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Immunization with Heat-Killed *Mycobacterium vaccae* Stimulates $CD8^+$ Cytotoxic T Cells Specific for Macrophages Infected with *Mycobacterium tuberculosis*

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Immune responses to *Mycobacterium tuberculosis* are analyzed in mice which have been immunized with *Mycobacterium vaccae* to examine novel ways of altering protective immunity against *M. tuberculosis*. The spleen cells of mice immunized with *M. vaccae* proliferate and secrete gamma interferon (IFN-γ) in response to challenge with live *M. tuberculosis* in vitro. Immunization with *M. vaccae* results in the generation of $CD8^+$ T cells which kill syngeneic macrophages infected with *M. tuberculosis*. These effector cytotoxic T cells (CTL) are detectable in the spleen at 2 weeks after immunization with *M. vaccae* but cannot be found in splenocytes 3 to 6 weeks postimmunization. However, *M. tuberculosis*-specific CTL are revealed following restimulation in vitro with heat-killed *M. vaccae* or *M. tuberculosis*, consistent with the activation of memory cells. These $CD8^+$ T cells secrete IFN-γ and enhance the production of interleukin 12 when cocultured with *M. tuberculosis*-infected macrophages. It is suggested that $CD8^+$ T cells with a cytokine secretion profile of the Tc1 class may themselves maintain the dominance of a Th1-type cytokine response following immunization with *M. vaccae*. Heat-killed *M. vaccae* deserves attention as an alternative to attenuated live mycobacterial vaccines.

Although tuberculosis is a preventable and curable disease, it is the major cause of death by a single infectious agent (3). Vaccination programs against tuberculosis have been established in many countries with the use of the attenuated strain of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). However, the immunoprotective efficacy of BCG in different trials over several decades has varied, and its value as a tuberculosis vaccine remains controversial (4).

Other mycobacterial strains which share cross-reactive antigens with *Mycobacterium tuberculosis* have also been considered as alternatives to *M. bovis* for vaccine use. One strain, *Mycobacterium vaccae*, has been tested as an immunotherapeutic agent in human tuberculosis (9, 14, 23). *M. vaccae* given as a single injection to patients within the first few weeks of starting chemotherapy for pulmonary tuberculosis appeared to improve the cure rate and reduce the number of deaths (23). Data from field studies suggest that *M. vaccae* should be investigated as an immunotherapeutic agent for tuberculosis patients who have not received adequate chemotherapy (24). It may also be useful as adjunctive therapy for patients with multidrug-resistant tuberculosis (9) or who are additionally infected with the human immunodeficiency virus (22). Compared with the results for control mice, immunization with heat-killed *M. vaccae* prior to infection of mice with *M. tuberculosis* reduced the number of mycobacterial CFU in spleens by one logarithm (1).

The mechanism by which *M. vaccae* influences the immune response to *M. tuberculosis* infection is unclear. In tuberculous lesions, macrophages infected with *M. tuberculosis* are surrounded by activated T cells (7). Both $CD4^+$ and $CD8^+$ T cells have been shown to lyse infected macrophages expressing mycobacterial antigens (8, 19). Depletion of $CD4^+$ or $CD8^+$ T cells in mice with monoclonal antibodies exacerbates infection with *M. tuberculosis* (13). Protection against infection can also be enhanced by the adoptive transfer of selected $CD4^+$ and $CD8^+$ T cells from infected mice (15). Mice with disrupted major histocompatibility complex class I genes and thus deficient in $CD8^+$ T cells (11) or with disrupted gamma interferon (IFN-γ) or IFN-γ receptor genes show increased susceptibility to tuberculosis (5, 25). IFN-γ also has an important role in immunity against human tuberculosis. Compared with healthy tuberculin reactors, lymphocytes from patients with tuberculosis showed diminished production of the Th1 cytokines, IFN-γ and interleukin-12 (IL-12) (26). At the same time there were no apparent changes in the production of the Th2 cytokines, IL-4, IL-10, and IL-13 (26). Following treatment for tuberculosis and with clinical recovery, patients' lymphocytes increased their production of IFN-γ (26).

