy ester (CFSE)-labeled macrophage material localized within dendritic cells in the draining lymph node after immunization with CFSE-labeled apoptotic peritoneal macrophages (pMac) containing heat-killed *H. capsulatum* (pMac [HK He]), depleting dendritic cells in immunized CD11c-DTR mice significantly reduced CD8+ T cell activation. Our results reveal that phagocyte apoptosis during the early growth phase in a *Histoplasma*-infected host is associated with CD8+ T cell activation and that immunization with apoptotic pMac (HK He) or with apoptotic peritoneal neutrophils (pNeu) containing heat-killed *H. capsulatum* (pNeu [HK He]) efficiently evokes a protective CD8+ T cell response. Thus, such an approach is a viable option to exploit apoptotic phagocytes as antigen donor cells in the development of vaccines that elicit strong protective CD8+ T cell as well as CD4+ T cell responses to *Histoplasma*.

### MATERIALS AND METHODS

**Mice**. Wild-type, inducible nitric oxide synthase-deficient (NOS−/−), and β2 microglobulin-deficient (β2m−/−) mice were originally obtained from the Jackson Laboratory (Bar Harbor, MA) and bred at the Laboratory Animal Center, National Taiwan University College of Medicine. CD11c-DTR transgenic mice (10) were provided by A. DeFranco (University of California at San Francisco, San Francisco, CA). All mice used were of the C57BL/6 background and were housed in sterilized cages fitted with filter cage tops and fed with sterilized food and water. Mice at 8 to 10 weeks of age were used in all the experiments in this study. The present study was carried out in strict accordance with the recommendations in the *Guidebook for the Care and Use of Laboratory Animals* (4a). The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Taiwan University College of Medicine (permit 20040318).

**Abs and medium.** Allophycocyanin (APC)-rat-anti-mouse CD8 (clone 53-6.7), fluorescein isothiocyanate (FITC)-rat-anti-mouse CD4 (clone RM4-5), phycoerythrin (PE)-hamster-anti-mouse CD3 (clone 145-2C11), FITC-anti-rat-Mouse B220 (clone RA3-6B2), PE-anti-rat-Mouse F4/80 (clone BM8), PE-anti-rat-Mouse Gr-1 (clone RB6-8C5), and PE-anti-IgG1 isotype (for immunofluorescence staining) antibodies (Abs) were purchased from eBioscience (San Diego, CA). FITC- and PE-hamster-anti-mouse CD11c (clone HL3) were from BD Pharminogen (San Diego, CA). Purified hamster-anti-CD3 (clone 145-2C11), purified hamster-anti-CD8 (clone 53-6.7), PE- and FITC-anti-Gr-1, PE- and FITC-anti-F4/80, and PE- and FITC-anti-CD11c, were from BD Pharminogen (San Diego, CA) and bred at the Laboratory Animal Center, National Taiwan University College of Medicine. CD11c-DTR transgenic mice (Bar Harbor, MA) were obtained from the Laboratory Animal Center, National Taiwan University College of Medicine. CD11c-DTR transgenic mice (Bar Harbor, MA) were obtained from the Laboratory Animal Center, National Taiwan University College of Medicine. CD11c-DTR transgenic mice (Bar Harbor, MA) were obtained from the Laboratory Animal Center, National Taiwan University College of Medicine.

**Preparation of and immunization with apoptotic phagocytes containing heat-killed *Histoplasma*.** Wild-type or β2m−/− mice were injected intraperitoneally with 4% thiglycolate (Difco). To obtain macrophages, peritoneal cells were harvested 4 days later. The cells (2 × 10^6) were allowed to adhere in RPMI 1640 medium overnight. Nonadherent cells were removed by washing with prewarmed Hank’s balanced salt solution (HBSS). Twenty million heat-killed *Histoplasma*...
yeast cells were added to the monolayer. After 2 h of incubation at 37°C, the monolayer was washed with prewarmed HBSS to remove free Histoplasma yeast cells. The culture was left at 37°C overnight. Apoptosis was induced by treatment with lipopolysaccharide (LPS) (Sigma-Aldrich) (1 µg/ml) at 37°C for 4 h, followed by addition of 5 mM ATP (Sigma-Aldrich) for another 45 min. The cells became nonadherent. They were harvested by gentle pipetting and washed with RPMI 1640 medium two times. One hundred percent of the cells were apoptotic as confirmed by a TUNEL assay. Apoptotic macrophages were suspended in 50 µl of RPMI 1640 medium and injected subcutaneously into mice on two sides of the base of the tail (1 × 10⁶ cells/injection) by two injections administered 2 weeks apart.

