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Enhanced Immunogenicity and Protective Efficacy Against *Mycobacterium tuberculosis* of Bacille Calmette-Guérin Vaccine Using Mucosal Administration and Boosting with a Recombinant Modified Vaccinia Virus Ankara[1]

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Heterologous prime-boost immunization strategies can evoke powerful T cell immune responses and may be of value in developing an improved tuberculosis vaccine. We show that recombinant modified vaccinia virus Ankara, expressing *Mycobacterium tuberculosis* Ag 85A (M.85A), strongly boosts bacille Calmette-Guérin (BCG)-induced Ag 85A specific $CD4^+$ and $CD8^+$ T cell responses in mice. A comparison of intranasal (i.n.) and parenteral immunization of BCG showed that while both routes elicited comparable T cell responses in the spleen, only i.n. delivery elicited specific T cell responses in the lung lymph nodes, and these responses were further boosted by i.n. delivery of M.85A. Following aerosol challenge with *M. tuberculosis*, i.n. boosting of BCG with either BCG or M.85A afforded unprecedented levels of protection in both the lungs (2.5 log) and spleens (1.5 log) compared with naive controls. Protection in the lung correlated with the induction of Ag 85A-specific, IFN-γ-secreting T cells in lung lymph nodes. These findings support further evaluation of mucosally targeted prime-boost vaccination approaches for tuberculosis. ***The Journal of Immunology,*** **2003, 171: 1602–1609.**

Tuberculosis is caused by the respiratory pathogen *Mycobacterium tuberculosis* and kills ~2 million people each year, predominantly in the developing world (http://www.who.int/gtb/publications/globrep01/index.html). The only licensed vaccine against *M. tuberculosis*, bacille Calmette-Guérin (BCG)[3] (1), is an attenuated strain of *Mycobacterium bovis*, which in developing countries is typically administered intradermally as a single dose to newborn infants. Review of many studies suggests that BCG vaccination is protective against childhood meningeal tuberculosis and systemic forms of the disease. However, protective efficacy is variable (ranging from 0–80%) (2) against adult pulmonary disease, the major global cause of tuberculosis mortality, and wanes with time (3). The basis of the variability is uncertain. Recent evidence suggests that exposure to high levels of environmental mycobacteria may diminish the protective effects of BCG (4). Even so, 80% of infants throughout the world receive BCG each year (http://www.who.int/inf-fs/en/fact104.html). This implies that trials of future tuberculosis vaccine candidates will probably be performed in BCG-vaccinated communities. Moreover, these vaccines should aim to protect where BCG fails, namely, against adult pulmonary tuberculosis.

The development of a new vaccine against tuberculosis has been aided by increased understanding of the immune responses induced following *M. tuberculosis* infection. Approximately 90% of people infected with this pathogen do not develop active disease, indicating that the host protective response can normally control bacterial growth. Protection from tuberculosis is associated with the maintenance of a strong cell-mediated response to infection involving both $CD4^+$ and $CD8^+$ T cells and the ability to respond with Th1-type cytokines, particularly IFN-γ (5, 6). BCG vaccination induces IFN-γ-secreting T cells, predominately of the $CD4^+$ T cell phenotype, and many that cross-react with *M. tuberculosis* proteins (7). Recent studies suggest that BCG delivered parenterally may fail to induce T cell immune responses in the lung mucosa, which may be critical for protection against pulmonary disease (8, 9). These studies show that long-lived T cell populations in the mucosa are best achieved by mucosal delivery of vaccines. They support *M. tuberculosis* challenge studies in macaques and guinea pigs that have shown aerosol administration of BCG to be more protective than parenteral (p.) vaccination (10, 11). BCG has also been given as an aerosol to healthy adults and children and repeatedly to patients with metastatic lung disease (12–14). Respiratory function tests and postmortem examination of cancer patients administered BCG by aerosol indicated no significant pulmonary dysfunction or disseminated BCG infection (13, 14).