In this study, immune responses to *M. tuberculosis* are analyzed in mice which have been immunized with heat-killed *M. vaccae*. Two striking effects have been observed: stimulation of $CD8^+$ cytotoxic T cells specific for target cells infected with *M. tuberculosis* and a cytokine secretion profile for $CD8^+$ T cells that reflects a Th1 phenotype. The significance of these findings to a human vaccine is discussed.

MATERIALS AND METHODS

Mice. All experiments were performed using 6- to 8-week old specific pathogen-free BALB/c mice purchased from the Department of Laboratory and Animal Science, University of Otago, Dunedin, New Zealand.

Bacterial immunization and infections. *M. vaccae* (ATCC 15483) was cultured in sterile medium 90 (yeast extract, 2.5 g/liter; tryptone, 5 g/liter; glucose, 1 g/liter) at 37°C. The bacteria were then harvested by centrifugation and transferred into sterile Middlebrook 7H9 (Difco Laboratories, Detroit, Mich.) medium with glucose at 37°C for 1 day. Bacteria cultures were centrifuged to pellet the bacteria, and the culture supernatant was removed. The bacterial pellet was resuspended in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml, equivalent to 10^{10} bacilli per ml. The cell suspension was autoclaved for 15 min

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at 120°C. Mice were immunized with a single dose of autoclaved *M. vaccae* by the intraperitoneal (i.p.) route.

The virulent strain of *M. tuberculosis*, H37Rv, supplied by AgResearch, Wallaceville, New Zealand, was grown in Middlebrook 7H9 medium supplemented with Tween 80 and oleic acid-albumin-dextrose-catalase (OADC). The bacteria were pelleted by centrifugation and suspended in PBS–0.05% Tween 80 and stored at −80°C. Mice were infected i.p. with live *M. tuberculosis* H37Rv at a dose of 5×10^5 CFU.

Spleen cell proliferation. Single cell suspensions of splenocytes were cultured in 0.2-ml volumes in 96-well tissue culture plates at 5×10^5 cells per ml with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 110 mg of sodium pyruvate per liter, 36 mg of L-arginine per liter, 116 mg of folic acid per liter, 60 mg of penicillin per liter, 100 mg of streptomycin per liter, and 2 mM glutamine. Live *M. tuberculosis* was added to experimental cultures at 50 μg/ml. After 6 days, the cultures were pulsed with 0.25 μCi of tritiated thymidine (26 Ci per mmol), incubated for a further 16 h, and then harvested. Thymidine uptake was determined by using the 1450 Microbeta Plus liquid scintillation counter (Wallac, Turku, Finland).

IFN-γ and IL-12 assays. IFN-γ concentrations in culture supernatants were measured by an enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated with a rat monoclonal antibody directed to mouse IFN-γ (R4-6A2; PharMingen, San Diego, Calif.) in PBS for 4 h at 4°C. Wells were blocked with PBS containing 0.2% Tween 20 for 1 h at room temperature. The plates were then washed four times in PBS–0.2% Tween 20, and samples diluted in culture medium in the ELISA plates were incubated overnight at room temperature. The plates were again washed, and a biotinylated monoclonal rat anti-mouse IFN-γ antibody (XMG1.2; PharMingen) diluted to 100 ng/ml in PBS–10% FCS was added to each well. The plates were then incubated for 1 h at room temperature and washed, and horseradish peroxidase-coupled avidin D (vector 2004) was added at a 1:4,000 dilution in PBS. After a further 1-h incubation at room temperature, the plates were washed, and an *o*-phenylenediamine substrate was added. The reaction was stopped after 10 min with 10% (vol/vol) HCl. The optical density was determined at 495 nm.

IL-12 concentrations in culture supernatants were measured by a similar ELISA technique with a rat anti-mouse IL-12 (p40/p70) monoclonal antibody (C15.6; PharMingen) as the capture antibody and a biotinylated rat anti-mouse IL-12 (p40/p70) monoclonal antibody (C17.8; PharMingen) as the detecting antibody.