To obtain neutrophils, peritoneal cells were harvested 4 h after thioglycollate injection. Seventy-five percent of the cells were Gr1⁺. Ten million of the heat-killed Histoplasma yeasts were added to culture containing 1 × 10⁶ neutrophils (multiplicity of infection [MOI = 10]). After overnight incubation, the cells were exposed to UV irradiation at 350 mJ/cm² to induce apoptotic cell death (7). Ninety percent of the cells were apoptotic after the treatment as confirmed by annexin V (BD Pharmingen) staining and flow cytometry analysis. Apoptotic cells were suspended in 50 µl of RPMI 1640 medium and injected subcutaneously into mice on two sides of the base of the tail (1 × 10⁶ cells/injection) by two injections administered 2 weeks apart.

To prepare CFSE-labeled apoptotic macrophages, thioglycollate-elicited peritoneal macrophages were allowed to take up heat-killed Histoplasma as described previously. Before induction of apoptosis, CFSE (Invitrogen, Carlsbad, CA) at a final concentration of 5 µM was added to the macrophage culture and let stand for 5 min. The monolayers were washed free of CFSE and treated with LPS and ATP to induce apoptosis. The detached cells were collected, washed, and injected subcutaneously into mice.

**Injection of apoptosis inhibitor.** Boc-D-FMK (pan-caspase inhibitor; Sigma-Aldrich) (0.2 µmol) or Z-FA-FMK (control peptide; Sigma-Aldrich) (0.2 µmol) was dissolved in 10 µl of dimethyl sulfoxide (DMSO) for storage and diluted with 190 µl of sterilized Dulbecco’s PBS immediately before injection. Mice were injected intraperitoneally with Boc-D-FMK or Z-FA-FMK at the time of intratrachal infection with Histoplasma and daily afterwards until the day of the experiment (2).

**In vivo cell depletion.** To deplete CD11c⁺ dendritic cells, CD11c-eDTR transgenic mice and wild-type control mice were injected with 0.1 µg of diphtheria toxin–200 µl PBS (Sigma-Aldrich) intraperitoneally 1 day before and 2 days after immunization with apoptotic MHC (HK). Diphtheria toxin treatment depleted >86% of the CD11c⁺ in the spleen as determined by flow cytometry at 3 to 4 days after the last dose of diphtheria toxin. To deplete CD4⁺ and/or CD8⁺ T cells, mice were injected intraperitoneally with concentrated supernatants from hybridoma GK1.5 (anti-CD4) or 2.43 (anti-CD8) or both at 1 day before and twice weekly after challenge with Histoplasma. The protein concentrations of concentrated hybridoma supernatants were 4.0 mg of GK1.5 and/or 2.5 mg of 2.43 per injection. The treatment depleted >95% of CD4⁺ and >95% of CD8⁺ T cells as determined by flow cytometry at 3 to 4 days after the last dose of the antibody treatment.

**Determination of fungal burden.** Mice immunized subcutaneously with live Histoplasma, apoptotic MHC (HK), or RPMI 1640 medium were challenged intravenously with 2.5 × 10⁶ live Histoplasma yeast cells at day 5 after the second immunization. Spleens were harvested and homogenized at day 10 after challenge; the homogenates were plated on cys-teine-blood-glucose agar and incubated at 30°C. Mycelial colonies were counted at 10 to 14 days after incubation. Fungal burdens in the spleens were expressed as the log₁₀ value of CFU per spleen as previously described (14).

