T

T   T

γ

-   CD4   CD8 T

CD8 T   CD4 T

90%   γ   CD4 T

85%   γ   CD8 T   CD44

γ   CD44

Vβ

T

Vβ   γ   Vβ

γ

CD8 T   CD4 T

CD8 T   CD8 T

CD4 T

MHC class II

CD8 T

CD8 T

γ　granzyme B　　　　CD8 T

IC-21　　　　　　　CD8 T

CD4 T

Journal of Immunology

：CD4 T　　　CD8 T　　　　　　　　　　T

Abstract

Part I.

Functional T cells are critical to host defense against infection. It has been reported that functional T cells as determined by their cytokine production represent antigen-specific T cells in infectious disease models. In this study, we enumerated *Histoplasma*-specific interferon γ-producing cells in bulk splenocyte culture and showed that infection with *Histoplasma capsulatum*, an intracellular pathogen of the macrophage, activated both CD4 and CD8 T cells. The magnitude of CD8 T cell response was lower than CD4 T cell, but the expansion and contraction of both cell types followed the same kinetics. Over 90% of interferon γ-producing CD4 T cells and >85% of CD8 T cells expressed CD44^{hi} phenotype. The strong correlation between interferon γ production and CD44^{hi} expression was observed not only at the peak of response but also throughout the course of infection. Moreover, a broad spectrum of Vβ populations responded to systemic as well as pulmonary infections, suggesting no obvious T cell receptor bias in primary immune response to histoplasmosis. While each Vβ population contributed to interferon γ production, several specific Vβ populations made up higher percentages of interferon γ-producing cells. Our study laid the groundwork for further investigations in immune response to histoplasmosis.

Part II.

The contribution of CD8 T cells in host defense against histoplasmosis is minor in the CD4-T-cell-intact mouse, as it has been shown that depleting CD8 T cells delays but does not affect fungal clearance. However, it remains to be determined whether the CD8 T cells are protective in a host lacking functional CD4 T cells. In this study, MHC class II-deficient mice infected with *Histoplasma* kept the fungus in check for up to 16 weeks, indicating CD8 T cells are able to limit fungal replication. *Ex vivo* studies showed that CD8 T cells from *Histoplasma*-infected mice expressed both intracytoplasmic interferon-gamma and granzyme B. Furthermore, CD8 T cells exhibited cytotoxic activity against IC-21 macrophage targets containing *Histoplasma*. Therefore, CD8 T cells are critical for host defense against the intracellular nonviral pathogen, especially in a host lacking functional CD4 T cells. (In revision)

Key words: CD4 T cells, CD8 T cells, *Histoplasma*, functional T cells.

Part I.

The paper has been published on International Immunology, 16: 1663-1673, 2004, the

PDF file of the publication is attached.

Part II.

Background and Aims

Infection with HIV generates an immune response that usually contains but does not eliminate the virus. Both HIV-specific CD4 Th cells and CD8 CTL cells are activated. However, with progressive HIV infection, the CD4 T-cell counts gradually decline and are finally lost, while total CD8 T-cell counts increase and remain constant for a prolonged period of time (1-3). Therefore, there is much to be learned about the function of CD8 T cells in the absence of CD4 T cells, especially in defense against opportunistic infections.

Histoplasma capsulatum is an opportunistic fungal pathogen that kills AIDS patients (4). Depletion (5) and adoptive transfer (6) studies have established the vital role of CD4 T cells in clearing *Histoplasma* in the mouse model. The functional role of CD4 T cells is to produce IFNγ (7-9). Depleting CD4 T cells dramatically decreases IFNγ production and accelerates mortality in mice (8, 9). On the contrary, CD8 T cell depletion or β_2m deficiency only marginally affects fungal clearance (9, 10). Since the role of CD8 T cells in a CD4-T-cell-intact host against histoplasmosis is comparatively minor, not much is known about CD8 T cell function or how they are activated in *Histoplasma* infection. Considering the importance of CD8 T cells in defense against infections in patients with low CD4 T cell counts, a thorough study of CD8 T cells against this fungal pathogen is warranted.