Isolation of $CD4^+$ and $CD8^+$ T cells. The spleen cells were passed through a $CD4^+$ or $CD8^+$ T-cell enrichment column (R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The spleen cells recovered from the column have been shown by flow cytometry to be enriched 85 to 90% for $CD4^+$ or $CD8^+$ T cells and contained less than 1% B cells and other major histocompatibility complex class II-positive cells, 1 to 2% γδ T cells, and 2 to 3% natural killer (NK) cells.

In some experiments, spleen cells were cultured with heat-killed *M. vaccae* or *M. tuberculosis* for 6 days prior to the isolation of $CD4^+$ or $CD8^+$ T cells.

Mice were injected with 1 ml of 3% thioglycolate i.p., and after 5 days macrophages were obtained from the peritoneal cavity. Macrophages were suspended in DMEM (without antibiotics)–10% FCS. The macrophages were infected with *M. tuberculosis* by culturing 10^5 macrophages per ml with 2×10^5 bacilli overnight. After washing of these cultures, there were 1 to 5 mycobacteria per infected macrophage, at least 50% of which were infected. In the coculture experiments, 10^6 $CD8^+$ or $CD4^+$ T cells were cultured with 10^5 infected or uninfected macrophages in 200 μl of DMEM (without antibiotics)–10% FCS for 72 h, after which the culture supernatants were harvested and assayed for IFN-γ and IL-12.

Cytotoxicity assay. For use as targets in cytotoxic assays, infected or uninfected macrophages were labelled with chromium-51 sodium chromate at 2 μCi per 10^4 macrophages in 100 μl of DMEM–10% FCS. The macrophages were washed and cultured overnight (16 h) with T cells enriched for $CD4^+$ or $CD8^+$ cells at various killer-to-target ratios. Specific killing was detected by the release of ^{51}Cr and expressed as the percentage of specific lysis, calculated from the counts per minute (cpm) by the following formula: [(cpm in test culture supernatant − cpm in control culture supernatant)/(total cpm − cpm in control culture supernatant)] × 100%.

RESULTS

Antigen-induced proliferation and IFN-γ production. Three groups of BALB/c mice were prepared: nonimmunized, immunized with 1 mg of heat-killed *M. vaccae*, and immunized with 1 mg of heat-killed *M. tuberculosis*. At 3 weeks following immunization, all mice were infected with 5×10^5 CFU of *M. tuberculosis*. One week later, spleen cells from mice were prepared and incubated in cell culture with live *M. tuberculosis* for 6 days. At that time, cell proliferation was measured by radioactive thymidine uptake, and cell supernatants were assayed for the presence of IFN-γ. The data presented in Fig. 1A show that the levels of radioactive thymidine uptake in spleen cell cultures prepared from mice immunized prior to infection with heat-killed *M. vaccae* or *M. tuberculosis* were similar. By comparison, the data presented in Fig. 1B show a difference in levels of IFN-γ in these culture supernatants. The IFN-γ levels were threefold higher in culture supernatants from spleen cells of mice immunized with killed *M. vaccae* than in supernatants from cultures of spleen cells of mice immunized with killed *M. tuberculosis.*

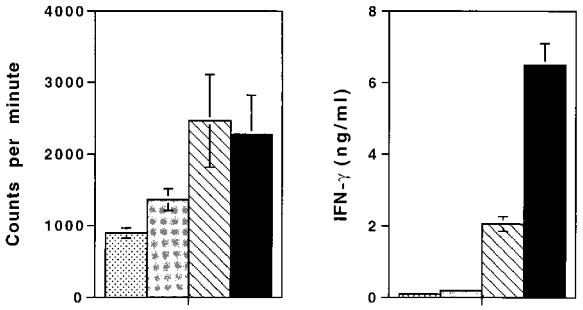

FIG. 1. Spleen cell in vitro response to live *M. tuberculosis* after immunization with *M. vaccae*. Mice were primed with 100 μg of heat-killed *M. vaccae* and 3 weeks later infected with 5×10^5 CFU of *M. tuberculosis* H37Rv. One week after infection, 10^5 spleen cells were cultured with 50 μg of live *M. tuberculosis* per ml in 200 μl of DMEM–10% FCS for 6 days, and their proliferation was measured. Supernatants from replicate cultures were harvested after 72 h and assayed for IFN-γ production. Values are the means and standard deviations for triplicate determinations from groups of three mice. ▨, nonimmunized, noninfected; ▤, *M. tuberculosis*-infected; ▧, killed *M. tuberculosis* immunized plus *M. tuberculosis* infected; ■, killed *M. vaccae* immunized plus *M. tuberculosis* infected.