Lungs from mice treated with caspase inhibitor or control peptide or left untreated were collected at day 10 after intratracheal inoculation of 2 × 10⁶ Histoplasma yeast cells. The lungs were homogenized, and the homogenates were plated on cys-teine-blood-glucose agar and incubated for 10 to 14 days. The CFU level in the control mice was assigned a value of 100%, and the CFU levels in the experimental groups were compared to the control level. The data are presented as percent CFU change.

**H and E staining.** The cryosections were fixed with 4% paraformaldehyde and stained with 0.1% hematoxylin and 2% eosin for 5 min.

**Statistics.** The comparisons between multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc Tukey test, performed using Prism 4.0 software (GraphPad Software). The comparisons between two groups were analyzed by Student’s t test. The level of statistical significance was defined as P < 0.05.

**RESULTS**

Apoptotic macrophages as antigen donors are efficient in delivering Histoplasma antigen to induce functional CD8⁺ and CD4⁺ T cell activation in vivo. In previous in vitro studies, we showed that apoptotic pMac (HK Hc) can deliver fungal antigens contained within them to dendritic cells, which in turn cross-present Histoplasma antigens to activate sensitized CD8⁺ T cells (14). Here we extended the study and immunized mice with apoptotic macrophages that had taken up Histoplasma yeasts. IFN-γ production as determined by intracellular cytokine staining was used as an indicator of functional activation of both CD8⁺ and CD4⁺ T cells (13). The results presented in Fig. 1A show that apoptotic pMac (HK Hc) at 2 × 10⁶ and 4 × 10⁶ efficiently induced activation of functional CD8⁺ and CD4⁺ T cells. Although the numbers of IFN-γ-producing CD8⁺ and CD4⁺ T cells did not reach the levels seen with the mice receiving live Histoplasma, they were significantly higher than those seen with mice receiving heat-killed Histoplasma alone (Fig. 1A) (P < 0.001 for CD8⁺ and P < 0.05 for CD4⁺). Moreover, CD8⁺ T cells expressed not only IFN-γ but also granzyme B (Fig. 1B). These results indicate that heat-killed Histoplasma antigens contained within apoptotic macrophages are efficiently delivered to cross-prime CD8⁺ T cells and activate CD4⁺ T cells in vivo.

The results presented in Fig. 2A further revealed that immunization with apoptotic macrophages mixed with heat-killed Histoplasma apoptotic (pMac + HK Hc), viable macrophages with ingested heat-killed Histoplasma pMac (HK Hc), apoptotic macrophages (apoptotic pMac), or viable macrophages (pMac) alone did not induce T cell responses at a level comparable to that of immunization with apoptotic pMac (HK Hc). It is noteworthy that apoptotic pMac simply mixed with heat-killed Histoplasma before injection (apoptotic pMac + HK Hc) did not induce a strong CD8⁺ or CD4⁺ T cell response (Fig. 2A), suggesting that Histoplasma antigens must be contained within apoptotic macrophages for antigen presentation. Moreover, immunization with apoptotic neutrophils containing heat-killed Histoplasma [apoptotic pNeu (HK Hc)] was as efficient as apoptotic pMac (HK Hc) at inducing a strong CD8⁺ or CD4⁺ T cell response (Fig. 2B), which indicates that, after Histoplasma yeasts are taken up by the phagocytes, the fungal antigen contained within the phagocytes can be presented to activate CD8⁺ and CD4⁺ T cells. Interestingly, immunizing mice with apoptotic pMac (HK Hc) lacking MHC-I molecules (β₂-m⁻/⁻) activated CD8⁺ T cells as efficiently as wild-type macrophages did (Fig. 2C), indicating that apoptotic cells serve as antigen donor cells to deliver Histoplasma antigens rather than as antigen-presenting cells in the immunized host.