In this study, we first established the importance of CD8 T cells in protecting mice lacking functional CD4 T cells against histoplasmosis. We also showed CD8 T cells function by both perforin-independent as well as –dependent mechanisms, because CD8 T cells isolated from *Histoplasma*-infected mice produce IFNγ as well as kill *Histoplasma*-containing IC-21 macrophage targets *in vitro* through

perforin-mediated mechanism. Therefore, CD8 T cells are functional and critical in host defense against histoplasmosis in the absence of CD4 T cells.

Materials and Methods

Mice

Wild type (WT), MHC class II-deficient (IIKO) (11), C57BL/6-*Prf1[tm1Sdz]* perforin-deficient mice (PKO, stock number 002407, (12)) and B6.129P2-*B2m[tm1Unc]* β_2m-knockout (β2m$^{-/-}$, stock number 002087, (13)) mouse breeders were obtained from The Jackson Laboratory (Bar Harbor, ME), and bred at the Laboratory Animal Center, National Taiwan University College of Medicine. Mice were housed in sterilized cages with sterilized beddings and filter cage tops and fed with sterilized food and water. Mice at 8 to 10 wk of age were used in all the experiments. All mice with different genetic deficiencies are on the C57BL/6 background.

Fungus and Infection

Histoplasma capsulatum strain 505 yeast cells were cultured at 37   on brain-heart infusion (BHI) agar supplemented with cysteine (1mg/ml) and glucose (20mg/ml). Fresh yeast cell suspensions were prepared in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) for injection. Mice were injected intravenously with 2.5×10^4 yeast cells.

Reagents

RPMI 1640 medium (GIBCO-BRL) was supplemented with 10% heat-inactivated FCS (Biological Industries, Israel), 1mM sodium pyruvate, 2mM L-glutamine, 0.1mM nonessential amino acid, 100U/ml penicillin, 100μ g/ml streptomycin, 5×10^{-5}

M 2-ME, and 25mM HEPES buffer. All supplements were obtained from GIBCO-BRL. Dulbecco's phosphate buffered saline (dPBS, Biological Industries) supplemented with 1% FCS and 0.1% NaN_3 (Sigma-Aldrich, St. Louis, MO) was used as staining buffer. Dulbecco's PBS supplemented with 1% FCS, 0.1% NaN_3 and 0.1% saponin (Sigma-Aldrich) was used as Perm/Wash buffer.

Quantitation of fungal load in the spleen

Spleens were harvested from mice at different time points after infection. The spleen was homogenized in a tissue grinder with 1ml of RPMI 1640 medium. One to 10 serial dilutions were made and 0.1ml was plated onto glucose-peptone agar. *Histoplasma* mycelial colonies were enumerated after incubation at 30   for 10 to 14 days.

Splenic T cell enrichment by nylon-wool passage and CD4 and CD8 T cell purification by MACS beads

Spleen cells were harvested from mice at day14 of infection. Single-cell suspensions were applied to pre-rinsed nylon-wool columns. After incubation in an upright position at $37^{o}C$ incubator for 90 min the nonadherent cells were eluted. Nearly 80% of the cells collected were $CD3^{+}$ T cells.

    To purify CD4 and CD8 T cells, nylon-wool-passaged cells were washed and resuspended in MACS buffer (PBS with 0.5% FCS) containing anti-CD4 or anti-CD8 magnetic microbeads (Miltenyi Biotec, Germany). The suspension was mixed and incubated at $4^{o}C$ for 30 min. The positive selection column (LS+/VS+ column for up to 10^{8} total cells) was prepared by washing with buffer. The cell suspension was

loaded onto the column and the negative cells were allowed to pass through the column in a magnetic field. To elute positive cells, the column was removed from the separator. A plunger which was supplied by the manufacturer was used to flush out the positive fraction. To release beads from positively selected cells, the cells were further incubated at 37 for 90 min followed by centrifugation. In the resuspended cell pellet, about 95% of cells was either $CD4^+$ or $CD8^+$ T cells after MACS purification.

In vivo cell depletion

To deplete $CD4^+$ or $CD8^+$ T cells, mice were treated with concentrated anti-CD4 (clone GK1.5) or anti-CD8 (clone 2.43) hybridoma supernatant at the time of infection and twice weekly until concluding the experiment. The *in vivo* depletion efficiency was confirmed by staining splenic and lymph node cells with anti-CD4 (clone RM4-4) or anti-CD8 (clone 53-6.7) mAb. The results showed that GK1.5 treatment depleted >95% CD4 T cells and 2.43 treatment depleted >99% CD8 T cells.

