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Immunology

Stimulation of protective CD8$^+$ T lymphocytes by vaccination with nonliving bacteria

(*Listeria monocytogenes*/cytolytic T cells/immunity)

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ABSTRACT **Infectious diseases caused by intracellular microbes are responsible for major health problems, and satisfactory control will ultimately depend on efficient vaccination strategies. The general assumption is that activation of protective immune responses against intracellular microbes dominated by CD8$^+$ T cells are achieved only by live vaccines. In contrast, we here demonstrate stimulation of protective immunity in mice against the intracellular pathogen *Listeria monocytogenes* by vaccination with heat-killed listeriae. Vaccine-induced immunity comprised cytolytic and interferon γ-producing CD8$^+$ T lymphocytes. CD8$^+$ T cells from vaccinated donor mice transferred protection against listeriosis. Moreover, vaccination with heat-killed listeriae induced protection in CD4$^+$ T-cell-deficient, *H2-Aβ* gene-disrupted mutant mice. We conclude that antigens from killed listeriae are introduced into the major histocompatibility complex class I pathway and thus are recognized by CD8$^+$ T cells. The practicability of killed vaccines against human infectious diseases therefore should be reevaluated.**

Prevention of infectious disease by vaccination is based on the activation of appropriate immune responses (1). Vaccination against many extracellular and toxin-producing bacteria has proven highly efficacious because the available vaccines induce the relevant immune response (2). In contrast, satisfactory vaccines are generally not available for control of intracellular bacteria and protozoa. This group of pathogens causes major health problems, including tuberculosis, leprosy, typhoid fever, trachoma, Chagas disease, leishmaniasis, and malaria (3). Hence, efficient vaccines would be highly desirable. Protection against bacteria and protozoa has been considered largely a domain of CD4$^+$ T cells producing T helper 1 (T_{H1})-like cytokines such as interferon γ (IFN-γ) (2). More recently, the importance of CD8$^+$ T cells in protective immunity to infections with intracellular microbes has been documented (4). Activation of protective CD8$^+$ T cells is considered to depend exclusively on viable microorganisms (4, 5). The central role of CD8$^+$ T lymphocytes in antimicrobial protection has been most extensively studied in experimental infection of mice with the Gram-positive bacterium *Listeria monocytogenes* (6). This pathogen is able to egress from the endosomal to the cytosolic compartment, where its antigens have access to the major histocompatibility complex (MHC) class I processing and presentation pathway that is essential for CD8$^+$ T-cell activation (6). The pore-forming cytolysin listeriolysin, which promotes egression into the cytosol, is considered central to listerial survival in macrophages, virulence, and activation of MHC class I-restricted CD8$^+$ T lymphocytes (7–10). Accordingly, neither ahemolytic nor killed listeriae should be able to activate protective CD8$^+$ T cells (7, 11, 12). The close linkage between listerial viability and virulence on the one hand, and protective immunity on the other hand, illustrates the general dilemma of vaccine design against intracellular microbes. In contrast to this currently held view, we have recently shown that antigens from killed wild-type *L. monocytogenes* and from ahemolytic *L. monocytogenes* mutant strains are processed and presented through the MHC class I pathway *in vitro* (13, 14). This is in accordance with presentation of endosomally derived bacterial antigens by MHC class I molecules (15). We therefore evaluated the feasibility of inducing a protective CD8$^+$ T-cell response *in vivo* by vaccination with heat-killed *L. monocytogenes* (HKL).

MATERIALS AND METHODS

Bacteria and Mice. *L. monocytogenes* strain EGD organisms were kept virulent by mouse passage. *L. monocytogenes* (aliquot of an infected liver) and *Streptococcus mutans* (patient isolate) were grown in trypticase soy broth overnight and samples were frozen at −70°C until used. For preparation of HKL or heat-killed *S. mutans* (HKS), bacteria were incubated at 70°C for 60 min, washed with phosphate-buffered saline, and frozen at −20°C until used. Control experiments excluded bacterial survival after heat treatment (detection limit, 10^{-13} organism per ml), showing that the inoculum of HKL or HKS was devoid of viable bacteria. All experiments were performed with 8- to 10-week-old female C57BL/6 mice and homozygous (−/−) β_2-microglobulin (*B2m*) and H-2I-A$_\beta$ (*H2-Ab*) gene-disrupted mutant mice kept under pathogen-free conditions at the animal facilities of the University of Ulm (16, 17). Mutant mice were bred on a C57BL/6 background and used in the seventh backcross.