CD8⁺ and CD4⁺ T cells make equal contributions to host defense after immunization with apoptotic macrophages containing heat-killed Histoplasma. Next, mice immunized with apoptotic pMac (HK Hc) were challenged with Histoplasma intravenously. Figure 3A shows that immunized mice had significantly lower fungal burdens in the spleen compared with uninunmized mice (CM; P < 0.01) at day 10 after challenge. Depleting either the CD8⁺ population T cell or the CD4⁺ T cell population in mice immunized with apoptotic pMac (HK Hc) at the time of challenge significantly increased the fungal
burden by 61-fold or 74-fold, respectively, and depleting both populations increased T cell fungal burden by 490-fold (Fig. 3B). These results, together with those presented in Fig. 1 and 2, demonstrate that immunization with apoptotic pMac (HK Hc) effectively induces activation of protective CD8+ cells and that the contribution of CD8+ T cells to host defense against histoplasmosis is comparable to that of CD4+ T cells.

**Reduction of macrophage apoptosis is due to iNOS deficiency decreases CD8+ T cell activation.** To explore the relationship between macrophage cell death and CD8+ T cell activation, iNOS−/− and wild-type mice were intratracheally infected with *Histoplasma*. Figure 4A shows that the number of apoptotic F4/80+ cells in the lungs of infected iNOS−/− mice was significantly lower than in the wild-type mice (*P < 0.05*). Coinciding with lower numbers of apoptotic F4/80+ cells in the lungs, the percentage of IFN-γ-producing CD8+ T cells was also significantly lower in iNOS−/− mice than in wild-type mice (Fig. 4B) (*P < 0.05*). Interestingly, the CD4+ T cell response in iNOS−/− mice was not affected by the reduction in numbers of apoptotic F4/80+ cells (Fig. 4B). The results of the macrophage phagocytosis assay and stimulation of CD8+ T cells with anti-CD3 and anti-CD28 antibodies showed that macrophages and CD8+ T cells from iNOS−/− mice were functionally comparable to those of wild-type mice (data not shown). Additionally, bone marrow-derived dendritic cells from iNOS−/− mice expressed more MHC-I and CD40 but fewer MHC-II molecules on the surface than cells from the wild-type mice after infection with *Histoplasma* yeasts (data not shown). These observations excluded the possibility of an intrinsic defect of macrophages or of the antigen presentation functions of dendritic cells in the iNOS−/− mice affecting CD8+ T cell activation. These results together indicated that iNOS, possibly through production of nitric oxide, is the cause of macrophage apoptotic cell death and that macrophage death is important for CD8+ T cell activation in cases of pulmonary infection by *Histoplasma*.