More recent strategies to induce enhanced T cell responses in tuberculosis vaccine research have harnessed recombinant DNA technology, using plasmid, bacterial, or viral vectors and recombinant protein to express *M. tuberculosis* Ags. Much attention has focused on the highly immunogenic Ag 85 complex. This is a family of proteins comprising Ags 85A, 85B, and 85C secreted by *M. tuberculosis*, BCG, and many other species of mycobacteria (15). Parenteral vaccination with either 85A or B as a protein with adjuvant or expressed by a plasmid or a viral vector has not

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[3] Abbreviations used in this paper: BCG, bacille Calmette-Guérin; MVA, modified vaccinia virus Ankara; CSP, circumsporozoite protein; FLN, facial lymph nodes; i.n., intranasal(ly); LLN, lung lymph nodes; p., parenteral(ly); PPD, purified protein derivative; P11, peptide 11; P15, peptide 15; SFC, spot-forming cells; TCH, thiophene-2-carboxylic acid hydrazide.

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achieved levels of protection greater than a single BCG immunization in animal models (16, 17).

We have previously shown that DNA85A vaccination boosted with a modified vaccinia virus Ankara (MVA) expressing Ag 85A (Ag85A) induced high frequencies of Ag85A-specific, IFN-γ-secreting T cells and afforded protection equivalent to BCG following *M. tuberculosis* challenge (18). Horwitz et al. (19) have recently developed a recombinant vaccine that elicited greater protection than low dose BCG in a guinea pig model. This vaccine is a recombinant BCG that overexpresses Ag 85B, suggesting that the inclusion of BCG may facilitate the design of future and more efficacious vaccines against tuberculosis.

In this study we investigate whether in a mouse *M. tuberculosis* aerosol challenge model, BCG-induced protection can be improved by mucosal lung delivery and/or boosting with a second dose of BCG or M.85A.

Materials and Methods

Animals and immunizations

Eight- to 10-wk-old female BALB/c (H-2^d) mice (Harlan Orlac, Blackthorn U.K.) were used in these studies. Generation of M.85A has been previously reported (18). Parenteral immunizations were given in the ear (25 μl/ear) or the left hind-footpad (50 μl). Intranasal (i.n.) immunizations were performed under light anesthesia (halothane), administered as a 100-μl bolus over 5 s. Immediately following i.n. BCG vaccination (Pasteur 1173P2D2) at doses of either 10^7 or 10^5 CFU, 1% of the total CFU delivered could be cultured from the lungs. In all the studies described, the MVA dose given was 10^6 PFU.

Cell culture and peptides

All cells were cultured in α-MEM (Life Technologies, Paisley, U.K.) supplemented with 10% FBS (Sigma-Aldrich, Watford, U.K.), 0.05 mM 2-ME (Life Technologies), 10 μM HEPES buffer (Life Technologies), 20 mM L-glutamine (Life Technologies), and 100 μg/ml penicillin/streptomycin (Life Technologies). The sequences of peptides used in assays are -P11-EWYDQSGLSV VMPVGGQSSF (aa 102–121) and P15-TFLTSELPGW LQANRHVKPT (aa 142–161) from Ag85A (GenBank accession no. CAA 17868), and PB9-SYIPSAEKI (aa 372–80) from circumsporozoite protein from *Plasmodium berghei* (GenBank accession no. AAA29541; Research Genetics, Huntsville, AL).

ELISPOT assays

Specific IFN-γ secretion by spleen, lung, or lymph node cell suspensions was assayed by ELISPOT following 18- to 20-h incubation of cells with Ag (20). Isolation of lymphocytes from perfused lungs has been described previously (21). The concentration of Ags used were purified protein derivative (PPD) at 10 μg/ml (SSI, Copenhagen, Denmark), Ag85A culture filtrate protein at 10 μg/ml (22), and individual peptides at 2 μg/ml. Results are expressed as the mean spot-forming cells per million cells (SFC/10^6 cells) ± SEM.

^{51}Cr release assays

Cells were stimulated with 2 μg/ml of peptide P11 and cultured for 7 days. Cells were fed 10 U/ml Lymphocult-T (Biotest, Dreieich, Germany) on day 3, harvested, then incubated for 4 h with ^{51}Cr-loaded P815 cells (TIB-64; American Type Culture Collection, Manassas, VA). Specific lysis was calculated as follows: 100 × [(experimental radioactivity − spontaneous radioactivity)/(total radioactivity − spontaneous radioactivity)]. Results are expressed as the mean percent specific lysis ± SEM.

M. tuberculosis *aerosol challenge*

M. tuberculosis strain H37Rv (Trudeau Mycobacterial Culture Collection 102) was grown in Dubos medium at 37°C for 21–28 days. The solution was centrifuged, suspended in a tryptic soy broth-glycerol, and stored at −70°C after titration. Stock solutions were sonicated before use.