Vaccination and Challenge Infection of Mice. Unless otherwise stated C57BL/6 mice were vaccinated by i.v. injection of 10^9 HKL three times at 5-day intervals. After another 5 days, mice were challenged with 1.5×10^4 live *L. monocytogenes* ($1.5 \times LD_{50}$, i.v.) and survival was monitored over 10 days. Control mice received 200 μl of the supernatant of the HKL preparation, received 10^9 HKS, or remained untreated before challenge infection. At day 10, spleens from vaccinated mice were removed and colony-forming units (CFU) were determined (18). Listerial growth was detectable neither on plates nor in liquid cultures, suggesting sterile clearance of listeriae in vaccinated mice. To assess the longevity of vaccine-induced protection, mice were challenge-infected with 10^5 live *L. monocytogenes* ($10 \times LD_{50}$, i.v.) 4 weeks after vaccination as described. Survival was monitored over 21 days. The *B2m*$^{-/-}$ and *Ab*$^{-/-}$ mice were vaccinated three times with 10^9 HKL i.v. and then infected with 5×10^3 live *L. monocytogenes* [$1 \times LD_{50}$ for these knockout mice (18)]. Nonvaccinated heterozygous littermates cleared bacterial challenge within 10 days (<10^2 CFU per spleen on day 10).

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Abbreviations: CFU, colony-forming unit(s); HKL, heat-killed *Listeria monocytogenes*; HKS, heat-killed *Streptococcus mutans*; IFN, interferon; MHC, major histocompatibility complex; T_H, T helper.

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Adoptive Transfer of Antilisterial Protection by Selected CD8$^+$ or CD4$^+$ T Lymphocytes. Spleen cells from HKL-vaccinated C57BL/6 mice were passed over nylon wool columns. Afterwards, 10^7 purified spleen cells were labeled with either 10 μl of Ly-2-MACS beads or L3T4-MACS beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and negatively sorted over a B2 MACS column (Miltenyi Biotec). Purity of CD4 or CD8 cells was >98%. Mice received i.v. 5×10^5 nylon wool-enriched cells as a source of CD4$^+$/CD8$^+$ T cells and MACS-sorted cells as a source of selected CD8$^+$ or CD4$^+$ T cells. Control mice received either 5×10^5 nylon wool-enriched CD4$^+$/CD8$^+$ T cells from naive donor mice or no cells. Immediately thereafter, mice were infected i.v. with 1.5×10^4 live *L. monocytogenes* ($1.5 \times LD_{50}$). After 3 days, CFU in spleens were determined (19).

IFN-γ Secretion and Cytolytic Activity of T Cells from HKL-Vaccinated Mice. Nylon wool-enriched cells (10^6 per ml) or MACS-sorted cells (10^6 per ml) were cultured with irradiated (3000 rad; 1 rad = 0.01 Gy) syngeneic spleen cells (10^6 per ml) and HKL (5×10^7 per ml) as described (14). Selected CD8$^+$ or CD4$^+$ T cells (purity > 98%) (10^6 per ml) were cultured in the presence of various antigen preparations (as indicated in figure legend) together with syngeneic antigen-presenting cells (10^6 per ml). As control, nylon wool-enriched cells from naive mice were used. After 4 days, IFN-γ production was measured by a conventional double sandwich ELISA (18). In unstimulated T-cell cultures, IFN-γ was not detectable. T cells were tested for cytolytic activities with bone marrow macrophages as targets in a conventional ^{51}Cr-release assay (14). For generation of bone marrow macrophages, bone marrow cells (10^5 per ml) from femurs of C57BL/6 mice were cultured at 37°C and 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, and 30% conditioned medium from L929 cells. These macrophages are virtually devoid of MHC class II molecules (14). Bone marrow macrophages were either infected or pulsed with HKL; control targets were pulsed with HKS or remained untreated.

