Vaccine Immunity to Pathogenic Fungi Overcomes the Requirement for CD4 Help in Exogenous Antigen Presentation to CD8\(^+\) T Cells: Implications for Vaccine Development in Immune-deficient Hosts

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Abstract
Systemic fungal infections with primary and opportunistic pathogens have become increasingly common and represent a growing health menace in patients with AIDS and other immune deficiencies. T lymphocyte immunity, in particular the CD4\(^+\) Th 1 cells, is considered the main defense against these pathogens, and their absence is associated with increased susceptibility. It would seem illogical then to propose vaccinating these vulnerable patients against fungal infections. We report here that CD4\(^+\) T cells are dispensable for vaccine-induced resistance against experimental fungal pulmonary infections with two agents, Blastomyces dermatitidis an extracellular pathogen, and Histoplasma capsulatum a facultative intracellular pathogen. In the absence of T helper cells, exogenous fungal antigens activated memory CD8\(^+\) cells in a major histocompatibility complex class I–restricted manner and CD8\(^+\) T cell–derived cytokines tumor necrosis factor \(\alpha\), interferon \(\gamma\), and granulocyte/macrophage colony-stimulating factor–mediated durable vaccine immunity. CD8\(^+\) T cells could also rely on alternate mechanisms for robust vaccine immunity, in the absence of some of these factors. Our results demonstrate an unexpected plasticity of immunity in compromised hosts at both the cellular and molecular level and point to the feasibility of developing vaccines against invasive fungal infections in patients with severe immune deficiencies, including those with few or no CD4\(^+\) T cells.

Key words: immunity • T cells • CD4 help • fungi • AIDS

Introduction
Opportunistic fungal infections have become increasingly common, especially in AIDS patients. Infections with Cryptococcus neoformans, Histoplasma capsulatum, Pneumocystis carinii, Coccidioides immitis, Candida albicans, and Blastomyces dermatitidis are collectively a major cause of morbidity and mortality in this patient population (1, 2). Treatment of these infections with amphotericin-B must be given for an extended duration, causes frequent and toxic side effects, and generally has to be followed by life-long suppressive therapy with antifungal azoles (2). No vaccines are available against fungi.

Cellular immunity mediated by T lymphocytes, and in particular CD4\(^+\) Th 1 cells, is considered the main defense against pathogenic fungi (3). The fact that AIDS patients with decreased CD4 T cells are predisposed to opportunistic fungal infections supports this premise (4). Furthermore, without CD4\(^+\) T cells, patients with disseminated cryptococcosis, even with the best available anti-fungal therapy, cannot clear the fungus and sterilize their tissues (5, 6). CD4\(^+\) T cells would appear to be crucial both for acquisition of protective immunity and combat against established opportunistic fungal infections.

CD8\(^+\) T cells play a pivotal role in immune responses against many viruses and tumors, and may also contribute to immunity to fungi (7–12). There are multiple paths for activating native CD8\(^+\) T cells. Most models have pointed to and clarified the requirement of CD4\(^+\) T helper cells for...
activation of naive CD8+ T cells. However, antiviral CTL have been induced with little or no requirement for CD4+ T helper cells (13–15), and CD8+ T cells have been shown to provide self-help in inducing antiviral peptide responses when the cells were present at a sufficiently high precursor frequency (16).

Herein, we tested, in immune-suppressed mice lacking CD4+ T cells, whether we could induce vaccine resistance against experimental infection with two of the principal systemic fungi B. dermatitidis and H. capsulatum, which cause disease in both immune-competent and immune-deficient hosts, with the latter being more susceptible. We report that CD4+ T cells are dispensable for induction of vaccine immunity against both fungal pathogens. CD8+ T cells compensate in the absence of CD4+ cells. Hence, CD8+ T cells alone, without CD4+ T cell help, can mediate efficient antifungal vaccine immunity. Vaccine resistance by CD8+ T cells was restricted by MHC class I and mediated by the production of TNF-α, and to a lesser extent, IFN-γ and GM-CSF. Although regulatory cytokines TNF-α and IFN-γ contributed significantly to the expression of CD8+ T cell immunity in wild-type mice, each of them also was shown to be dispensable for vaccine immunity in TNF-α−/− and IFN-γ−/− mice.

Our results underscore the plasticity of immune responses in the immune-compromised, CD4+ T cell–deficient host. Our findings demonstrate that residual elements of a compromised immune system can be recruited effectively against opportunistic and invasive fungal infections. Our study has general implications for immunologists and others trying to develop vaccine strategies for people with compromised immunity, including AIDS.

Materials and Methods

**Fungi.** Strains used were American Type Culture Collection 26199 (17), the isogenic, attenuated mutant lacking BAD1, designated strain #55 (18), and H. capsulatum strain G217B. Isolates of B. dermatitidis were maintained as yeast on Middelbrook 7H10 agar with oleic acid-albumin complex (Sigma-Aldrich) at 37°C; H. capsulatum was maintained at 37°C on Brain Heart Infusion Agar.

**Mouse Strains.** Inbred strains of mice including C57BL/6, the T lymphocyte specific Thy 1.1 allele carrying congeneric C57BL/6 strain B6.P–Thy1+Cy (stock #000406; reference 19), CD4-deficient C57BL/6–CD4−/Mak (stock #002663; reference 20), β-2-microglobulin–deficient B6.129P2–B2mH10 (stock #002087; reference 21), transporter associated with antigen processing (TAP)1-deficient B6.129S2–Abcm1Ap (stock #002944; reference 22), IFN-γ-deficient C57BL/6–Ifngm1Ts (stock #002287; references 23–25), and TNF-α–deficient B6.129–Tnfm1Gat (stock #003008; reference 26) and control B6129S2F2 (stock #010145) were obtained from The Jackson Laboratory and MHC class II–deficient C57BL/6Tac–Abbm1 (27) and C57BL/6 wild-type control mice were purchased from Taconic. Male mice 6–7 wk of age at the time of purchase were housed and cared for throughout these experiments according to guidelines of the University of Wisconsin Animal Care Committee, who approved all aspects of this work.