Four weeks after the second immunization, mice were challenged by aerosol with *M. tuberculosis* using a modified Henderson apparatus, which was enclosed within a category III isolator (23). Mice were individually restrained to allow exposure of only the nose during challenge (ADG Developments, Hitchin, Hertfordshire, U.K.). Deposition in the lungs was estimated 24 h after challenge to be 250 CFU/lung. Six weeks after challenge, bacterial loads in the lungs and spleens were calculated by plating 10-fold serial dilutions of organ homogenates on both Middlebrook 7H11 agar and thiophene-2-carboxylic acid hydrazide (TCH; 5 μg/ml)-supplemented Middlebrook plates. TCH (Sigma-Aldrich, Poole, U.K.) at this concentration prevents BCG growth (24). Protection results are expressed as $\log_{10}$(mean CFU) ± SEM (n = 9–12).

Statistical analysis

The SPSS statistics package (SPSS, Chicago, IL) was used for all analysis. Student's t tests were used to compare the bacterial loads of different vaccinated groups following challenge. Linear regression analysis or Student's t tests were performed to compare T cell responses between groups in ELISPOT and chromium release assays.

Results

Ag 85A is immunodominant following BCG vaccination

The magnitude of PPD- and 85A-specific T cell responses induced by p. BCG vaccination was compared over the course of 1 year (Fig. 1). The level of response to 85A protein ranged between 20–60% of the overall PPD response. PPD and 85A protein T cell responses were abrogated by $CD4^+$ T cell depletion, but were not affected by $CD8^+$ T cell depletion (data not shown). Responses to Ag85A class I- and II-restricted epitopes (P11 and P15, respectively) (25) were also assayed. T cell responses specific for the major $CD4^+$ epitope in Ag85A, the P15 peptide, accounted for the majority of the T cell response to the whole protein at all time points. T cells specific for the $CD8^+$ epitope were not detected in either ELISPOT or chromium release assays performed on cultured splenocytes (data not shown).

Intranasal BCG immunization induces strong T cell responses in spleen, lung, and lung lymph nodes (LLN)

The frequency and localization of IFN-γ secreting T cells induced by either p. or i.n. delivered BCG were compared. Intranasal BCG and p. BCG vaccination produced comparable levels of PPD- and Ag85A peptide-specific $CD4^+$ T cell responses in the spleen (Fig. 2*A*), while p. BCG immunization in the ear produced specific IFN-γ secretion in the draining facial lymph nodes (FLN), intranasal delivery produced responses exclusively in the LLN, comprising cells pooled from the paratracheal and mediastinal lymph nodes (Fig. 2, *B* and *C*). No responses were detected in the inguinal lymph nodes, which drain the lower flanks, following either route of immunization (data not shown). Parenteral BCG immunization induced strong specific T cell responses in the lung (~300 SFC/10^6 to PPD stimulation), but i.n. BCG induced a 5-fold higher response (Fig. 2*D*). The total number of cells isolated from the lungs did not significantly differ between naive mice and mice given BCG by either route (data not shown). The specific lung

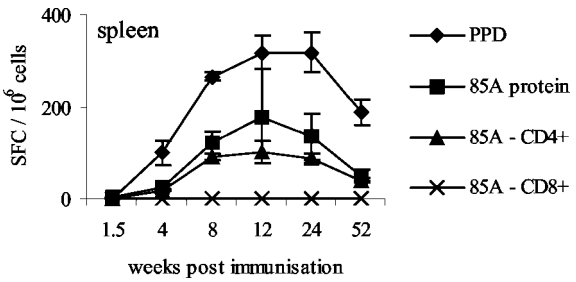

FIGURE 1. Ag 85A is immunodominant following BCG immunization. BALB/c mice (n = 3–6) were immunized with 5 × 10^5 CFU of BCG in the footpad. At the weeks indicated, spleens were assayed by ex vivo ELISPOT for responses to PPD, 85A protein, and 85A-$CD4^+$ (P15) and 85A-$CD8^+$ (P11) peptides. Responses are expressed as the mean number of IFN-γ-secreting SFC per 10^6 cells ± SEM.