Intracytoplasmic cytokine, granzyme B staining and flow cytometry

One million nylon-wool-enriched T cells, or MACS-purified $CD8^+$ T cells were cultured in flat-bottom 96-well plates in medium containing 2.5×10^4 live *Histoplasma* yeast cells for 24 h. At 6 h before harvest, monensin (2μ M, Sigma-Aldrich) was added. Cells were harvested and stained with allophycocyanin-conjugated anti-CD4 (clone GK1.5) or anti-CD8 (clone 53-6.7) at 4 in the dark for 30 min. Cells were washed and then fixed in Cytofix (4% paraformaldehyde in dPBS) for 20 min on ice and permeabilized by two washes with Perm/Wash solution. To stain intracellular

granzyme B, the cells were treated with purifed 2.4G2 antibody directed against mouse Fcγ II/III receptors (Fc Block) and stained with PE-labeled mouse anti-human granzyme B mAb (Caltag, Burlingame, CA) of which the specificity has been confirmed (14). PE-labeled mouse IgG1 was used as isotype control. To stain for intracellular cytokine, FITC- or PE-conjugated anti-IFNγ mAb (clone XMG1.2) in the Perm/Wash buffer was added and cells were left on ice in the dark for 30 min. Cells were washed with Perm/Wash buffer and fixed in staining buffer/2% paraformaldehyde. FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) was used for cell acquisition. The data were analyzed by CellQuest (BD Biosciences). All antibodies except granzyme B were purchased from eBioscience (San Diego, CA). Dead cells were excluded from analysis by both forward- and side-scattering.

Cytotoxicity assay

Mouse macrophage cell line IC-21 (ATCC TIB-186, C57BL/6 origin, SV40 transformed) was used as target cells. IC-21 cells at 1×10^4 per well were cultured in triplicate or quadruplicate in 96-well round bottom plate. After 2 h adhesion, heat-killed (1×10^5) *Histoplasma* yeasts were added to the wells for another 2 h. Free extracellular yeasts were removed by several washes with warm HBSS. The cultures were incubated for 16-18 h. Splenic T cells were obtained from normal or infected mice pre-treated with anti-CD4 mAb (clone GK1.5) two days prior to experiment. After nylon-wool enrichment, CD8 T cells were purified by MACS as described above. Different numbers of CD8 T cells were added to the wells containing antigen-pulsed IC-21 cells to obtain different E/T ratios. After 6 h of incubation, 50μl of culture supernatant was removed from the wells and transferred to an ELISA plate.

An equal volume of substrate provided in CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) was added. The coupled-enzymatic assay detects lactate dehydrogenase (LDH) in the culture supernatnts. The plates were left at room temperature in the dark for 30 min to allow conversion of a tetrazolium salt (INT) into a red formazan product. The reaction was stopped by Stop Solution and color absorbance was read at 492 nm. The amount of color formed is proportional to the number of lysed cells. Thus, % cytotoxicity was calculated from the absorbance values as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100\%$$

Statistics

Student's *t* test was used to compare the difference between groups.

Results

CD8 T cells are critical in host defense against histoplasmosis in the absence of CD4 T cells

To address whether CD8 T cells are protective against histoplasmosis, MHC class II-deficient mouse (IIKO) were infected with a sublethal dose of *Histoplasma* yeast cells. The IIKO mice lacking mature CD4 T cells were able to contain the fungal burden at the same level as that at wk 1 through wk 16 (Fig. 1*A*). To further confirm that CD8 T cells and not other cells in the IIKO mice were responsible for maintaining the chronic state, IIKO mice were depleted of CD8 T cells and infected with the same dose of *Histoplasma* yeast cells as was given to IIKO mice that were not depleted of CD8 T cells. Depleting CD8 T cells not only significantly increased the fungal burden ($p < 0.05$, Fig. 1*B*) but also resulted in 100% mortality (Fig. 1*C*). These results clearly show that CD8 T cells alone, although not able to clear the infection, are functional in limiting fungal replication and are protective in the absence of CD4 T cells.