**In Vivo Cell Depletion and Neutralization of IFN-γ, TNF-α, and GM-CSF.** CD4+ and CD8+ T cells were depleted by mAb treatment. mAb GK1.5 (rat IgG2b anti-CD4) was purchased from American Type Culture Collection. mAb 2.43 (rat IgG2b anti-CD8) was provided by Dr. A. Rakhmilevich, UW-Madison, Madison, WI; mAb XMGI.2 (rat IgG1 anti–IFN-γ) was provided by R. Seder, National Institutes of Health, Bethesda, MD; and mAbs XT22.1 and MP1–22E9 (rat IgG2a anti–TNF-α and rat IgG2a anti–GM-CSF, respectively) were provided by Dr. G. Deepe, University of Cincinnati, Cincinnati, OH with permission of Dr. J. Abrams, DNAX Research Institute, Palo Alto, CA. Ascites was made in BALB/c Nu/Nu males. Rat IgG in ascites was ammonium-sulfate precipitated and quantified by measuring OD280. For depletion, mice received 250 μg anti-CD4 mAb or anti-CD8 mAb intravenously a day before infection and weekly afterward. Cell depletion analyzed by FACS showed >95% depletion of desired subsets in the peripheral blood and lung (data not depicted). For neutralization of IFN-γ, TNF-α, and GM-CSF, mice were injected intravenously with 1 mg, 0.5 mg, and 0.5 mg mAb, respectively, 4 to 6 h before infection, and then intraperitoneally every other day (IFN-γ) or every 3 d (TNF-α and GM-CSF) afterward with mAb doses above. Controls were given 500 μg of rat IgG (Sigma-Aldrich) by a similar schedule.

**Real Time RT-PCR.** Lung cells were obtained by crushing the organs (n = 6–8 mice/group) in 40 μM cell strainers (Becton Dickinson) to obtain single–cell suspensions. Erythrocytes were lysed with NH4Cl–Tris solution and washed twice. Total RNA was isolated from 1–5 × 10^6 lung cells using the RNeasy Mini Kit (QIAGEN). RNA was purified over RNase-free DNase Set (QIAGEN). 0.5 to 1 μg RNA in a final volume of 20 μl was reverse transcribed using random hexamers and the TaqMan RT-PCR Kit (Applied Biosystems). 5 μl of a 1:10 dilution of cDNA was amplified in a final volume of 25 μl PCR reaction using SYBR Green Supermix (Bio-Rad Laboratories). The following primers were used at a final concentration of 100 nM: IFN-γ (forward CCTGCGGCTGATGCTCGA; reverse CAGCAGAAACAGCCCATGAG), TNF-α (forward TGCCCTCCTCTCTCATGTT; reverse TCCCTGCCTTGGTGTTTTC), GM-CSF (forward GGGCCGCTTGAAAGATGAC; reverse TTGGTTTTCACGTTGCT), and 18S RNA as an endogenous control gene (forward CGCGCCTAGGTTGAATCT; reverse CGAACCCTCGACTTTCGTTTCT). Amplification was performed in an iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories) and assayed under the same conditions for all targets: 5 min at 95°C, 45 cycles of 15 s at 95°C, and 45 s at 60°C. Transcript quantity was calculated using the comparative Cۚ method (28) and reported as n-fold difference relative to a calibrator cDNA (i.e., sample from unvaccinated mice). Data represent average of two independent experiments.

**Intracellular Cytokine Staining.** Lung cells were obtained as described above. An aliquot of isolated cells was stained for surface CD4 and CD8 using anti-CD4 and anti-CD8 CyChrome mAbs (clone H129.19 and clone 53–6.7; BD Biosciences) to determine the percentage of CD4+ and CD8+ T cells. The numbers of CD4+ and CD8+ T cells per lung were derived by multiplying the percentage of cells by the total number of lung cells isolated. The rest of the cells were stimulated for 4 h with anti-CD3 (clone 145–2C11; 0.1 μg/ml) and anti-CD28 (clone 37.51; 1 μg/ml) in the presence of 2 μM monensin (Sigma-Aldrich) to halt egress of cytokines from the cells. After cells were washed and fixed in 2% parafomaldehyde at 4°C overnight, they were permeabilized with 0.1% saponin in PBS containing 0.1% bovine serum albumin and 0.1% sodium azide. Permeabilized cells were stained with phycoerythrin-conjugated antibodies and analyzed by FACScan.
mAbs and isotype controls (BD Biosciences) for IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22), and GM-CSF (clone MP1-22E9) in 20% mouse serum for 30 min at 4°C, washed, and analyzed by FACSc. Lung cells were harvested serially during infection and stained intracellularly for cytokines. Lymphocytes were gated on CD4 and CD8 and cytokine expression within each gate analyzed. The number of cytokine producing CD4+ and CD8+ T cells per lung was calculated by generating the product of percent cytokine producing cells and the number of CD4+ and CD8+ cells in the lung. Density of cytokine producing T cells in the lung was calculated by dividing the total number of type 1 cytokine producing T cells by the total number of lung hematopoietic cells.