**Inhibition of apoptotic cell death by a caspase inhibitor decreases CD8+ T cell responses and exacerbates pulmonary histoplasmosis.** To further demonstrate the importance of apoptosis of antigen donor cells in activation of CD8+ T cell responses after pulmonary infection, we treated infected mice with the caspase inhibitor Boc-D-FMK (26). The number of apoptotic F4/80+ cells seen at the early phase after infection was significantly (*P < 0.05*) reduced in mice treated with Boc-D-FMK compared with the number seen after treatment with CD4+ T cells in the total CD8+ or CD4+ T cell population. The data shown represent the means ± standard deviations (SD) of the results obtained with 5 mice used in 3 independent experiments.
FIG. 2. Apoptotic phagocytes containing Histoplasma induce T cell responses independent of donor cell MHC-I. (A) Thioglycolate-elicited peritoneal macrophages were allowed to ingest heat-killed Histoplasma before treatment with LPS and ATP. Mice were subcutaneously inoculated with 2 x 10^6 of apoptotic pMac (HK Hc) [Apoptotic pMac (HK Hc)] or nonapoptotic pMac [pMac (HK Hc)] containing heat-killed Histoplasma, apoptotic pMac [Apoptotic pMac] or nonapoptotic pMac (pMac) without ingested heat-killed Histoplasma, or apoptotic pMac mixed with heat-killed Histoplasma [Apoptotic pMac + HK Hc] at days −14 and 0. Cells from inguinal lymph nodes were harvested at day 5 and stained with FITC-anti-CD4, APC-anti-CD8, and PE-anti-IFN-γ antibodies. IFN-γ-producing CD8^+ and CD4^+ T cells were analyzed by flow cytometry. Data shown represent the means ± SD of the results obtained with 4 mice used in 3 independent experiments. The *P values were obtained by comparing the percentages of IFN-γ-producing cells in the five groups by a post hoc Tukey test. *P < 0.05; **P < 0.01; ****P < 0.001. (B) Peritoneal macrophages (pMac) or neutrophils (pNeu) were allowed to ingest heat-killed Histoplasma [pMac (HK Hc) or pNeu (HK Hc)] or mix with heat-killed Histoplasma [pMac + HK Hc] or [pNeu + HK Hc]) before apoptosis was induced by LPS and ATP treatment or UV irradiation. Mice were subcutaneously inoculated with the apoptotic cells as described for panel A, and the CD8^+ and CD4^+ T cell responses elicited were analyzed. Mice inoculated with PBS or live Histoplasma served as experimental controls. Data shown represent the means ± SD of the results obtained with 4 mice used in 2 independent experiments. The *P values were obtained by comparing the percentages of IFN-γ-producing cells in the four groups of mice receiving apoptotic cells by a post hoc Tukey test. *P < 0.05; NS, not significant. (C) Peritoneal macrophages from wild-type (WT) or β2 microglobulin-deficient (β2m^-/-) mice were allowed to ingest heat-killed Histoplasma before treatment with LPS and ATP. Wild-type mice were immunized with apoptotic pMac (HK Hc) that had been obtained from either wild-type or β2, microglobulin-deficient mice. The immunization schedule, time of experiment, and staining were the same as described for panel A. Cells harvested from mice receiving wild-type macrophages cultured in medium only (CM) served as controls. Data shown represent the means ± SD of the results obtained with 4 mice used in 3 independent experiments. ****P < 0.001; NS, not significant.
Z-FA-FMK control peptide (23) (Fig. 5A). Interestingly, and consistent with what we observed in iNOS−/−/iNOS−/− mice, the CD8+ T cell response but not the CD4+ T cell response was reduced in mice whose macrophage apoptosis was inhibited (Fig. 5B). Notably, Boc-D-FMK treatment resulted in a higher fungal burden than Z-FA-FMK treatment (Fig. 5C). These results together indicate that macrophage apoptosis is critical for CD8+ T cell activation and subsequent fungal clearance in Histoplasma infection.

CD8+ T cell responses in mice subcutaneously immunized with viable Histoplasma yeasts correlate with infiltrating cell death. It was previously reported that subcutaneous immunization with live Histoplasma yeast cells elicits protective CD8+ T cell responses even in the absence of CD4+ T cells (32). Whether induction of a protective CD8+ T cell response correlates with antigen donor cell death in this mouse model was a question to be addressed. Immunofluorescence analysis revealed that the cellular infiltrates in the subcutaneous tissues

FIG. 3. Immunization with apoptotic macrophages containing Histoplasma confers CD8+ and CD4+ T cell-dependent protection against challenge. (A) Wild-type mice were inoculated subcutaneously with live Histoplasma (Live Hc) (2 × 10^6 yeast cells/mouse), apoptotic macrophages containing heat-killed Histoplasma [Apoptotic pMac (HK Hc)] (2 × 10^6 cells/mouse), or medium (CM) as described for Fig. 2A. At day 5, mice were challenged intravenously with 2.5 × 10^4 live Histoplasma yeasts. Fungal burden in the spleen was assessed 10 days after challenge. (B) Wild-type mice were inoculated subcutaneously with apoptotic pMac (HK Hc) as described for panel A. At the day before challenge and twice weekly thereafter, mice were treated with depleting antibodies against either CD4 or CD8 or both. Fungal burden in the spleen was assessed 10 days after challenge. (A and B) Data represent the means ± SD of the results obtained with 5 or 6 mice per group. The P values were obtained by comparing the results by a post hoc Tukey test (**, P < 0.01; ****, P < 0.001; NS, not significant).