CD8 T cell-mediated protection is perforin-dependent as well as perforin–independent

We reasoned that CD8 T cells could be contributing to holding *Histoplasma* in check by (i) production of IFN   which would activate macrophages to limit its intracellular growth and/or (ii) via a perforin/granzyme B-mediated cytotoxic effect on the infected macrophages. To determine the mechanism(s) whereby CD8 T cells defend the host against histoplasmosis, we compared perforin-deficient (PKO) mice

to those with MHC class I deficiency ($\beta_2 m^{-/-}$) in clearing the fungus. The results show that both $\beta_2 m^{-/-}$ and PKO mice had greater fungal burden than WT controls ($p < 0.05$) (Fig. 2A), confirming that CD8 T cells are involved in limiting fungal replication. These results also showed that perforin was important in controlling the fungus. However, fungal counts in $\beta_2 m^{-/-}$ mice were higher than in PKO mice at day21 ($p < 0.05$) of infection, suggesting a CD8 T cell-mediated perforin-independent mechanism was also operative in controlling the fungus. Together these results strongly demonstrate that CD8 T cell-mediated perforin-dependent as well as -independent mechanisms are operative in controlling the fungus.

To better define the protective role of perforin in the absence of CD4 T cells, the PKO mice were treated with anti-CD4 Ab and their ability to clear the fungus was evaluated. The results show that CD4 T cell depletion increased fungal burden in both PKO mice and WT mice as it has been reported ($p < 0.05$, Fig. 2B) (5, 9). More importantly, PKO mice depleted of CD4 T cells had greater fungal counts than WT mice with the same treatment, indicating perforin-dependent cytotoxic activity was critical in restricting the growth of *Histoplasma* in mice without CD4 T cells ($p < 0.05$, Fig. 2B).

CD8 T cells from Histoplasma-*infected mice have cytotoxic function*

The cytotoxic granules of CD8 T cells contain both pore-forming perforin and granzymes, which belong to the family of serine proteases (15, 16). Upon delivery to target cells, granzyme B, the most abundant granzyme in the granules, induces caspase-dependent as well as -independent (15, 16) cytoxicity. The appearance of intracytoplasmic granzyme B in Ag-specific CD8 T cells correlates well with their cytotoxic function (14). Since the specificity of the anti-granzyme B mAb has been

confirmed (14) and the isotype control was negative (Fig. 3*A*), we used granzyme B expression as a functional marker for CD8 T cell cytotoxicity. The results show that CD8 T cells from *Histoplasma*-infected mice expressed granzyme B with or without further antigenic stimulation *in vitro* whereas CD8 T cells from normal uninfected mice did not. It is noticeable that a non-CD8 T population also expressed granzyme B. These cells were identified to be NK1.1⁻, B220⁻, CD11c⁻, and CD4⁻ (data not shown). Importantly, CD8 T cells from infected WT mice at an effector:target cell ratio of 160:1, killed 25% of macrophage IC-21 targets containing *Histoplasma* yeasts, while CD8 T cells from infected PKO mice did not exhibit cytotoxity (Fig. 3*B*). Taken together, these data demonstrated the role of cytotoxicity by CD8 T cells as a protective mechanism in *Histoplasma*-infected mice.

CD8 T cells from Histoplasma-*infected mice produce IFN*

Since IFN   is critically important in host defense against histoplasmosis (7-9, 17, 18), we examined whether CD8 T cells in *Histoplasma*-infected mice were activated to produce IFN   . Intracytoplasmic cytokine and surface staining revealed that CD8 T cells, similar to CD4 T cells, produced IFN   (Fig. 4), although the CD8 T cell response was less than that of CD4 T cell. These data together with those in Fig. 2*A* demonstrate that activated CD8 T cells produce IFN   which could account, at least in part, for the perforin-independent anti-*Histoplasma* activity.

Discussion

In CD4-T-cell-intact mice, the contribution of CD8 T cells to defense against histoplasmosis is minor (9, 10). Thus, the functional role of CD8 T cells in *Histoplasma* infections has not been thoroughly studied. In the present study, we demonstrate that CD8 T cells alone in the absence of functional CD4 T cells, as in the situation of opportunistic infection in patients with low CD4 T cell counts, provide protection against *Histoplasma* infection. We further showed the functions of CD8 T cells in normal mice include IFN production and killing antigen-containing macrophage targets.