Vaccination and Experimental Infection with B. dermatitidis. Mice were vaccinated as described (29) twice, 2 wk apart, each time receiving a subcutaneous injection of 10^4 #55 yeast at each of two sites, dorsally and at the base of the tail, unless otherwise stated. To generate immune CD8+ T cells for adoptive transfer, mice were depleted of CD4+ T cells with mAb during vaccination to evoke CD8+ T cell immunity. These mice were immunized subcutaneously with strain #55 yeast as above, but three times, 2 wk apart. 2 wk after the final vaccination, mice received 200 μg of soluble yeast cytosol extract (YCE)* (29) or soluble hen egg lysozyme (HEL) as a control antigen emulsified in complete Freund's adjuvant. 12 d later, draining lymph node cells and splenocytes were harvested and CD8+ T cells purified using aCD8-coated immuno-magnetic beads (Miltenyi Biotec). CD8+ T cells isolated from Thy 1.1+ mice were transferred intravenously to irradiated (5.5 gray [Gy]) Thy 1.2+ β2M−/− and β2M+/+ mice, whereas CD8+ T cells from Thy 1.2+ mice were transferred into nonirradiated Thy 1.2+ recipients. Irradiated mice were rested for 8 wk before infection, nonirradiated mice were challenged the day after transfer. Transferred Thy 1.1+ CD8+ T cells were monitored by FACSc analysis in the spleen, lymph nodes, and the lung of recipients during the period of recovery and infection.

Mice were infected intratracheally with 2 × 10^2 to 2 × 10^3 wild-type strain 26199 yeast as described (29). Infected mice were monitored for survival or analyzed 2 wk after infection for extent of lung infection, determined by plating of homogenized lung and enumeration of yeast CFU on Brain Heart Infusion (BHI; Difco) agar.

Vaccination, Depletion of T Cells, and Challenge with H. capsulatum. Mice were immunized twice, 2 wk apart, each time receiving a subcutaneous injection of 2 × 10^4 H. capsulatum yeast strains, strain G217B, that were suspended in 0.1 ml of HBSS, at the base of the tail. 2 wk after the second immunization, mice were challenged intranasally with 1.25 × 10^7 yeast. For depletion of CD4+ cells, mice were administered 100 μg of mAb to CD4 (clone GK 1.5) intraperitoneally beginning on days −7, −3, and on the day of immunization and weekly thereafter. One group of CD4-depleted mice also received 100 μg of mAb to CD8 (clone 2.43) intraperitoneally beginning 1 d before challenge with 1.25 × 10^6 yeasts and on the day of challenge. Administration of mAb to CD8 was continued weekly (30). Controls received an equal amount of rat IgG. Lungs and spleens were removed and homogenized in HBSS, and 100 μl of homogenate was dispensed onto plates containing brain heart infusion agar supplemented with 5% defibrinated sheep erythrocytes, 1% glucose, and 0.01% cysteine hydrochloride (wt/vol). Plates were incubated at 30°C for 7 d, and colony forming units enumerated (9).

Histology. Lung tissue was fixed in 10% formalin and embedded in paraffin wax. Sections 5-μm thick were stained with hematoxylin and eosin and Gomori's methenamine silver. Areas of pneumonia consolidation were measured at a final projected magnification of 8.8 and expressed as a percentage of total lung areas in sections. The number of yeast was counted in 20 fields with a 60X objective, projected on a TV screen, and expressed as yeast/high power field.

Statistical Analysis. Kaplan Meier survival curves were generated (31). Survival times of infected mice alive by the end of the study were regarded as censored. Time data were analyzed by the log rank statistic (Mantel-Haenszel test; reference 32) and exact P values were computed using the statistical packaged Stat Xact-3 by CYTEL Software Corporation. The number of TNF-α, IFN-γ, and GM-CSF producing CD4+ and CD8+ T cells and differences in number of CFU were analyzed using the Wilcoxon Rank test for nonparametric data (31). A P value of < 0.05 is considered statistically significant.

Results

CD4+ T Cells Are Dispensable during Induction of Vaccine Immunity. Depletion of T cell subsets during the period of vaccination (defined as the induction phase of vaccine immunity) and throughout the period after infection (defined as the expression phase of vaccine immunity) gave unanticipated results. Mice depleted of either CD4+ or CD8+ T cells acquired levels of vaccine immunity similar to that of rat IgG-treated controls, as assessed by lung CFU analysis (Fig. 1A). Animals depleted of both CD4+ and CD8+ T cells were as susceptible as unvaccinated controls. Thus, T cells are required for vaccine immunity, but CD4+ T cells appear to be dispensable when absent during induction of vaccine immunity. CD8+ T cells are essential in a CD4+ T cell–deficient host.

Survival analysis of CD4-depleted wild-type animals supported the above findings and indicated that lung CFU data are a reliable predictor of survival (33). Mice depleted of CD4+ T cells during induction and expression of vaccine immunity survived significantly longer than unvaccinated mice (mean, 123 ± 11 d vs. 23 ± 2 d; P < 0.0001). At 75 d after infection, all unvaccinated mice were dead, whereas 80% of CD4+ T cell–depleted mice were still alive. Ultimately, 35% of CD4–depleted mice survived a lethal challenge with virulent, wild-type yeast over a prolonged period of 200 d after infection; all of the survivors had no detectable CFU in their lungs (detection limit = 5 CFU), indicating that they had acquired sterilizing immunity. In comparison, 100% of vaccinated, non-T cell–depleted mice survived the lethal challenge and cleared the infection. Thus, in the absence of CD4+ T cells, vaccination greatly prolonged survival and a significant proportion of those animals survived and acquired sterilizing immunity.

Vaccine immunity evoked in the absence of CD4+ T cells was durable and persisted for at least 8 wk after vaccination. CD4-depleted mice that were rested for 8 wk after vaccination (but maintained CD4 deficient) remained

*Abbreviations used in this paper: β2M, beta-2-microglobulin; DC, dendritic cell; HEL, hen egg lysozyme; ODN, oligodeoxynucleotides; YCE, yeast cytosol extract.
highly resistant to lethal infection. 6 out of 10 CD4-depleted mice acquired sterilizing immunity and the remaining four mice had 20–200 lung CFU 25 d after infection. In contrast, unvaccinated mice appeared moribund with a lung burden of $5.2 \pm 2.9 \times 10^6$ CFU at this time point. All vaccinated wild-type mice had cleared the infection. Thus, resting of vaccinated CD4-depleted mice indicated durable immunity and even greater resistance to infection.