$CD8^+$ T cells from *M. vaccae* immunized mice secrete IFN-γ. To identify the cell type producing IFN-γ in the experiment for which the results are shown in Fig. 1, mice were immunized with 1 mg of heat-killed *M. vaccae* and their spleen cells were obtained at intervals from 1 to 4 weeks later. $CD4^+$ and $CD8^+$ splenic T cells were prepared separately by using T-cell enrichment columns as described in Materials and Methods. $CD8^+$-enriched splenic T cells were then cultured with uninfected peritoneal macrophages or macrophages infected with *M. tuberculosis* for 72 h, and the culture supernatants were harvested and assayed for IFN-γ. The data in Fig. 2 show that *M. vaccae*-primed $CD8^+$ T cells secreted IFN-γ when cultured with infected macrophages but not with uninfected macrophages. A fourfold increase in IFN-γ secretion was observed 2 weeks following immunization with *M. vaccae* and remained 30- to 40-fold higher for a further 2 weeks. In four separate experiments, only minimal amounts of IFN-γ (0 to 3.8 ng/ml) were detected when $CD8^+$ T cells, infected macrophages, or uninfected macrophages were cultured.

Similar experiments were carried out with spleen cells depleted of $CD8^+$ T cells and enriched for $CD4^+$ T cells. When cocultured with infected macrophages, $CD4^+$ T cells from unimmunized mice secreted 20 to 40 ng of IFN-γ per ml. No increase in IFN-γ secretion was observed in cocultures of infected macrophages with $CD4^+$ T cells isolated at 1 or 2 weeks after immunization with *M. vaccae* (data not shown).

$CD8^+$ T cells are cytotoxic for macrophages infected with *M. tuberculosis*. To determine whether *M. vaccae* stimulates $CD8^+$ T cells capable of killing macrophages infected with *M. tuberculosis*, $CD8^+$-enriched T cells prepared from mice immunized with 1 mg of heat-killed *M. vaccae* were assayed for cytotoxic T-cell (CTL) activity. CTLs which specifically lysed macrophages infected with *M. tuberculosis* were detected only in

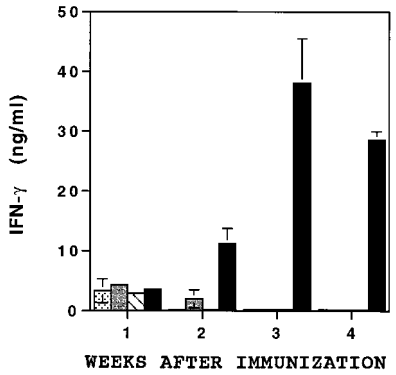

FIG. 2. IFN-γ secretion by $CD8^+$ T cells after exposure to *M. tuberculosis*-infected macrophages. Peritoneal macrophages (10^5) infected 18 h earlier with live *M. tuberculosis* were incubated with 10^6 $CD8^+$-enriched T cells from *M. vaccae*-primed mice, and IFN-γ secretion was measured after 72 h. IFN-γ secreted by *M. vaccae*-primed $CD8^+$ T cells alone was undetectable, and the amount secreted by naive $CD8^+$ T cells incubated with infected or uninfected macrophages did not differ from that secreted by infected (▤) or uninfected (▨) macrophages alone. Primed $CD8^+$ T cells were cocultured with infected (■) or uninfected (▧) macrophages. The data show means for triplicate estimations; error bars indicate standard deviations.

splenocytes from mice immunized 2 weeks previously with *M. vaccae*. The specificity and potency of the $CD8^+$ CTLs were demonstrated by their ability to give 95% specific lysis of infected target cells at a killer-to-target cell ratio of 10:1, whereas the specific lysis of uninfected targets at this killer-to-target cell ratio was not detectable (Fig. 3).