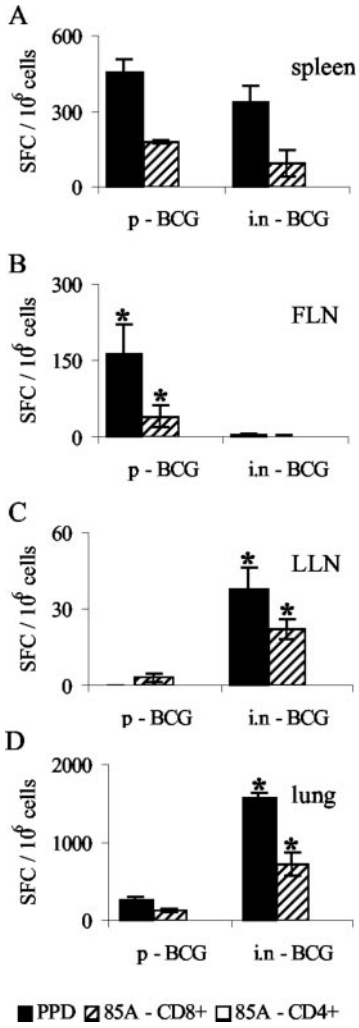

FIGURE 2. Intranasal BCG immunization induces higher frequencies of specific T cells in the lung compartment than p. BCG (p-BCG) immunization. BALB/c mice were immunized with 10^7 CFU of BCG either i.n. or p. in the ear. Twelve weeks later, spleens (*A*), FLN (*B*), draining LLN (*C*), and lungs (*D*) were assayed by ELISPOT for responses to PPD and the 85A-CD4$^+$ and 85A-CD8$^+$ epitopes. Spleens and lungs were assayed individually. Results for these organs are the mean ± SEM of two independent experiments with four mice per group. For the lymph node assays, nine mice were divided into three subgroups from which nodes were pooled. Results are expressed as the mean of the three subgroups ± SEM. *, Significant differences ($p < 0.05$) by Student's *t* tests in the induction of T cell responses by the different routes of vaccination.

immune responses induced by the different routes of vaccination paralleled the pattern observed in the LLN (Fig. 2*C*). This suggests that T cell responses in lung are compartmentalized and dependent on the route of vaccination. In summary, while both i.n. and p. BCG produce strong splenic T cell responses, i.n. BCG elicits strong long-lived T cell immunity in the LLN and significantly higher specific responses in the lung parenchyma.

M.85A boosts both CD4$^+$ and CD8$^+$ T cell responses induced by high dose BCG vaccination

We examined whether we could specifically boost the strong Ag85A T cell response induced by BCG with MVA expressing 85A (M.85A). Mice were boosted with M.85A 14 wk after p. BCG vaccination. Parenteral boosting with M.85A doubled the PPD response and increased the Ag85A-specific CD4$^+$ and CD8$^+$ T cell responses 4- and 5-fold, respectively, in the spleen (Fig. 3*A*). Immune responses in the FLN showed a similar pattern of boosting in PPD and Ag85A CD4$^+$ and CD8$^+$ peptide assays (data not shown). To determine whether BCG was specifically priming the Ag85A CD8$^+$ T cell response, parallel experiments were conducted in which mice were immunized with BCG and boosted with an irrelevant MVA expressing a malarial *P. berghei* circumsporozoite protein (M.CS) Ag (Fig. 3*B*). The frequency of CS-specific CD8$^+$ T cells was not significantly different between BCG/M.CS and M.CS-only groups, suggesting that the Ag85A CD8$^+$ T cell response (while undetectable following the BCG immunization) is specifically boosted by M.85A. Conversely, M.CS immunization did not affect the frequency of 85A-specific T cell responses induced by BCG (data not shown).

Similar to the wholly parenteral BCG/M.85A prime-boost regimen described above, parenteral M.85A boosting of i.n. BCG-immunized mice significantly increased the level of PPD and Ag85A CD4$^+$ and CD8$^+$ T cell responses in the spleen (Fig. 3*C*). Intranasal M.85A boosting of i.n. BCG immunized mice produced a 3- to 5-fold increase in the level of Ag85A CD4$^+$ T cells in the LLN (Fig. 3*D*), but did not significantly increase the Ag85A-specific T cell response in the spleen (Fig. 3*C*).