FIG. 4. iNOS deficiency reduces F4/80+ cell apoptosis and weakens CD8+ T cell response in pulmonary histoplasmosis. Wild-type and iNOS−/−/iNOS−/− mice were infected intratracheally with 2 × 10^5 live Histoplasma. (A) At day 5 after infection, lung cells were isolated and stained with PE-anti-F4/80 antibody and TUNEL reagents containing FITC-dNTP. F4/80+ TUNEL+ apoptotic cells were analyzed by flow cytometry. Data shown represent the means ± SD of the total numbers of apoptotic F4/80+ cells determined for 3 mice used in 3 independent experiments. (B) At day 10 after infection, the mediastinal lymph nodes were harvested and cells were stained with PE-anti-IFN-γ and FITC-anti-CD4 or APC-anti-CD8 antibodies. Data shown represent the means ± SD of the percentages of IFN-γ-producing CD8+ or CD4+ T cells in the total CD8+ or CD4+ T cell population determined for 6 mice used in 3 independent experiments. Cells harvested from uninfected wild-type mice served as controls. The P values were obtained by comparing the results determined for pairs of groups (linked by a bracket) using Student’s t test (*, P < 0.05; NS, not significant).
(Fig. 6A) were mostly Gr-1<sup>+</sup> and F4/80<sup>+</sup> cells, some CD11c<sup>+</sup> cells, and very few B220<sup>+</sup> and CD3<sup>+</sup> cells (Fig. 6B and C). A single-cell suspension prepared from the digested subcutaneous tissues contained mainly Gr-1<sup>+</sup> (68.9%) and F4/80<sup>+</sup> (18.9%) cells (Fig. 6B), confirming that granulocytes and macrophages represent the major cell populations at the site of subcutaneous inoculation. It is noteworthy that both infiltrating granulocytes and macrophages became apoptotic (Fig. 6D) and that granulocytes and macrophages constituted 77.6% and 17.2%, respectively, of the total apoptotic cell populations at day 5 after inoculation (Fig. 6E). Moreover, subcutaneous inoculation of iNOS<sup>−/−</sup> mice with viable *Histoplasma* induced CD4<sup>+</sup> T cell but not CD8<sup>+</sup> T cell activation (Fig. 6F). Together, these results point to the importance of apoptotic cell death during the early phase of *Histoplasma* infection in inducing protective CD8<sup>+</sup> T cell responses and fungal clearance.

**CD11c<sup>+</sup> cells are required for CD8<sup>+</sup> T cell cross-priming.**

We further investigated the role of host dendritic cells in presenting antigens contained within apoptotic macrophages to activate CD8<sup>+</sup> T cells. Mice were subcutaneously immunized with CFSE-labeled apoptotic pMac (HK Hc). Five days after immunization, CFSE-labeled apoptotic macrophage cytoplasmic materials were found inside CD11c<sup>+</sup> cells in the draining lymph nodes (Fig. 7A). Furthermore, depleting CD11c<sup>+</sup> cells in CD11c-DTR transgenic mice by diphtheria toxin treatment before inoculation of apoptotic pMac (HK Hc) significantly reduced the percentage of IFN-γ-producing CD8<sup>+</sup> by 6.5-fold compared with the results seen with wild-type mice treated with diphtheria toxin (Fig. 7B). These results indicate that host CD11c<sup>+</sup> dendritic cells at the inoculation site take up the fungal antigens contained within the apoptotic macrophages after immunization with apoptotic pMac (HK Hc) and migrate to the draining lymph nodes to cross-prime CD8 T cells.