For comparison, the CTL activity of $CD4^+$-enriched splenocytes from mice immunized with *M. vaccae* was tested. These $CD4^+$ cells also lysed infected target cells but only achieved 40% specific lysis at a killer-to-target cell ratio of 50:1 (Fig. 3). The $CD4^+$-enriched CTLs also did not lyse uninfected macrophages. $CD8^+$ T cells isolated from spleens of mice at 3 and 4 weeks after immunization with *M. vaccae* were no longer cytotoxic for infected macrophages.

To determine whether $CD8^+$ CTLs detected 2 weeks after immunization with *M. vaccae* had given rise to memory cells,

TABLE 1. Cytotoxicity of functional memory $CD8^+$ T cells for *M. tuberculosis*-infected macrophages[a]

Killer-to-target cell ratio	% CTL specific lysis of macrophage targets	
	Uninfected	Infected
10:1	0.1 ± 2.5	41.2 ± 6.4
2.5:1	5.3 ± 0.9	27.6 ± 1.4
0.625:1	9.7 ± 3.8	ND

[a] Mice were immunized with 1 mg of heat-killed *M. vaccae*, and 3 weeks later spleen cells were obtained and stimulated with *M. vaccae* in vitro for 6 days. $CD8^+$ T cells were isolated from the spleen cells and tested for cytotoxicity against 10^4 *M. tuberculosis*-infected macrophages. Results are expressed as the percent specific lysis (means ± standard deviations). The spontaneous release of ^{51}Cr was 40.8 and 17.3% for infected and uninfected macrophages, respectively. ND, not detected.

the following experiment was performed. Mice were immunized with 1 mg of heat-killed *M. vaccae*, and 3 weeks later spleen cells were prepared and restimulated in vitro with *M. vaccae* for 6 days. At that time, spleen cells were harvested and separated into two fractions. One fraction was tested directly for cytotoxicity against macrophages infected with *M. tuberculosis* and gave 25% specific lysis at a killer-to-target cell ratio of 30:1 (data not shown). The second fraction was passaged through a $CD8^+$ enrichment column prior to the cytotoxicity assay. The $CD8^+$-enriched population gave 40% specific lysis at a killer-to-target cell ratio of 10:1 (Table 1). The specific lysis of uninfected macrophages by these CTL was less than 10%.

IL-12 production in cocultures of $CD8^+$ T cell and macrophages. The ability of $CD8^+$ T cells from *M. vaccae*-immunized mice to influence IL-12 production was examined by the following experiment. Four groups of mice were immunized with 1 mg of heat-killed *M. vaccae*, and their spleens were removed at 1, 2, 3, or 4 weeks after immunization. $CD8^+$ T cells were prepared from the spleens and cocultured for 72 h with uninfected macrophages or macrophages infected with *M. tuberculosis*. In four separate experiments, cultures of 10^5 uninfected macrophages were found to secrete only 0 to 8 pg of IL-12. Macrophages infected with *M. tuberculosis* were found to secrete 19 to 124 pg of IL-12. The addition of $CD8^+$ T cells from

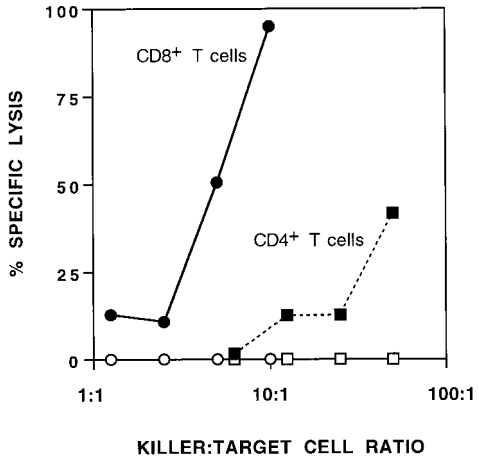

FIG. 3. CTL activity of T-cell subsets from *M. vaccae*-primed mice. $CD4^+$ (squares)- or $CD8^+$ (circles)-enriched splenocytes from *M. vaccae*-primed mice were incubated with 10^4 infected (closed) or uninfected (open) macrophages for 18 h. The spontaneous ^{51}Cr release for infected and uninfected macrophages was 39 and 40%, respectively. The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.