RESULTS

Protection Against *L. monocytogenes* After Vaccination with HKL. Mice were vaccinated i.v. with 10^9 HKL three times at 5-day interval and, after another 5 days, challenge-infected with a high dose of viable *L. monocytogenes*. As expected, naive mice succumbed to listeriosis, whereas all vaccinated mice survived and achieved sterile clearance of infection (Fig. 1 *A* and *B*). Vaccination was effective over a broad range from 10^6 HKL to 10^{12} HKL, whereas lower doses ($<10^4$ HKL) failed to induce protection (data not shown). Immunization with peptides allows direct loading of surface-expressed MHC class I molecules and hence may bypass antigen processing (20, 21). We exclude such exogenous loading of peptides because immunization with supernatants of the HKL preparation failed to protect mice against subsequent *L. monocytogenes* challenge (Fig. 1*A*). Nonspecific macrophage activation contributes to resistance against *L. monocytogenes*. To assess possible influences of nonspecific effects of vaccination, mice were vaccinated with a killed preparation of a related, though distinct, bacterial species, *S. mutans*. All HKS-vaccinated mice succumbed to challenge infection with *L. monocytogenes*, excluding a major influence of nonspecific resistance to HKL-induced protection (Fig. 1*B*). To determine the longevity of vaccine-induced protection, HKL-vaccinated mice were challenge infected with a high dose of *L. monocytogenes* ($10 \times LD_{50}$) 4 weeks later. Whereas all nonvaccinated mice died rapidly, mice were partially protected even 4 weeks after HKL vaccination (Fig. 1*C*).

Adoptive Protection by CD4$^+$ and CD8$^+$ T Cells from HKL-Vaccinated Mice. Although CD8$^+$ T cells are central to antilisterial immunity, partial protection is afforded by CD4$^+$ T lymphocytes (18). We determined the contribution of CD4$^+$ and CD8$^+$ T cells to vaccine-induced protection in an adoptive transfer system. A low number of negatively selected CD4$^+$ and CD8$^+$ T lymphocytes from mice which had been vaccinated with HKL were transferred to recipients that were then