**DISCUSSION**

*Histoplasma capsulatum* is a facultative intracellular fungal pathogen of the macrophage (16). It has long been recognized that the macrophage serves as the host cell as well as the
A effector cell for *Histoplasma* yeasts (17, 30, 31). The results of our in vitro studies showed that ingestion of *Histoplasma* yeasts 

**Fig. 6.** CD8$^+$ T cell responses in mice subcutaneously immunized with *Histoplasma* correlate with infiltrating cell death. (A to E) Wild-type mice were injected subcutaneously with live *Histoplasma* at day 0, and subcutaneous tissues were collected at day 5. (A) The subcutaneous tissues at the inoculation site were harvested. The tissues were subjected to cryosectioning and stained with hematoxylin and eosin (H&E) (magnification, \( \times 40 \)). The circled area shows cellular infiltration in the inoculation site. (B) Subcutaneous tissues of the inoculation sites were treated with type I collagenase to obtain single cells. Isolated cells were stained with FITC-anti-CD11c and PE-anti-Gr-1 or PE-anti-F4/80 antibodies and analyzed by flow cytometry. The numbers indicate the percentages of CD11c$^+$, Gr-1$^+$, or F4/80$^+$ cells in the total cell population. The data shown are from one mouse and are representative of the results of two independent experiments. (C) Cryosections of the subcutaneous tissues at the inoculation site were prepared, and the cell populations were determined by staining with PE-anti-Gr-1, PE-anti-F4/80, or PE-anti-CD3 (red) and FITC-anti-CD11c or FITC-anti-B220 (green) antibodies and Hoechst stain 33258 (blue). Images were viewed under a confocal microscope. Bars, 40 \( \mu \text{m} \) (magnification, \( \times 630 \)). (D) Cryosections of the subcutaneous tissues at the inoculation site were stained with TUNEL reagents containing FITC-dNTP and PE-anti-Gr-1 or PE-anti-F4/80 antibodies. Images were viewed under a confocal microscope. The arrowheads point to apoptotic granulocytes or macrophages where TUNEL-reactive nuclei are adjacent to Gr-1$^+$ or F4/80$^+$ staining. Bars, 40 \( \mu \text{m} \) (magnification, \( \times 630 \)). (E) Cells isolated from subcutaneous tissues at days 3, 5, and 7 after inoculation of *Histoplasma* were stained with TUNEL reagents and PE-anti-F4/80 or PE-anti-Gr-1 antibodies. The percentages of apoptotic macrophages (TUNEL$^+$ F4/80$^+$) and granulocytes (TUNEL$^+$ Gr-1$^+$) were analyzed by flow cytometry. The bar graphs show the means \( \pm \) SD of the percentages of apoptotic macrophages or granulocytes in the total TUNEL$^+$ apoptotic cell populations. (F) Wild-type and iNOS$^-$/iNOS$^-$/mice were injected subcutaneously with live *Histoplasma* at days \(-14\) and 0. Cells were isolated and stained as described for Fig. 1. Data shown represent the means \( \pm \) SD of the results obtained with 6 mice used in 3 independent experiments (***, \( P < 0.005 \); NS, not significant by Student’s t test).

effecting killed yeasts [pMac (HK Hc)] does not efficiently activate CD8$^+$ and CD4$^+$ T cells (Fig. 2A and B). In infection by *Histoplasma*, iNOS deficiency and apoptosis inhibitor treatment decrease the number of apoptotic macrophages and weaken CD8$^+$ T cell but not CD4$^+$ T cell responses. The results of our study demonstrate that apoptosis of phagocytes is an immune mechanism that is of critical importance to CD8$^+$ T cell activation in histoplasmosis.

Infection-induced apoptosis is a newly recognized immune function important to antimicrobial immunity, especially in
defense against phagosome-enclosed pathogens (21, 27, 33). Different host and fungal factors are known to be involved in defense against phagosome-enclosed pathogens (21, 27, 33). Different host and fungal factors are known to be involved in Histoplasma-induced macrophage death. Our unpublished data showed that Histoplasma yeasts induced macrophage iNOS expression. Coinciding with those results, we have shown in the present report that Histoplasma infection-induced macrophage apoptosis is significantly reduced in iNOS−/− mice compared to wild-type mice (Fig. 4). These findings together demonstrate that iNOS induction contributes to macrophage death. On the fungus side, both the yeast cell wall component α-(1,3)-glucan and the secreted calcium-binding protein (CBP) have been previously identified as Histoplasma virulence factors (19). Silencing α-(1,3)-glucan biosynthesis reduces the ability of Histoplasma yeast to cause macrophage-like P388D1 cell death (18), and a cbp1-null mutant lost the ability to kill P388D1 cells in culture in vitro (11). Although it is not clear whether Histoplasma yeast cell α-(1,3)-glucan and secreted CBP cause macrophages to produce nitric oxide, it appears that a built-in system exists in the interaction between the fungus and macrophage to ensure macrophage death, which we showed is essential to CD8+ T cell cross-priming.