Perforin-mediated host defense against histoplasmosis has been reported (9). Perforin$^{-/-}$ mice exhibit increased fungal burden and accelerated mortality after infection (9). However, *in vitro* CD8 T cell cytotoxicity activity has never been demonstrated. Nor has it been clarified whether perforin-dependent anti-*Histoplasma* activity was attributed to CD8 T cells or NK cells. In this study, we showed specifically that CD8 T cells from *Histoplasma*-infected mice express granzyme B and exhibit cytotoxic activity against *Histoplasma*-containing IC-21 macrophage targets. These results provide strong evidence that CD8 T cells in mice infected with *Histoplasma* have cytotoxic function.

Histoplasma yeast cells are facultative intracellular organisms that reside within the macrophages (8, 17, 19). It has been reported that macrophages activated by IFN are armed to inhibit the intracellular growth of the fungus (20-22). CD8 T cell killing target cells by cytotoxic activity through granule exocytosis kills the intracellular pathogen (16, 23). Thus, by producing IFN and exhibiting cytotoxic activity against macrophage targets, the CD8 T cells in *Histoplasma*-infected mice contribute to restricting intracellular growth and killing of the fungus within the

macrophage.

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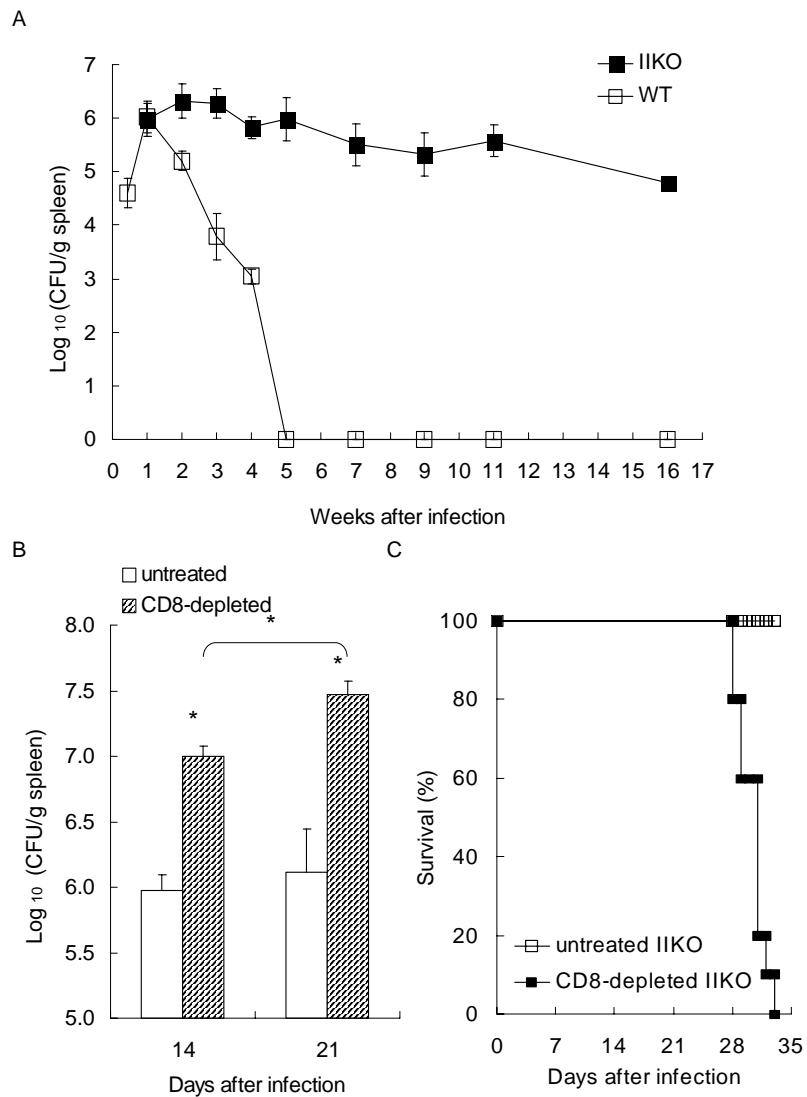23. Russell, J. H., and T. J. Ley. 2002. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol 20:323*.