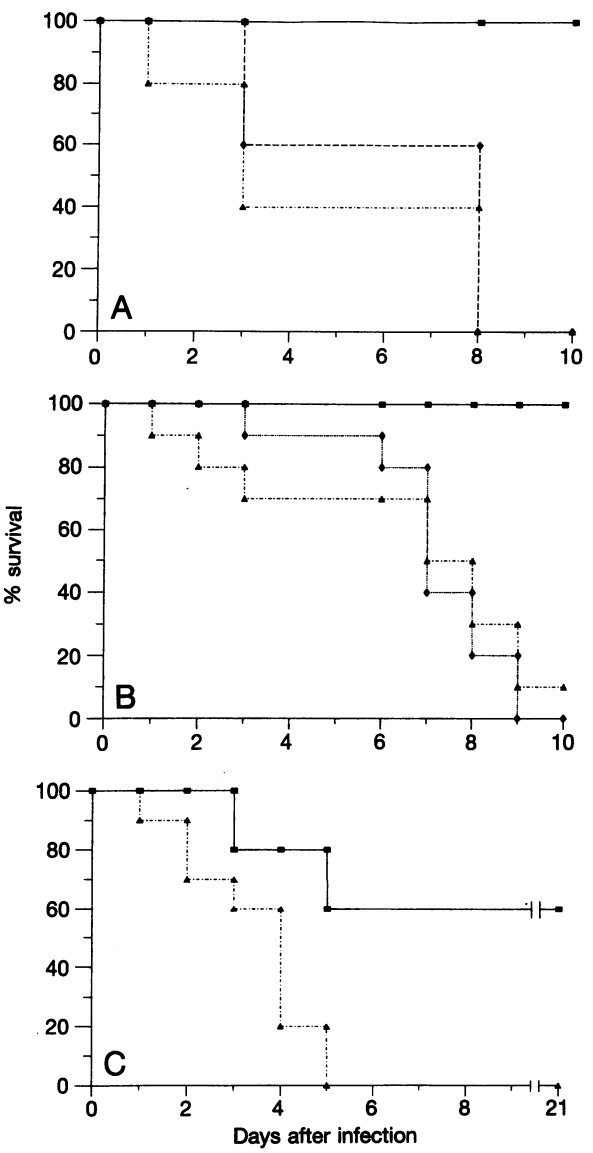

FIG. 1. (*A*) Protection of mice from *L. monocytogenes* infection by HKL vaccination. C57BL/6 mice were vaccinated (i.v.) with 10^9 HKL three times at 5-day intervals (■). After another 5 days, mice were challenged with 1.5×10^4 live *L. monocytogenes* ($1.5 \times LD_{50}$, i.v.) and survival was monitored over 10 days. Control mice received 200 μl of the supernatant of the HKL preparation (◆) or remained untreated (▲) before challenge. At day 10, spleens from vaccinated mice were removed and CFU were determined (18). Vaccination was equally successful in C57BL/6 mice and BALB/c mice (data not shown). Experimental groups comprised 5 animals. Experiments were performed three times with similar results. (*B*) Specific protection of mice after vaccination. Mice were vaccinated three times with 10^9 HKL (■) or HKS (◆) at 5-day intervals or remained untreated (▲). Five days after the last vaccination, mice were challenged with 1.5×10^4 live *L. monocytogenes* and survival was monitored over the next 10 days. Experimental groups comprised 10 animals. Experiments were performed three times with similar results. (*C*) Induction of long-term immunity. Mice were vaccinated with 10^9 HKL (■) as described or remained untreated (▲). After 4 weeks, animals were challenged with 10^5 live *L. monocytogenes* ($10 \times LD_{50}$, i.v.). The experiment was performed twice with 10 animals per experimental group.

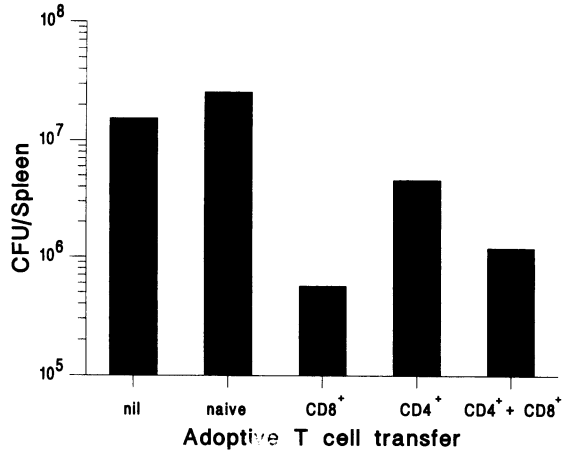

FIG. 2. Adoptive transfer of antilisterial protection by selected $CD8^+$ or $CD4^+$ T lymphocytes. Mice received i.v. 5×10^5 nylon wool-enriched $CD4^+/CD8^+$ T cells or negatively sorted $CD8^+$ or $CD4^+$ T cells from vaccinated mice. Control mice received 5×10^5 nylon wool-enriched $CD4^+/CD8^+$ T cells from naive mice (naive) or no cells (nil). Immediately thereafter, mice were infected with 1.5×10^4 live *L. monocytogenes* ($1.5 \times LD_{50}$). After 3 days, CFU in spleens were determined (19). Log_{10} differences and *P* values (Student's *t* test) of experimental groups versus untreated control are shown. Significant differences ($P < 0.05$) were determined for groups receiving unselected or selected $CD8^+$ T cells from vaccinated mice. Experiments were done twice with comparable results.

challenged with *L. monocytogenes*. Unselected and selected $CD8^+$ T lymphocytes from vaccinated mice transferred protection, whereas transfer of naive T cells showed no such effect (Fig. 2). In the particular experiment depicted in Fig. 2, 5×10^5 $CD8^+$ T cells conferred 27-fold (Δlog 1.22) protection, in contrast to $CD4^+$ T cells, which at these low cell numbers failed to transfer a significant degree of protection (Δlog 0.52). Superior protection by $CD8^+$ over $CD4^+$ T cells is consistent with the major role of $CD8^+$ T cells in antilisterial resistance (4, 5). We conclude that nonliving bacteria can activate protective $CD8^+$ T lymphocytes. We assume that denaturation of listerial proteins by heat and the presence of components