We have showed in the present report that macrophages and granulocytes in subcutaneous tissues as well as macrophages in the lungs constitute the major cell populations during the early phase of subcutaneous and pulmonary infection, respectively, and undergo apoptosis (Fig. 6 and 4). The percentages of infiltrating CD3+ T cells, in contrast, are low at early time points (data not shown). Allen and Deepe analyzed the apoptotic cell populations in the lungs of Histoplasma-infected mice at days 7, 14, and 21 after infection (2), which represent the activation and contraction phases of T cell response (13), and found that apoptotic CD3+ T cells were the major apoptotic cells (2, 13). Their study showed that inhibition of apoptosis is associated with elevation of interleukin-4 (IL-4) and IL-10 levels and that the release of those cytokines exacerbates the severity of infection. We found that administration of Boc-D-FMK at the beginning of infection reduced the number of apoptotic macrophages by about 50% at day 4 and dampened the subsequent CD8+ T cell but not the CD4+ T cell response (Fig. 5A and B). This causal relationship demonstrates that macrophage apoptotic cell death at the early phase of infection, the time when antigen processing, presentation, and cross-presentation take place, modulates the protective CD8+ T cell response. Our results and those of Allen and Deepe together stress that apoptosis at the antigen processing and presentation phases as well as the T cell contraction phase is critical for protective immune responses in histoplasmosis.

It has been reported that immunization of mice with apoptotic vesicles prepared from BCG-OVA-infected macrophages induces adoptively transferred OT-1 CD8+ T cell activation and that such mice are protected against challenge by Mycobacterium tuberculosis (28). Injection of apoptotic antigen-pulsed dendritic cells induces both humoral and cellular immune responses and confers protection against Toxoplasma gondii presented as an oral challenge (4). In this study, we showed that immunization with apoptotic pMac (HK Hc) induces activation of functional CD8+ and CD4+ T cells and that CD8+ and CD4+ T cells contribute equally to efficient clearance of Histoplasma. These results together suggest that apoptotic cells delivering antigens to elicit functional and protective CD8+ and CD4+ T cell responses can be used to immunize against intracellular bacterial and parasitic as well as fungal pathogens.

After subcutaneous injection of live Histoplasma, there is massive granulocyte infiltration and 60 to 80% of the cells are apoptotic at days 3 to 7 after inoculation (Fig. 6A to E). Apoptotic neutrophils are as efficient as apoptotic macrophages at delivering Histoplasma antigens to activate CD8+ and CD4+ T cells (Fig. 2B). Granulocyte infiltration in the spleen after systemic infection or in the lungs after intracheal infection is not as pronounced as it is in the subcutaneous tissues after local infection (S.-H.H. personal observation). The contribution of apoptotic neutrophils to cross-presentation in infection through the natural routes may not be as important as that of the macrophages. However, utilizing...
apoptotic granulocytes or neutrophils as antigen donors for immunization is also a feasible option.

In summary, we showed that phagocyte apoptosis during the early phase of *Histoplasma* infection is important for activation of functional CD8⁺ T cells. Immunization with apoptotic pMac (HK Hc) or, alternatively, apoptotic pNeu (HK Hc) activates both CD8⁺ and CD4⁺ T cells and protects against fungal challenge. Thus, exploiting the role of apoptotic phagocytes as antigen donor cells is a viable strategy for the development of efficacious vaccines against histoplasmosis.

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