FIGURE 1. CD8 T cells are critical in controlling *Histoplasma* infection in the absence of CD4 T cells. (*A*) Fungal counts in the spleen. (*B*) Fungal counts in IIKO mice depleted of CD8 T cells. IIKO mice were treated with anti-CD8 mAb twice weekly from day0 (* *p* < 0.05, *n* = 3 in each group). (*C*) Survival of IIKO mice with or without CD8 T cell depletion. IIKO mice were giving the same dose of *Histoplasma* yeast cells as in (*A*). *n* = 10 in each group.
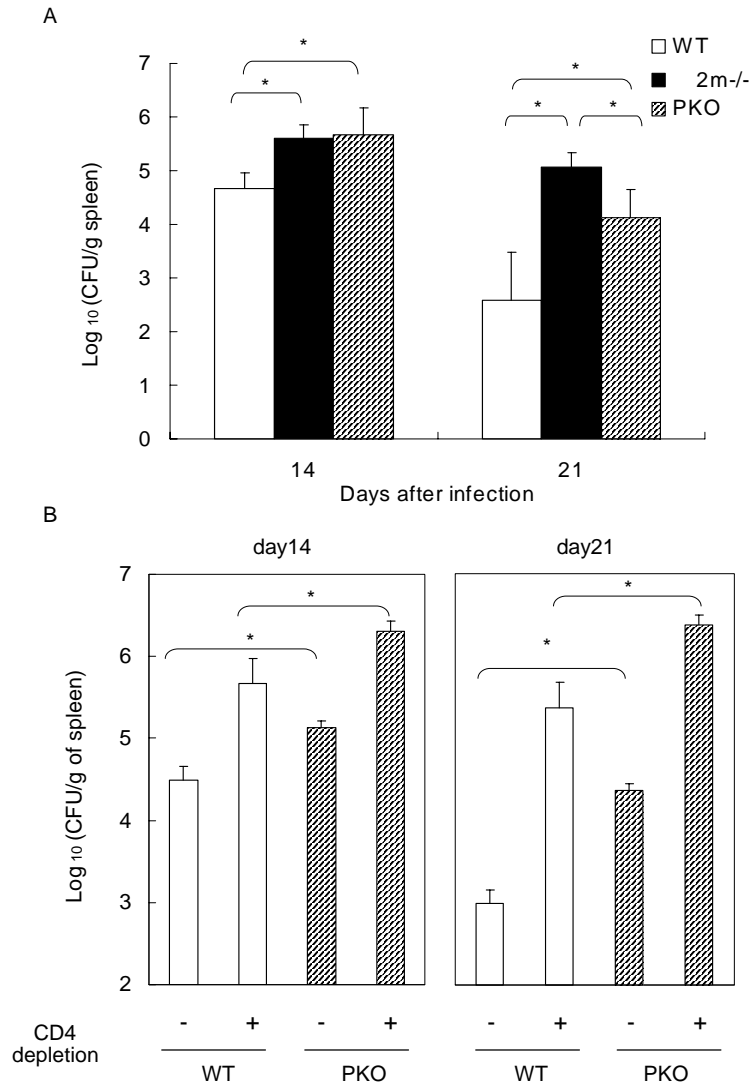
FIGURE 2. CD8 T cells limit *Histoplasma* replication by perforin-dependent as well as –independent mechanisms. (*A*) Perforin or ₂m deficiency impairs fungal clearance. (* $p < 0.05$, $n = 3$-5). (*B*) Perforin-dependent mechanism contributes to fungal clearance in the absence of CD4 T cells. WT and PKO mice (3/group) were treated with anti-CD4 mAb twice weekly from day0 (* $p < 0.05$). Experiment was repeated two times.
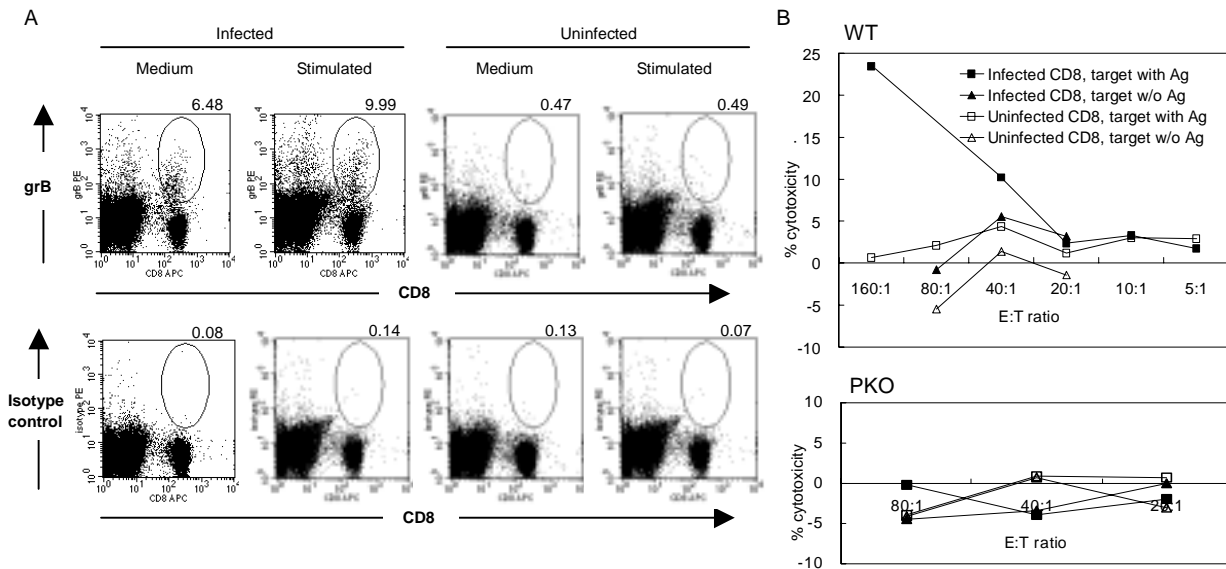
FIGURE 3. CD8 T cells in *Histoplasma*-infected mice are cytotoxic. (*A*) Granzyme