CD4⁻/⁻ Mice Acquire Robust Vaccine Immunity. Because depletion of CD4 T cells might not be complete, and residual cells could provide sufficient help for other effector cells, we investigated vaccine immunity in CD4⁻/⁻ mice.

Remarkably, all vaccinated CD4⁺ T cell-knockout mice survived an extended period after infection (Fig. 1 B); 9 out of 11 had in fact acquired sterilizing immunity, and the other two had low numbers of CFU in their lungs (200 and 280 CFU). Survival data generated with class II-knockout mice were similar to those reported for CD4-depleted wild-type mice. Thus, the results obtained using mice congenitally deficient in CD4⁺ cells support the data using CD4⁺ T cell depletion and the notion that CD4⁺ T cells are dispensable.

We examined the histological appearance of inflammation in the lungs of infected mice. Unvaccinated CD4 knockout mice and wild-type mice each had 50% of their lungs replaced with granulomas, which contained 20.7 yeast/mm² of tissue. By contrast, vaccinated, CD4 knockout mice and wild-type mice had only 7 and 0.8% of their lung tissue inflamed with granulomas, which contained 1.9 and 3.6 yeast/mm², respectively (Table I). The architecture and cellular composition of the granulomas in vaccinated mice was unaffected by absence of CD4⁺ T cells (data not depicted). Hence, the extent and microscopic appearance of granulomatous inflammation in the lung was dependent on the resistance phenotype, rather than on the cellular subset mediating resistance.

CD4⁺ T Cells Are Dispensable in Vaccine Immunity to H. capsulatum. We determined whether our findings could be extended to other pathogenic and opportunistic fungi, and tested this in an experimental model of pulmonary histoplasmosis, a fungal disease that causes frequent and life-threatening opportunistic infection in AIDS patients. All the mice that had been vaccinated in either the presence or absence of CD4⁺ T cells survived a lethal pulmo-

![Image](https://via.placeholder.com/150)

**Table I.** Histological Analysis of B. dermatitidis Infection and Granulomatous Inflammation

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Granuloma/area¹</th>
<th>No. yeast²/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 knockout</td>
<td>Unvaccinated</td>
<td>49%</td>
<td>20.7</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Unvaccinated</td>
<td>51%</td>
<td>20.7</td>
</tr>
<tr>
<td>CD4 knockout</td>
<td>Vaccinated</td>
<td>7%</td>
<td>1.9</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Vaccinated</td>
<td>0.8%</td>
<td>3.6</td>
</tr>
</tbody>
</table>

¹Percentage of microscopic field inflamed with granulomas.
²Number of yeast per square millimeter of lung tissue section viewed under 400X magnification.
³C57BL/6 mice (4/group) were depleted of CD4⁺ T cells during the induction and expression phase of vaccine immunity.
⁴In addition to CD4⁺ T cell depletion as described above, CD8⁺ T cells were depleted during vaccine expression.
nary challenge with the wild-type virulent strain, and acquired sterilizing immunity by 50 d after infection (detection limit of 200 CFU; Fig. 1 C). In contrast, unvaccinated mice and CD4+ T cell–depleted mice that were depleted of CD8+ T cells after infection died within 10–20 d after infection. Thus, vaccine resistance also can be raised against pulmonary histoplasmosis in the absence of CD4+ T cells.

CD8+ T Cells Are Required during the Efferent Phase of Vaccine Immunity. Studies above (Fig. 1 A) showed that CD8+ T cells must be present during vaccine induction when CD4+ T cells are absent, but did not address whether CD8+ T cells are required and responsible for immunity during the expression or efferent phase. To determine if CD8+ T cells serve as effectors during the efferent phase, we used two approaches. First, mice in whom CD4+ T cells were depleted during induction and expression of vaccine immunity were depleted of CD8+ T cells upon infection and afterward. Elimination of CD8+ T cells after infection reduced resistance 150-fold, compared with controls depleted only of CD4+ T cells (Fig. 2 A). Similarly, elimination of CD8+ T cells after infection reduced the mean survival time from 200 ± 0 d to 49 ± 5 d (P = 0.007) in vaccinated CD4 knockout mice (Fig. 1 B) and from 123 ± 11 d to 49 ± 5 d (P < 0.0001) in vaccinated CD4-depleted wild-type mice. The corresponding survival rates in these two respective groups went from 100 to 40% at 200 d after infection, and from 80 to 0% at 75 d after infection. Vaccine induced resistance against pulmonary histoplasmosis in CD4-depleted mice depended exclusively on CD8+ T cells. Elimination of CD8+ cells in those mice reduced the survival rate from 100 to 0%.

In a second approach, we adoptively transferred immune CD8+ T cells that had been generated in wild-type mice in the absence of CD4+ T cells. Blastomyces immune CD8+ T cells lowered lung CFU 10- to 15-fold compared with mice that got HEL-CD8+ T cells or no T cells, respectively (Fig. 2 B, Exp. 1). Resistance was adoptively transferred by Blastomyces-immune CD8+ T cells in a dose-dependent manner, using cell numbers from 106 to 2 × 107 (Fig. 2 B, Exp. 2).

Taken together, our findings indicate that, when CD4+ T cells are absent, CD8+ T cells are required during the induction phase and participate as effectors during the efferent phase of vaccine immunity against B. dermatitidis and H. capsulatum infection.