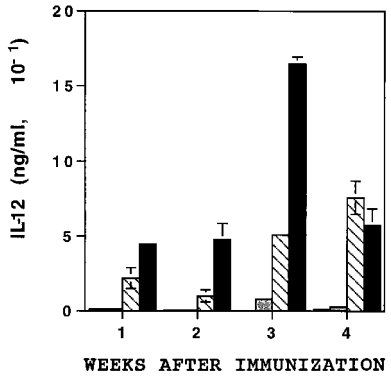

FIG. 4. $CD8^+$ T-cell stimulation of IL-12 secretion from *M. tuberculosis*-infected macrophages. Peritoneal macrophages (10^5) infected 18 h earlier with live *M. tuberculosis* were incubated with 10^6 $CD8^+$-enriched T cells from *M. vaccae*-primed mice, and IL-12 secretion was determined 72 h later. IL-12 secreted by infected or uninfected macrophages incubated with naive $CD8^+$ T cells did not differ from that secreted by the infected or uninfected macrophages alone (▤ or ▨, respectively). Primed $CD8^+$ T cells were cocultured with infected (■) or uninfected (▧) macrophages. The data are means for triplicate estimations ± standard deviations.

TABLE 2. $CD8^+$ T-cell in vitro response to *M. tuberculosis*-infected macrophages 6 weeks after in vivo infection[a]

Treatment of mice	Mean $CD8^+$ T-cell response ± SD					
	IFN-γ (ng/ml)		IL-12 (pg/ml)		% CTL specific lysis by restimulated T cells[b]	
	Fresh	Restimulated	Fresh	Restimulated	Uninfected macrophages	Infected macrophages
Nonimmunized	2.4 ± 0.01	27.8 ± 0.16	77.2 ± 0.02	72.8 ± 0.01	10.8 ± 4.1	ND
M. vaccae immunized	1.58 ± 0.07	16.5 ± 0.26	101.6 ± 0.04	74.3 ± 0.01	4.9 ± 5.7	13.9 ± 2.3

[a] Mice were immunized i.p. with *M. vaccae* and three weeks later infected i.p. with 5×10^5 *M. tuberculosis* CFU. Six weeks after infection, spleen cells from immunized and nonimmunized mice were prepared as follows: either the $CD8^+$ T cells were isolated from fresh splenic cells or the spleen cells were restimulated in vitro with killed *M. tuberculosis* for 6 days prior to isolation of $CD8^+$ T cells. $CD8^+$ T cells (10^6) were cultured with 10^5 macrophages infected with *M. tuberculosis*, and the supernatants were assayed for IFN-γ and IL-12 concentrations after 3 days. In addition, 10^5 $CD8^+$ T cells were also assayed for CTL activity against 10^4 infected or noninfected macrophages. ND, not detected.

[b] CTL specific lysis in fresh splenic cells was not detectable in both nonimmunized and *M. vaccae*-immunized mice.

mice immunized with *M. vaccae* to the infected macrophage cultures increased the amount of IL-12 in the supernatants by two- to fourfold (Fig. 4). The enhanced IL-12 secretion in the cocultures was noted after the addition of $CD8^+$ enriched cells obtained as early as one week following immunization and peaked with $CD8^+$ T cells prepared from spleens 3 weeks after immunization. There was no increase in IL-12 production in cocultures of $CD8^+$ T cells from mice immunized with *M. vaccae* and uninfected macrophages or in cocultures of naive $CD8^+$ T cells and infected macrophages.

Effect of immunization with *M. vaccae* on $CD8^+$ T cells of mice infected with *M. tuberculosis*. Mice were immunized with 1 mg of heat-killed *M. vaccae* and 3 weeks later infected with 5×10^5 CFU of *M. tuberculosis*. Their spleens were removed after a further 6 weeks. One fraction of splenic cells was passaged through a $CD8^+$ T-cell enrichment column, and splenic $CD8^+$ T cells were isolated. These freshly isolated $CD8^+$ T cells were cultured for 3 days with macrophages infected with *M. tuberculosis*, and the coculture supernatant was assayed for IFN-γ and IL-12 production. The data in Table 2 reveal that the secretion levels of both IFN-γ and IL-12 were similar in cocultures of infected macrophages with $CD8^+$ T cells from immunized and nonimmunized infected mice. The cytotoxicity of the freshly isolated splenic $CD8^+$ T cells was also assayed but found to lack activity cytolytic to macrophages infected with *M. tuberculosis.*