B expression. Spleen cells from *Histoplasma*-infected WT mice were stained with

mouse anti-human granzyme B (grB) mAb. PE-conjugated mouse IgG1 was used as

the isotype control. The numbers indicate the percentage of CD8 T cells expressing

granzyme B ($CD8^+grB^+/CD8^+$). The data shown are from one representative mouse.

Three mice were used in each experiment and the experiment was repeated three

times. (*B*) CD8 T cells are cytotoxic against *Histoplasma*-containing IC-21

macrophages. $CD8^+$ T cells were isolated from uninfected or infected WT (upper

panel) or PKO (lower panel) mice. IC-21 macrophages with or without internalized

heat-killed *Histoplasma* yeasts were used as targets. Cytotoxicity was determined by

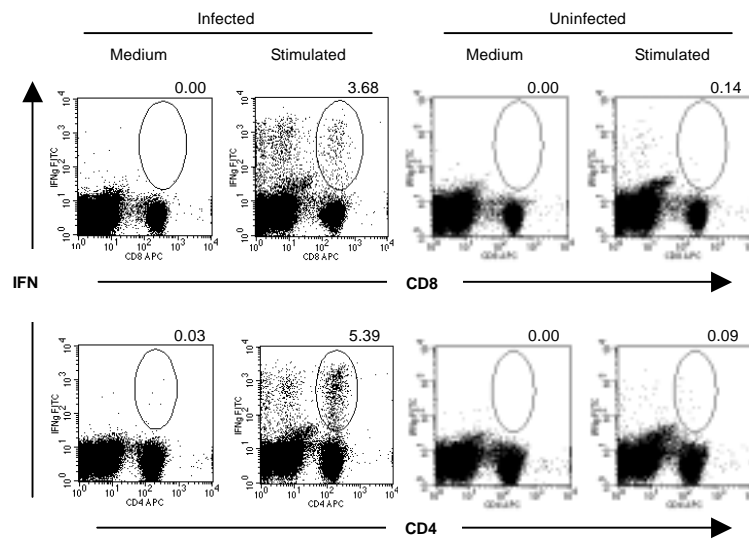LDH release assay. The data shown are representative of five separate experiments.

FIGURE 4. *Histoplasma* infection induces CD8 T cell IFNγ production. Spleen cells were harvested from infected or uninfected WT mice and cultured in medium alone or medium containing live *Histoplasma* yeast cells for 24 h. The splenocytes were stained for intracytoplasmic IFNγ. The numbers at the upper-right corner are the percentages of CD4 or CD8 T cells that are producing IFNγ (CD4$^+$IFNγ$^+$/CD4$^+$ or CD8$^+$IFNγ$^+$/CD8$^+$). The data shown are from one of eight independent experiments.