Antigen Presentation to CD8 T Cells Requires Class I Molecules. To investigate how CD8+ T cells exert vaccine-induced resistance against B. dermatitidis, a predominantly extracellular pathogen, we considered the following two possibilities. Protective CD8+ T cells either recognized fungal antigen on the yeast’s surface directly and in the absence of MHC restriction, as previously hypothesized for other fungi (34), or alternatively, they became activated by cross-presentation of exogenous antigens displayed on MHC class I molecules. To determine whether MHC class I is necessary for CD8+ T cell vaccine immunity, we adoptively transferred CD8+ T cells from vaccinated CD4+ T cell-depleted, wild-type mice into naive class I–deficient mice. Immune CD8+ T cells reduced the lung CFU by a factor of 440 in β2M-sufficient mice, but had no effect on lung CFU after transfer into β2M-deficient mice (Fig. 3 A). We replicated these findings in β2M+/+ and β2M−/− mice that had been sublethally irradiated before transfer and infected eight weeks later to avoid the possibility that transferred CD8+ cells from β2M-sufficient mice could have been rejected in β2M-deficient mice (35). In this scheme, adoptively transferred, immune CD8+ T cells were found to persist and expand in β2M+/+ and β2M−/− mice during the period of recovery and infection (data not depicted); they reduced lung CFU in β2M−/− mice by 165-fold, but again had no effect in β2M+/+ mice. Thus, MHC class I molecules are required and likely cross-present exogenous fungal antigens to vaccine induced CD8+ T cells.

Molecular Mechanisms of CD8+ T Cell Vaccine Immunity. Effector mechanisms of CD8+ T cells include, but are not limited to, the production of type 1 regulatory cytokines. By real-time PCR of lung cells 48 h after infection, cyto-

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**Figure 2.** CD8+ T cells mediate resistance in the absence of CD4+ T cells. (A) Depletion of CD8+ T cells impairs resistance. C57BL/6 mice were depleted of CD4+ T cells alone or together with CD8+ T cells. Controls were vaccinated mice treated with rat-IgG (C) or unvaccinated mice. Mice were infected with 2 × 103 yeast and analyzed for lung CFU 14 d later. *, P < 0.0001 vs. CD4-depleted mice. Data represent an average of two independent experiments (n = 10 mice/group). (B) CD8+ T cells transfer resistance. In experiment 1, naive mice (8/group) received either Blastomyces immune (YCE) CD8+ T cells, no cells, or control HEL-CD8+ T cells. Mice were infected 1 d later with 103 yeast, and lung CFU analyzed 2 wk after infection. *, P < 0.001 vs. mice receiving either HEL-CD8+ T cells or no cells. Data show one representative experiment of three independent experiments. In experiment 2, naive mice (8–9/group) received various numbers of YCE-CD8+ T cells or no cells and were infected with 2 × 103 yeast. 2 wk after infection, lung CFU was analyzed. *, P < 0.004 vs. mice receiving either 106 YCE-CD8+ T cells or no cells.
kine transcript was elevated in vaccinated (CD4 depleted and wild-type) mice compared with unvaccinated mice; TNF-α transcript was 3–4-fold higher, IFN-γ, 24–29-fold higher, and GM-CSF 10–24 fold higher (P < 0.001). Therefore, we investigated whether CD8+ T cells mediate their effects via production of TNF-α, GM-CSF, or IFN-γ (Fig. 3 B). Mice were depleted of CD4+ T cells during vaccination and throughout infection to evoke CD8+ T cell immunity. After infection, the mice were neutralized alone or in combination for TNF-α, IFN-γ, and GM-CSF or given rat IgG as a control. Neutralization of GM-CSF, IFN-γ, and TNF-α independently, or IFN-γ and TNF-α together, sharply reduced resistance, compared with treatment with rat IgG. Neutralization of IFN-γ and TNF-α together yielded lung CFU values comparable to those in unvaccinated control mice. Hence, these two type 1 cytokines appear to be the most critical in mediating CD8+ T cell immunity to B. dermatitidis infection.

Rapid Influx of Cytokine Producing CD8+ T Cells Into Lung Coincides with CFU Reduction. To determine whether CD8+ (and CD4+) cells produce TNF-α, IFN-γ, and GM-CSF, we monitored the expression of these cytokines by intracellular staining of lung T cells ex vivo. The number of lung CD4+ and CD8+ cells combined that produced TNF-α, IFN-γ, and GM-CSF rose sharply between 2 and 4 d after infection in vaccinated wild-type mice and vaccinated CD4-depleted mice versus unvaccinated mice (Fig. 4 A). In vaccinated wild-type mice, cytokine producing T cells were largely CD4+ cells, whereas CD8+ cells contributed to a lesser extent as assessed by the frequency (Fig. 4 B) and number of cytokine-producing T cells (Ta-

Figure 3.  Essential role of MHC class I and type 1 cytokine production in protective CD8+ T cells. (A) Adoptive transfer of CD8+ T cells into β2M-deficient mice. 1.8 × 10⁷ CD8+ T cells isolated from spleen and lymph node of CD4-depleted wild-type mice were transferred into β2M−/− and β2M+/+ recipients. Mice were infected with 4 × 10⁴ yeast the next day, and analyzed for lung CFU 20 d later. Data show one representative experiment of two independent experiments (n = 10 mice/group). *, P = 0.0005 vs. untransferred β2M−/− mice; **, P = 0.112 vs. untransferred β2M−/− mice. (B) Neutralization of TNF-α, GM-CSF, and IFN-γ. C57BL/6 mice were depleted of CD4+ T cells during induction and expression of vaccine immunity. mAbs against TNF-α, GM-CSF, and IFN-γ alone or together, GM-CSF, CD8+ T cells, or rat IgG control (C) as indicated in histogram bars were administered after and throughout infection. Mice were infected with 2 × 10⁸ yeast, and lung CFU was analyzed 14 d later. Data represent an average of three independent experiments (n = 10 mice/group). *, P < 0.008 vs. all groups.

Figure 4. (continues on facing page)
Although not statistically significant, the number of cytokine producing CD8$^+$ cells showed a tendency to be increased in vaccinated CD4$^-$depleted mice, as compared with vaccinated wild-type mice. Most importantly, in both vaccinated groups of mice, the number of cytokine producing T cells peaked at 4 d after infection, whereas in unvaccinated mice, comparable numbers of cytokine producing T cells did not appear until 8–12 d after infection. The peak influx of cytokine producing T cells in vaccinated mice coincided with a significant reduction in lung CFU in those mice compared with unvaccinated mice (Fig. 4 C). At day 4, the number of lung CFU in vaccinated mice was already 15-fold lower than in unvaccinated mice, perhaps a critical reduction in burden of lung infection that set the stage for the final outcome. By day 26, unvaccinated mice appeared moribund and harbored $3 \times 10^6$ yeast in their lungs.