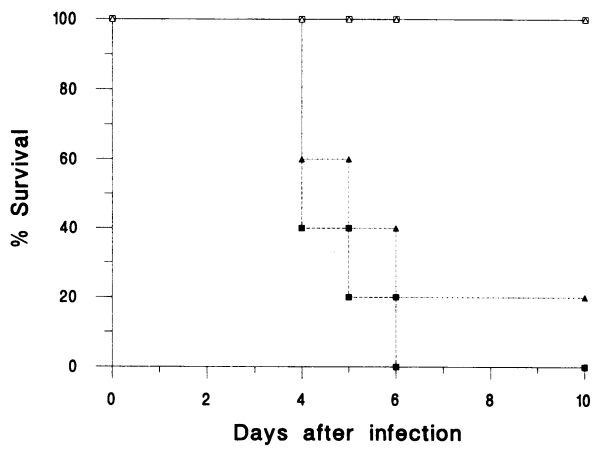

FIG. 3. Antilisterial protection in $B2m^{-/-}$ and $Ab^{-/-}$ knockout mice by vaccination with HKL. The $B2m^{-/-}$ mice (□) or $Ab^{-/-}$ mice (△) were vaccinated as described (open symbols) or remained untreated (filled symbols). For challenge, mice were infected with 5×10^3 live *L. monocytogenes* [$1 \times LD_{50}$ for these knockout mice (18)]. Nonvaccinated heterozygous controls survived the challenge inoculum of 5×10^3 *L. monocytogenes*, confirming an impact of both MHC II- and MHC I-dependent T-cell responses on protective immunity to this pathogen (18). The experiment was performed twice with comparable results.

with adjuvant activity facilitated MHC class I processing and subsequent $CD8^+$ T-cell activation (22–24).

Protection by Autonomous $CD4^+$ or $CD8^+$ T Lymphocytes. Activation of protective $CD4^+$ and $CD8^+$ T lymphocytes by the killed vaccine independent from the alternative subset was analyzed in $B2m^{-/-}$ and $Ab^{-/-}$ mice. These mutants lack conventional $CD8^+$ or $CD4^+$ T lymphocytes, respectively (16, 17). The $B2m^{-/-}$ or $Ab^{-/-}$ mutants were vaccinated with HKL and subsequently challenged with *L. monocytogenes* (Fig. 3). This challenge infection was lethal for all naive $B2m^{-/-}$ mutants and the majority of $Ab^{-/-}$ mutants. Importantly, $B2m^{-/-}$ and $Ab^{-/-}$ mutants were effectively protected by HKL vaccination against challenge infection. We conclude that vaccination activated protective $CD4^+$ and $CD8^+$ T lymphocytes independent from each other. This finding points to the feasibility of $CD8^+$ T-lymphocyte activation by nonliving microbial vaccines in situations of $CD4^+$ deficiency, as for example in AIDS patients (25).

IFN-γ Production and Cytotoxic Activities by Vaccine-Induced T Cells. Production of IFN-γ and specific lysis of infected target cells are considered essential for protective immune responses against intracellular microbial pathogens (4, 26, 27). IFN-γ is required for activation of antimicrobial capacities in macrophages. Both $CD4^+$ and $CD8^+$ T cells from HKL-vaccinated mice produced high amounts of IFN-γ after restimulation with the homologous antigen preparation (Fig. 4*A*). $CD4^+$ T cells, but not $CD8^+$ T cells, also produced IFN-γ

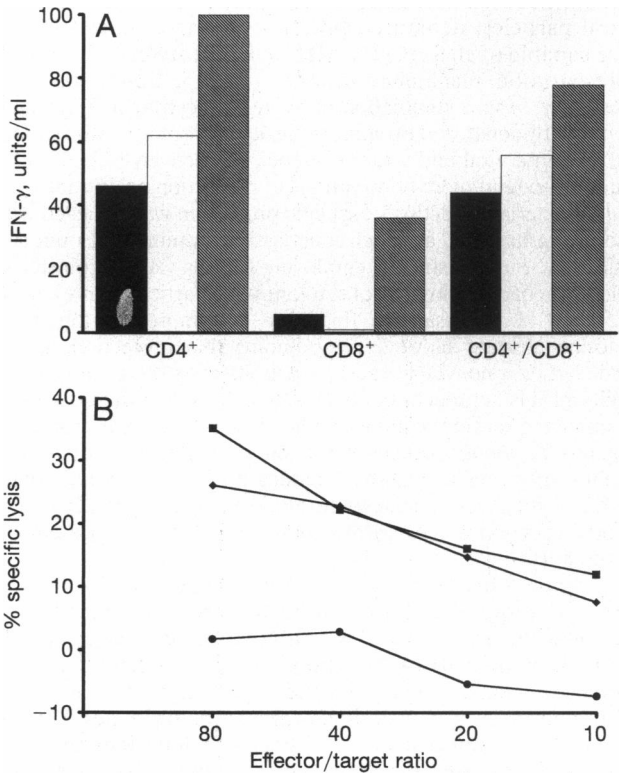

FIG. 4. IFN-γ secretion and cytolytic activity by T cells from HKL-vaccinated mice. (*A*) Selected $CD8^+$ or $CD4^+$ T cells (purity > 98%) (10^6 per ml) were cultured in the presence of listerial culture filtrate (5 μg/ml, filled bars), listerial lysate (5 μg/ml, open bars), or HKL (10^7 per ml, hatched bars) together with syngeneic antigen-presenting cells (10^6 per ml). After 4 days, IFN-γ production was measured. In unstimulated T-cell cultures, IFN-γ was not detectable. T cells from naive C57BL/6 mice were used as control. (*B*) T cells were tested for cytolytic activities with bone marrow macrophages as targets in a conventional ^{51}Cr-release assay (14). The macrophages were either infected (◆) or pulsed (■) with HKL; control targets remained untreated (●). Experiments were repeated three times with similar results.