To determine whether the $CD8^+$ T-cell population contained precursors specific for *M. tuberculosis*, the second fraction of spleen cells removed 6 weeks after infection were cultured with heat-killed *M. tuberculosis* for 6 days, following which $CD8^+$ T cells were isolated. The restimulated $CD8^+$ T cells from infected mice produced similar amounts of IFN-γ and IL-12 in the in vitro coculture response to infected macrophages, regardless of whether the $CD8^+$ T cells came from immunized or unimmunized mice. Restimulated $CD8^+$ T cells prepared from infected mice which had been immunized with *M. vaccae* resulted in the generation of cells that were specifically cytotoxic for infected macrophages (Table 2). The restimulation of $CD8^+$ T cells prepared from nonimmunized infected mice did not develop cells cytotoxic for infected macrophages.

DISCUSSION

Prior exposure to various mycobacteria can potentially alter immune responses in a subsequent infection with *M. tuberculosis*. In this paper we describe the properties of *M. tuberculosis*-reactive $CD8^+$ T cells that are generated in mice immunized with heat-killed *M. vaccae.*

Two weeks following immunization of mice with heat-killed *M. vaccae*, $CD8^+$ T cells were found in spleens that were specifically cytotoxic for syngeneic macrophages infected with live *M. tuberculosis* but not for uninfected macrophages (Fig. 2). However, this effector activity rapidly disappeared at 3 and 4 weeks following immunization; these effector $CD8^+$ CTLs were no longer found in the spleen. By incubating spleen cells in culture with *M. vaccae*, $CD8^+$ CTL activity reappeared. This finding suggests that the effector cells have reverted to a memory state and that with appropriate activation signals it is possible to stimulate *M. vaccae*-primed memory cells to effector CTLs capable of recognizing and killing *M. tuberculosis*-infected cells.

The $CD8^+$ CTLs were also strong producers of IFN-γ, and this cytokine profile indicates that these cells may be classed as the Tc1 type of CTLs (20). In addition to secreting IFN-γ, CTLs from *M. vaccae*-immunized mice enhance IL-12 production in coculture with *M. tuberculosis*-infected macrophages. IL-12 is produced by macrophages early in the immune response, and it enhances IFN-γ production by T cells. The $CD8^+$ T cells from as early as 1 week after immunization enhanced IL-12 production. The peak production of both IFN-γ and IL-12 occurred 3 weeks after immunization with *M. vaccae*. This finding is consistent with the interdependence of IFN-γ-producing $CD8^+$ T cells and IL-12-producing macrophages (10). Memory $CD8^+$ T cells restimulated with specific antigen were also capable of enhancing IL-12 when cocultured with infected macrophages. In these experiments, NK cells are present at 2 to 3% in the $CD8^+$-enriched T-cell populations, but preliminary experiments indicate that 10- to 100-fold of this number of NK cells are required to account for the levels of IFN-γ observed. If NK cells contributed to the enhancement of IFN-γ secretion and the resulting $CD8^+$ Tc1 response, this cooperative role for NK cells was not evident in T-cell populations enriched for $CD4^+$ cells.

The CTLs that appear in unimmunized mice following infection with *M. tuberculosis* differ from those generated in mice immunized with *M. vaccae*. Orme et al. detected in the spleens of mice infected with *M. tuberculosis* $CD4^+$ T cells cytotoxic for peritoneal macrophages pulsed with *M. tuberculosis* culture filtrate proteins (17). *M. tuberculosis*-specific memory cells have been found in the $CD4^+$ splenic T-cell population (2). $CD4^+$ T cells from infected mice produce more IFN-γ than does the $CD8^+$ T-cell subset (17). This is in contrast to the results for the mice immunized with *M. vaccae* whose $CD8^+$ T

cells were more cytotoxic when challenged with *M. tuberculosis*-infected macrophages than their $CD4^+$ counterpart. *M. vaccae*-primed $CD4^+$ T cells produced amounts of IFN-γ to equivalent to those of naive $CD4^+$ T cells. Compared with naive $CD8^+$ T cells, *M. vaccae*-primed $CD8^+$ T cells challenged with macrophages infected with *M. tuberculosis* showed greater specific enhancement of IFN-γ.