Figure 4. Intracellular production of TNF-$\alpha$, IFN-$\gamma$, and GM-CSF by lung T cells coincides with reduction in lung CFU. (A) Total number of type-1 cytokine producing lung T cells after B. dermatitidis infection in vaccinated and unvaccinated mice ($n$ = pool of 6–12/group at each time point). TNF-$\alpha$, IFN-$\gamma$, and GM-CSF producing cells are combined for CD4$^+$ and CD8$^+$ T cells at each time point, which represent an average of four independent experiments. *, $P = 0.004$, and **, $P = 0.03$ vs. unvaccinated mice for all three cytokines. (B) Type 1-cytokine response by CD4$^+$ and CD8$^+$ T cells at day 2 and 4 after infection. Analyses are gated on CD4$^+$ and CD8$^+$ T cells; numbers represent the percentage of CD4$^+$ and CD8$^+$ T cells positive for IFN-$\gamma$, TNF-$\alpha$, and GM-CSF. Data show a representative experiment ($n$ = pool of 6–8 mice/group at each time point) of four independent experiments. (C) Kinetics of lung CFU clearance. Mice were infected with $10^2$ yeast and analyzed for lung CFU serially after infection (detection limit = 5 CFU). Number with sterilizing immunity (undetectable CFU) is depicted as a fraction of those tested ($n$ = 4 mice/group). *, $P < 0.03$ vs. vaccinated mice and vaccinated, CD4$^-$depleted mice. Similar results were found when the experiment was repeated with a higher inoculum ($10^3$ yeast). (D) Density of cytokine producing T cells in lung ($n$ = pool of 6–12 mice/group at each time point). Total number of type-1 cytokine expressing T cells (CD4$^+$ and CD8$^+$ cells combined) expressed as a fraction of total lung hematopoietic cells. *, $P < 0.03$ vs. unvaccinated mice for all three cytokines. **, $P < 0.08$ vs. unvaccinated mice for TNF-$\alpha$ and GM-CSF.
lungs, whereas both groups of vaccinated mice had largely cleared the infection.

To explore why unvaccinated mice failed to resist infection even though they eventually produced comparable numbers of cytokine expressing lung T cells by day 8 to 12, we analyzed the density of cytokine producing effector cells in the lung (Fig. 4 D). Vaccinated wild-type mice and vaccinated, CD4-depleted mice reached their highest densities of effector cells as early as day 4 and maintained them over the period investigated. Despite the significant increase in number of cytokine producing cells after day 8 postinfection, unvaccinated mice never reached the same levels of effector cell density. This was largely a consequence of the increasing number of hematopoietic cells migrating into the lungs (from one million at day 0 up to 20 million cells at day 12), presumably the result of increasing CFU over time in unvaccinated mice. In contrast, in both vaccinated groups of mice, total lung hematopoietic cells increased rapidly to three million cells and remained at that level over the period investigated. Lung CFU fell reciprocally in proportion to the high density of lung effector cells (data not depicted). Thus, vaccine-induced, protective CD8+ cells likely mediate their effect(s) via production of TNF-α, IFN-γ, and GM-CSF.

CD8+ T Cells Show Plasticity in Cytokine Repertoire for Vaccine Immunity. To ascertain the absolute requirements for IFN-γ and TNF-α in CD8+ T cell vaccine immunity, we vaccinated transgenic mice deficient in these cytokines in the absence of CD4+ T cells. Remarkably, in both transgenic mouse strains, CD8+ T cells compensated for the absence of either cytokine (Fig. 5 A). We analyzed the compensatory mechanisms. In CD4-depleted IFN-γ−/− mice, neutralization of TNF-α or GM-CSF, or depletion of CD8+ T cells, during vaccine expression increased lung CFU 980-fold, 206-fold, and 1385-fold, respectively, compared with the rat IgG control (Fig. 5 A). Hence, TNF-α, and to a lesser extent GM-CSF, regulate expression of CD8+ T cell immunity in the absence of IFN-γ. By contrast, in vaccinated, CD4-depleted TNF-α−/− mice, neutralization of GM-CSF, but not IFN-γ, after infection

<p>| Table II. Number (×10^6) of Type 1 Cytokine-producing CD4+ and CD8+ T Cells in Lung after B. dermatitidis Infection as Measured by Intracellular Cytokine Staining |
|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Day</th>
<th>IFN-γ CD4+</th>
<th>CD8+</th>
<th>TNF-α CD4+</th>
<th>CD8+</th>
<th>GM-CSF CD4+</th>
<th>CD8+</th>
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<tbody>
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<tr>
<td>0</td>
<td>5 ± 2</td>
<td>4 ± 2</td>
<td>13 ± 8</td>
<td>17 ± 9</td>
<td>4 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>1 ± 0</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>5 ± 2</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>14 ± 4</td>
<td>13 ± 4</td>
<td>70 ± 33</td>
<td>79 ± 31</td>
<td>20 ± 8</td>
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<tr>
<td>6</td>
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<td>263 ± 147</td>
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</tr>
<tr>
<td>8</td>
<td>186 ± 99</td>
<td>299 ± 166</td>
<td>508 ± 342</td>
<td>879 ± 64</td>
<td>172 ± 91</td>
<td>33 ± 16</td>
</tr>
<tr>
<td>12</td>
<td>279 ± 105</td>
<td>424 ± 161</td>
<td>1,438 ± 578</td>
<td>2,108 ± 857</td>
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<td>246 ± 82</td>
</tr>
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</tr>
<tr>
<td>0</td>
<td>23 ± 14</td>
<td>10 ± 6</td>
<td>64 ± 48</td>
<td>40 ± 30</td>
<td>31 ± 20</td>
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<tr>
<td>2*</td>
<td>92 ± 88</td>
<td>36 ± 30</td>
<td>178 ± 197</td>
<td>87 ± 88</td>
<td>87 ± 80</td>
<td>16 ± 13</td>
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<tr>
<td>4b</td>
<td>325 ± 257</td>
<td>226 ± 192</td>
<td>1,355 ± 981</td>
<td>954 ± 743</td>
<td>593 ± 426</td>
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<td>6</td>
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<td>111 ± 61</td>
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<td>8</td>
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<td>25 ± 13</td>
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<tr>
<td>2*</td>
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<tr>
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<td>1,234 ± 758</td>
<td>263 ± 162</td>
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</tbody>
</table>