Parenteral BCG immunization, although a poor inducer of specific T cell responses in the LLN, did elicit specific T cell responses in the lung parenchyma (Fig. 2, *C* and *D*). We therefore examined whether T cells induced in the lung compartment by p. BCG immunization could be boosted by i.n. M.85A vaccination. Intranasal M.85A boosting did not significantly increase T cell responses in the spleen (data not shown). However, i.n. M.85A immunization did boost p. BCG-induced 85A CD4$^+$ T cell responses in the LLN from background levels to >125 SFC/10^6 cells (Fig. 3*E*).

M.85A boosts 85A-specific CD4$^+$, but not CD8$^+$, T cell responses following low dose BCG vaccination

M.85A boosting of 85A-specific T cells induced by vaccination with lower doses of BCG was investigated. Mice were immunized i.n. with BCG doses ranging from 10^2 to 10^6 CFU. Eight weeks later, groups were boosted either i.n. or p. with M.85A (Fig. 4, *A* and *B*). No PPD-specific T cell response was detected following vaccination with 10^2 and 10^3 CFU BCG (data not shown). T cell responses to PPD were detected after vaccination with 10^4 CFU BCG, but M.85A immunization did significantly increase 85A-specific CD4$^+$ or CD8$^+$ T cell responses (data not shown). The 85A CD4$^+$ T cell response was significantly boosted following vaccination with 10^5 and 10^6 CFU of BCG, but no boosting of the P11-specific CD8$^+$ T cell response was observed (compare with Fig. 3*C*). This suggests that lower doses of BCG either do not induce or inadequately induce 85A-specific CD8$^+$ T cells that can be expanded by subsequent M.85A vaccination.

Two doses of BCG afford greater protection than one in BALB/c mice

We tested whether the higher levels of specific IFN-γ produced by BCG/M.85A vaccination and the induction of specific T cells in the LLN produced by i.n. immunization impacted on the levels of protection against *M. tuberculosis* in an aerosol challenge model (Fig. 4). There was no significant difference in the number of mycobacterial colonies on plates with or without TCH, indicating no detectable BCG in the lungs or spleens 6 wk after challenge (data not shown). Compared with naive controls, significant protection was conferred in the lung, but not the spleen, by a single parenteral low dose of BCG administered 22 wk before challenge in BALB/c mice (lung, $p < 0.01$; spleen, $p = 0.84$). In previous studies BCG