after stimulation with soluble secreted or somatic listerial antigens (Fig. 4*A*). This is consistent with the notion that nonliving bacteria are capable of charging both the MHC class II and the MHC class I pathway *in vitro*, whereas soluble proteins are largely restricted to MHC class II processing and presentation (5, 21). In contrast, interleukin 4 was not detected under any stimulation condition (data not shown). Hence, HKL vaccination induced $CD4^+$ T cells of T_{H1} rather than T_{H2} type (28). Lysis of infected target cells seems to be required for releasing microbial pathogens from inefficient or MHC class II-deficient host cells and subsequent uptake by more-potent effector cells (5). The $CD8^+$ T cells from vaccinated mice lysed target cells which had been pulsed with killed or viable *L. monocytogenes* (Fig. 4*B*). In contrast, bone marrow macrophages pulsed with HKS were not lysed by these T cells (data not shown). Thus, vaccination with HKL induced $CD8^+$ T lymphocytes expressing the two biological functions essential for protective immunity—i.e., IFN-γ production and target cell lysis.

DISCUSSION

The experiments described are of interest to both basic immunology and vaccinology for the following reasons. Exogenous protein antigens are generally prevented from entering the cytosolic pathway and are processed/presented by MHC class I-expressing cells only after artifical introduction into the cytosol—e.g., by osmotic shock (29). More recent findings, however, have revealed that bacteria remaining in the endosome, as well as nonliving bacteria, killed viral particles, denatured proteins, or particulate antigens, are capable of charging the MHC class I antigen-processing/presentation machinery *in vitro* (14, 15, 22–24, 30, 31). Recently, *in vivo* stimulation of protective cytotoxic T lymphocytes with denatured protein or particulate antigen was demonstrated in a viral and a tumor model, respectively (23, 32). Our findings extend these observations to protection against intracellular bacteria. Induction of specific protection was achieved with nonliving bacteria, although long-lasting immunity was not satisfactory. Further studies, employing various vaccination schedules and coadministration of cytokines—in particular, interleukin 12—will be necessary to find ways for improving long-term vaccine efficacy. However, our finding that protection can be induced by a nonviable vaccine demonstrates the general feasibility of this approach. In contrast to our finding the efficacy of a nonliving vaccine to induce $CD8^+$ T-cell-dependent protection against *L. monocytogenes* has been questioned (11, 12). The failure of a previous study to induce protective immunity with HKL might have been due to the mode of application (12). In that study, injection was performed only once and not repeatedly as done here.

Infectious diseases caused by intracellular microbial pathogens are responsible for major health problems, and $CD8^+$ T lymphocytes are essential for complete protection against most of these pathogens (4, 5). The vital role of $CD8^+$ T cells in protection against many of these pathogens has provided major obstacles to conventional vaccination strategies. Current attempts to overcome these obstacles include immunization with peptides or naked DNA as well as application of recombinant live bacterial or viral vectors (33–35). Each of these strategies has its own merits and drawbacks. Our finding that nonliving bacteria are potent inducers of protective $CD8^+$ T cells—provided an appropriate vaccination schedule is chosen—adds one of the oldest vaccine types to the list of future vaccination strategies. Application of nonliving vaccines is not restricted to bacterial infections, because the use of killed recombinant bacteria expressing foreign protein antigens extends the scope of this type of vaccination to viral and protozoan diseases. Nonliving whole bacterial vaccines have several advantages, including built-in adjuvanticity and independence from MHC polymorphism in the target population, which are major problems for peptide-based vaccines (36). Moreover, any safety concerns regarding reversion to virulence, disease development in immunocompromised patients, integration of naked nucleic acids into the host genome, or the general use of live recombinant vaccines can be excluded (37). Future vaccination strategies, therefore, should reevaluate the practicability of killed vaccines in infectious diseases which are under the control of complex immune responses dominated by $CD8^+$ T lymphocytes.

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