We have also shown that the Tc1 characteristics of $CD8^+$ T cells from mice immunized with *M. vaccae* persist in mice after infection with *M. tuberculosis*. Six weeks after infection, the $CD8^+$ T cells from the spleens of immunized and unimmunized mice produced comparable amounts of IFN-γ and IL-12 in response to *M. tuberculosis*-infected macrophages. As expected, no mycobacterium-specific $CD8^+$ effector CTLs were detected, presumably because such cells are distributed to organs other than the spleen or that at this stage the numbers of activated effector CTLs have subsided. However, memory-type CTLs specific for *M. tuberculosis*-infected macrophages were detected but only in spleens of mice immunized with *M. vaccae.*

There are numerous problems in the development of protective vaccines against tuberculosis or immunotherapy for patients with clinical disease. The first is defining the cellular components of a protective response. The second is the recall of these components when cells are confronted with infection and maintenance of dominance of appropriate effector mechanisms during the development of clinical disease. Experimental data from mice suggest that adoptively transferred T-cell clones that were the most effective at providing protection against *M. tuberculosis* infection were the ones that were most cytotoxic (21). On the other hand, recent data from perforin and granzyme gene knockout mice suggest that the role of $CD8^+$ T cells in controlling *M. tuberculosis* infection may rely on mechanisms such as cytokine secretion and not on their lytic activity (6, 12).

Experimental data from infected mice is often criticized because animals do not normally develop clinical disease. Nevertheless, the reasons why mice exhibit such resistance to this disease are important in understanding protective immunity. Experiments in the 1980s indicated that several nonliving mycobacterial preparations tested in vivo in mice conferred the ability to mount a delayed-type hypersensitivity response to tuberculin (18). However, none of such preparations generated protective T cells capable of adoptive immunization against virulent tuberculosis (16). The idea developed that immunity to live mycobacteria was not directed against constitutive proteins but against proteins secreted by live organisms. It is thus striking that in mice immunized with heat-killed *M. vaccae* the number of mycobacteria in spleens was reduced following infection with live *M. tuberculosis* (1).

There are two aspects to the experiments described here. The first is that heat-killed *M. vaccae* stimulates $CD8^+$ T cells which are specifically cytotoxic for syngeneic macrophages infected with live *M. tuberculosis* and thus retains antigens which are cross-reactive with those of live *M. tuberculosis*. The second is that the use of heat-killed *M. vaccae* stimulates $CD8^+$ T cells which secrete IFN-γ and enhance IL-12 production by infected macrophages. Recently, Abou-Zeid et al. showed that immunization of mice with heat-killed recombinant *M. vaccae* expressing the 19-kDa lipoprotein of *M. tuberculosis* is more effective than immunization with the same antigen in incomplete Freund's adjuvant in producing an antigen-specific Th1 response (1). These observations suggest that heat-killed *M. vaccae* also contains adjuvant substances which preferentially stimulate T cells with a Th1 cytokine secretion profile. Immunization with heat-killed *M. vaccae* appears to promote the dominance of IL-12, IFN-γ, and the Tc1 loop.

Our data provide the immunologic basis for defining further the components of *M. vaccae* that may be useful as a vaccine for tuberculosis. It is not clear whether the antigenic and adjuvant properties of *M. vaccae* reside within the same molecular component. The enhancement of the $CD8^+$ T-cell subset may be a significant advantage for *M. vaccae* over *M. tuberculosis* or other mycobacterial strains in immunizations. Although whole autoclaved *M. vaccae* may be effective, it is possible that selected antigenic proteins and adjuvant components may perform better as vaccines due to their Th1- or Tc1-inducing properties. We are currently cloning the genes of several *M. vaccae* proteins for development in protein-based subunit vaccines or as DNA vaccines.

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