Data represent an average of four independent experiments (lungs of 6–12 mice per group were pooled at each time point). Mice were infected with 2 × 10^5 yeast and analyzed serially after infection.

*P ≤ 0.01 vs. unvaccinated mice for all three cytokines.

*P ≤ 0.005 vs. unvaccinated mice for all three cytokines.

*C57BL/6 mice were depleted of CD4+ T cells during the induction and expression phase of vaccine immunity.
sharply increased lung CFU compared with the rat IgG control (Fig. 5B). These results illustrate that CD8+ T cells are flexible in the mechanisms at their disposal for mediating vaccine immunity in the absence of type 1 cytokines IFN-γ and TNF-α.

Discussion

AIDS patients manifest impaired or defective CD4+ T cell responses and are predisposed to opportunistic microbes, suggesting that CD4+ T cells play an essential role in host resistance (1, 2). Other lines of evidence indicate that resistance against such microbes including fungi is largely mediated by protective CD4+ T cells (29, 30, 33, 36, 37). Thus, it would seem impossible to vaccinate this growing population of immune-compromised patients against opportunistic fungi. To test this premise, we explored whether in immune-deficient mice lacking CD4+ T cells we could harness residual cellular and molecular elements in vaccine immunity to fungi.

Herein, we provide evidence that CD4+ T cells are dispensable in vaccine immunity against experimental pulmonary blastomycosis and histoplasmosis. CD8+ T cells alone, in the absence of CD4+ T cells, could induce and mediate vaccine resistance against lethal infections with these pathogenic fungi. We used multiple independent approaches to support our findings. First, we depleted CD4+ T cells in wild-type mice during vaccination and throughout infection. Both survival and burden of infection outcomes indicated that CD4+ T cells are dispensable for vaccine immunity. Vaccine resistance in CD4-depleted wild-type mice was durable and persisted at least 8 wk after vaccination. Second, vaccination of CD4− or class II-knockout mice genetically deficient in CD4+ T cells confirmed our findings in CD4-depleted wild-type mice. Vaccinated CD4 knockout mice were as resistant as vaccinated wild-type mice and survived a lethal infection. Furthermore, vaccinated CD4-deficient mice mounted a granulomatous inflammatory response in lungs that was comparable to that in wild-type mice. Although granuloma formation is considered a form of delayed-type hypersensitivity requiring principally MHC class II-restricted CD4+ T cells, other cells and antibodies can replace them. During Mycobacterium tuberculosis infection, granuloma formation in CD4-deficient mice was delayed by about one week vs. wild-type mice, but at later times, the numbers of granulomas in lung and liver sections were comparable (38). Third, elimination of CD8+ T cells in vaccinated CD4-deficient mice during vaccine expression largely abolished resistance, indicating that CD8+ T cells are responsible for the resistance. Fourth, resistance by immune CD8+ T cells could be adoptively transferred to naive mice.

We saw a subtle difference in the amount of lung inflammation of vaccinated CD4-deficient mice vs. depleted mice versus vaccinated wild-type mice. The former groups had slightly more lung inflammation, but the lung tissue of both immune-deficient groups was still largely devoid of inflammatory cells (93 and 96%, respectively). Pathogenesis and tissue injury is the net result of microbial growth versus the host response to pathogen and collateral tissue damage (39). Though vaccine induces protection in CD4-deficient hosts, increased inflammatory response could reflect loss of subsets of T cells important in regulating inflammation.

CD8+ T cells have been reported to mediate secondary immunity or vaccine immunity in the absence of CD4+ T cells; for example, in secondary immunity to H. capsulatum (30) and vaccine immunity to Leishmania major (40). In those studies, experimental animals had an intact immune system upon initial infection or vaccination, and CD8+ T cells were shown to play a prominent role in protection after rechallenge during the expression phase of immunity. By contrast, in our study, animals congenitally lacked CD4 T cells or had them depleted before they were vaccinated. Hence, they were immune-deficient hosts lacking CD4+ T cells during the induction phase of vaccine immunity (and also during the expression phase). Nevertheless, CD8+ T cells alone were sufficient both for the induction and expression of immunity, and robust resistance
was demonstrable against both extracellular and intracellular fungal pathogens.

According to dogma, induction of CD8+ T cell responses against exogenously processed antigens requires CD4+ T cell help (41–43). T helper cell–induced stimulation of CD40 on macrophages and dendritic cells (DCs) is a commonly described mechanism that leads to up-regulation of costimulatory molecules and induction of cytokines, such as IL-12, to “condition” an APC to stimulate naive CD8+ T cells. Recent reports provide evidence of some flexibility for CD4+ T cell requirement. Harty and colleagues (44) reported that during *Listeria monocytogenes* infection bacterial antigens that did not have access to endogenous MHC class I processing pathways were able to overcome the requirement for CD40L in exogenous antigen presentation to CD8+ T cells. Bachmann and colleagues (45) showed that DC maturation occurred in vivo after infection with LCMV and vesicular stomatitis virus in the absence of CD40 and T helper cells. This maturation did not require viral infection of DCs but was mediated by peptide-specific CD8+ T cells. Wang and colleagues (16) showed that, in the absence of preactivated APCs or of inflammatory signals that substitute for help during bacterial and viral infections (46, 47), CD8+ T cells can help for other responding CD8+ T cells, when present in sufficient numbers. Besides CD8+ T cells, possibly other cells (e.g., NK cells) too can substitute for CD4+ Th cells under appropriate conditions.