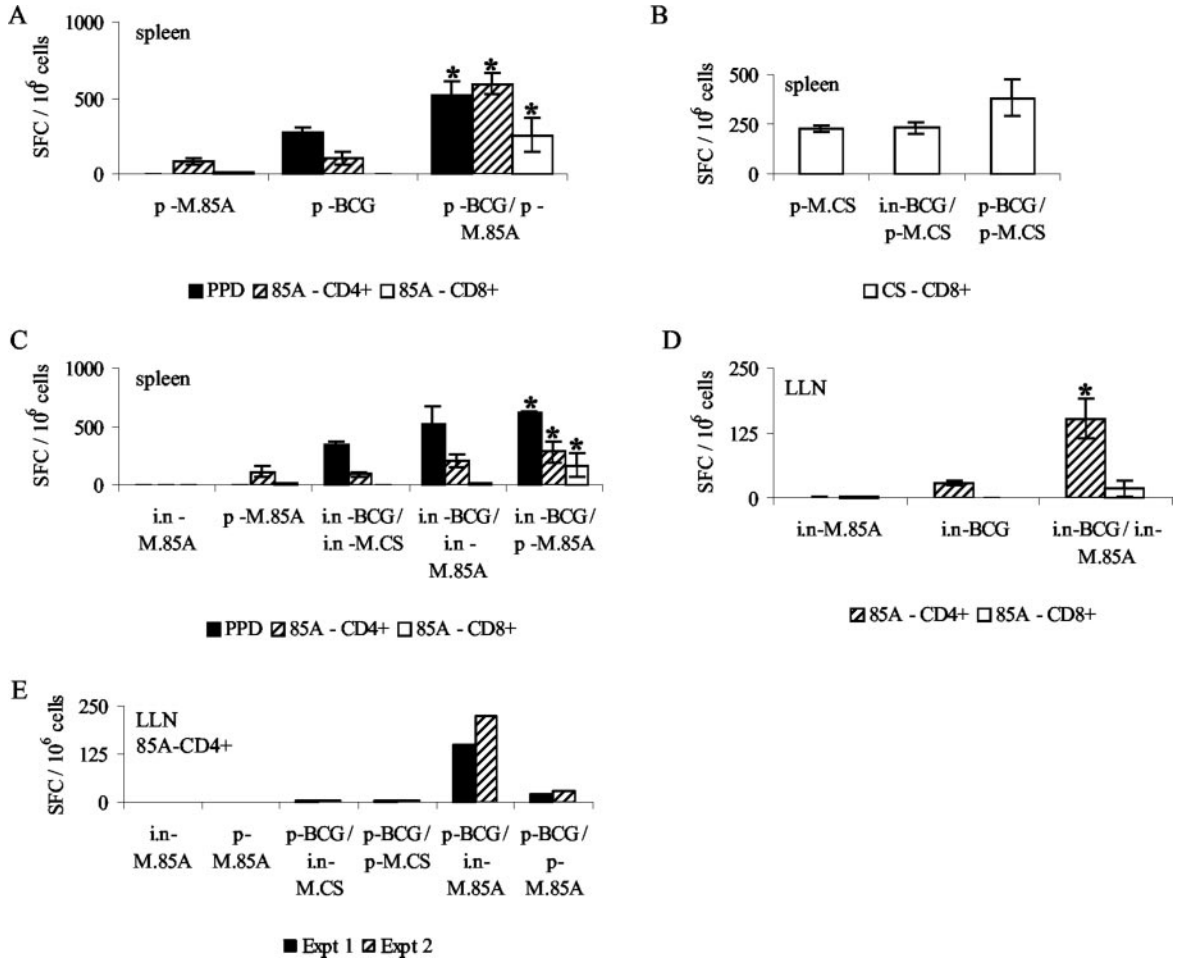

FIGURE 3. M.85A specifically boosts both 85A $CD4^+$ and $CD8^+$ T cell responses induced by BCG. BALB/c mice were p. immunized with 10^7 CFU of BCG. Fourteen weeks later, mice were p. boosted (25 μl/ear) with 10^6 PFU of M.85A (*A*) or 10^6 PFU of M.CS (*B*). M.85A- or BCG-only controls were included. Ten days after boost, spleens were assayed by IFN-γ ELISPOT. Results are the mean $\pm$ SEM of two experiments with three or four animals per group. Mice were immunized i.n. with 10^7 CFU of BCG and after 14 wk were boosted i.n. or p. with 10^6 PFU of M.85A. Ten days later, spleens (*C*) and LLN (*D*) were assayed by ELISPOT. Spleens were assayed individually, and results shown are representative of three experiments. For LLN, results are expressed as the mean $\pm$ SEM of LLN pooled from three or four mice from three experiments. *, $p < 0.05$, as determined by Student's *t* test comparisons between groups receiving BCG or M.85A only. Mice were immunized p. with 10^7 CFU of BCG and after 14 wk were boosted i.n. or p. (25 μl/ear) with 10^6 PFU of M.85A or M.CS. Ten days later LLN (*E*) were assayed by IFN-γ ELISPOT. LLN were pooled from three or four mice. Results show 85A-$CD4^+$-specific T cell responses in two independent experiments.

immunization 4 wk before aerosol challenge yielded highly significant protection in both spleen and lungs (data not shown), indicating that the protective effect of BCG in spleen diminishes with time (26). When mice were immunized with a second dose of BCG, protection in both lungs and spleens was highly significant compared with that in naive controls (lung, $p < 0.001$; spleen, $p = 0.001$) and that in the 1× p. BCG group (lung, $p = 0.022$; spleen, $p = 0.001$; Fig. 5, *A* and *B*).

Intranasal BCG offers greater protection than intradermal BCG in BALB/c mice

A single i.n. BCG immunization (0.5 log CFU lower than the parenteral dose) resulted in significant protection in the lung compared with the naive control group ($p < 0.001$), but this was not significantly different from the 1× p. BCG group ($p = 0.065$) (Fig. 5*A*). Numbers of bacilli in the spleen were similar in the i.n. BCG, p. BCG, and naive control groups (all comparisons, $p > 0.5$; Fig. 5*B*). When a second i.n. BCG immunization was given, protection in both lung and spleen was significantly improved compared with the naive, 1× i.n. BCG, and 1× p. BCG groups (all comparisons: lung, $p < 0.001$; spleen, $p < 0.001$). Two doses of i.n. BCG also showed significantly greater protection in the lung than immunization with 2× p. BCG ($p < 0.001$).