Recently, CD8+ effector T cells have been generated by DNA or protein/CpG oligodeoxynucleotides (ODN) vaccines in a CD4- and CD40L-independent manner. For example, an immunostimulatory DNA-based vaccine induced CTL to the model antigen OVA through a T helper–independent mechanism (48). Vaccine-induced CTLs were able to suppress in vivo growth of the E.G7 cell line transfected with OVA. This study offered early evidence that, in the absence of T cell help, cross priming of CD8 cells by an ODN vaccine induces CTL that lyse “tumor” targets and suppress their growth when they express the model antigen OVA.

The authors above speculated on whether ODN-based vaccines and cross priming might allow vaccination of immune-deficient hosts. Here, we provide unequivocal experimental evidence of T helper–independent vaccination of immune-deficient hosts against infectious diseases, and prove this principle in two separate models of systemic fungal diseases that can commonly afflict AIDS patients. The profile of CD8 immunity raised in our study differed from that where ODN was used. Cho et al. (48) failed to detect type 1 cytokine and Horner et al. (49) detected weak responses using ODN vaccines without T cell help. In our study, CD4-independent, CD8+ T cells generated IFN-γ, TNF-α, and GM-CSF, which were linked with and essential in vaccine resistance. Maintenance of type 1-cytokine responses may be crucial for control of fungal and other eu-karyotic intracellular infections, but dispensable in CTL control of tumor or viral processes.

An enigma is how CD8+ T cells become activated during infections with *B. dermatitidis*, a predominantly extra-cellular pathogen, and with *H. capsulatum*, an intracellular pathogen that resides in the phagolysosome with no demonstrated access to the cytosol. To elucidate how CD8+ T cells are activated, we explored the requirements for MHC class I. An unconventional mechanism of CD8+ T cell immunity to *C. albicans* and *C. neoformans* has been proposed involving direct binding of T cells to the fungi in the absence of MHC and accessory cells. Direct in vitro antimicrobial activity of T cells has been described with *C. albicans* and *C. neoformans* (24), and against parasites *Toxoplasma gondii* (50), *Schistosoma mansoni* (51), and *Entamoeba histolytica*. While the above examples show that T cells can directly inhibit or kill various pathogens in vitro, the circumstances by which direct T cell–mediated antimicrobial activity contributes to host defenses in vivo remain unclear.

In our study, adoptively transferred CD8+ T cells protected wild-type mice, but not class I–deficient mice, suggesting that CD8+ T cell immunity against *B. dermatitidis* requires MHC class I for antigen presentation and protection.

Although cross-presentation of exogenous antigens to MHC class I molecules is observed in various infections, the route of uptake and processing, and the compartment where peptides combine with MHC class I may differ. Our recent experiments have shown that protective CD8+ T cells recognize exogenous fungal antigens largely in a TAP-independent manner (unpublished observations), suggesting a nonclassical pathway for antigen presentation. It will be important to investigate the pathway(s) used for cross-presentation of *B. dermatitidis* and *H. capsulatum* antigens on MHC class I. Their understanding is relevant for the development of vaccines meant to induce protective CD8+ T cell immunity to benefit immune-compromised patients.

Vaccine resistance mediated by CD8+ T cells required the production of type 1 cytokines. Independent and combined neutralization of TNF-α, IFN-γ, and GM-CSF during the expression phase of CD8+ T cell immunity greatly impaired resistance. TNF-α neutralization reduced vaccine immunity 5-fold or 30-fold more than IFN-γ or GM-CSF neutralization, respectively, indicating the hierarchical contribution of these cytokines. On analysis by real time PCR and intracellular staining, lung CD8+ T cells demonstrated significantly enhanced expression of these type 1 cytokines between 2 to 4 d after infection in vaccinated mice compared with unvaccinated mice. This rapid increase of cytokine expression by CD8+ T cells in CD4-depleted mice (and by both CD4+ and CD8+ T cells in vaccinated wild-type mice) early after infection coincided with a sharp reduction in lung CFU. Although CD8+ T cells are one important source of these products, we cannot exclude other cellular sources. Even though quantification of cytokine producing T cells in lung revealed significant differences between vaccinated and unvaccinated mice, more refined analysis of the lung microenvironment by laser capture micro-dissection could magnify these differences or demonstrate other cells and products that contribute to vaccine immunity (52, 53).

As IFN-γ and TNF-α were key regulators of CD8+ T cell–mediated resistance, it was surprising that robust vac-
cine immunity could be induced and expressed in both IFN-γ−/− and TNF-α−/− mice. Either reciprocal cytokine or alternatively GM-CSF could compensate, indicating that CD8+ cells show plasticity similar to CD4+ cells in their capacity to regulate vaccine immunity (33). Hence, IFN-γ and TNF-α are independently regulated and dispensable in vaccine immunity to B. dermatitidis infection in a CD4+ T-cell–deficient host.

In conclusion, we offer firm evidence, in two independent experimental animal models, that fungal vaccines can protect hosts that are immune-compromised at both the cellular and molecular level. Vaccinated animals acquired potent and durable cellular immunity in the absence of CD4+ cells and cytokines critically important for resistance in a normal host. Immune-deficient hosts relied on alternative mechanisms for vaccine immunity. Our findings provide encouragement that vaccines can be designed that will protect even immune-deficient hosts.

We thank Drs. Suresh Marulasiddappa and Matyas Sandor at UW-Madison for helpful advice and discussions.

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