M.85A boosting of BCG provides comparable protection to two BCG immunizations and 2.5 log protection over naive group

Boosting i.n. BCG, either i.n. or p. with M.85A, produced significantly higher levels of protection against challenge compared with the 1× i.n. BCG group (Fig. 5). Protection in the i.n. M.85A-boosted group was striking, showing the same levels of protection as the 2× i.n. BCG group (lung, 2.5 logs; spleen, 1.5 logs). Compared with the i.n. BCG group, the mice boosted parenterally with M.85A did not show increased protection in the lung ($p = 0.494$; Fig. 5A). However, the level of protection in the spleen was significantly more than that in the 1× i.n. BCG group ($p < 0.05$) and was comparable to that in the 2× i.n. BCG and i.n. BCG/i.n. M.85A groups (Fig. 5*B*).

Protection in the lung correlates with detection of T cell responses in the LLN

On the day of challenge (4 wk after second immunization), four mice from each group were sacrificed for immunogenicity studies.

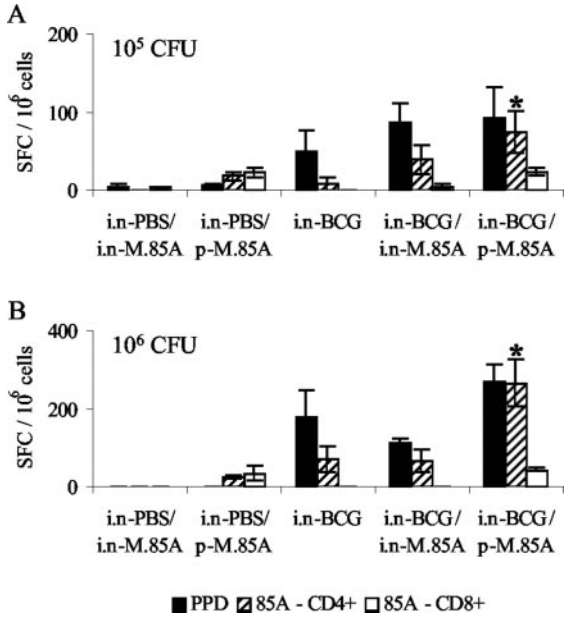

FIGURE 4. BCG induces 85A-specific $CD8^+$ T cells following high, but not low, dose vaccination. BALB/c mice were vaccinated i.n. with 10^5 (*A*) or 10^6 (*B*) CFU of BCG. After 8 wk, mice were boosted either i.n. or p. with M.85A (10^6 PFU). Ten days later, spleens were assayed for responses to PPD and 85A-$CD4^+$ and 85A-$CD8^+$ peptides by IFN-γ ELISPOT. Spleens were assayed individually, and results are expressed as the mean ± SEM (SFC per 10^6 cells). *, $p < 0.05$, as determined by Student's *t* test comparison to i.n. BCG- and p. M.85A-only groups.

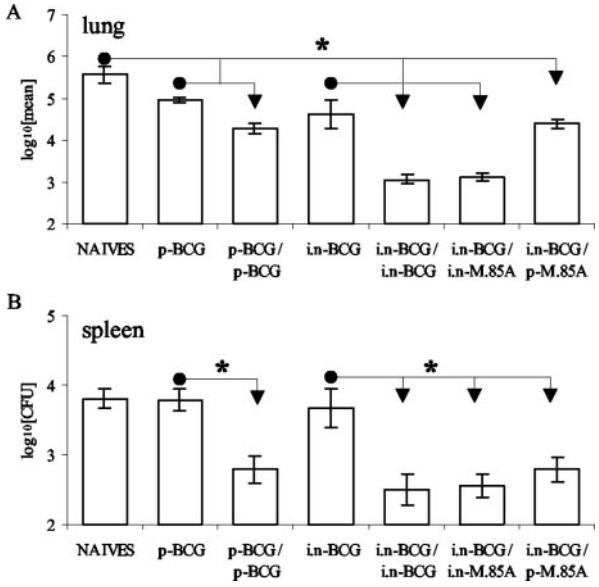

FIGURE 5. Protection of BALB/c mice against aerosol *M. tuberculosis* challenge is afforded by BCG boosting regimens. Mice were immunized with BCG given either i.n. (10^5 CFU) or p. in the footpad (5×10^5 CFU). Twenty-two weeks later, groups were boosted either i.n. or p. (footpad) with BCG or M.85A (10^6 PFU). Four weeks following the second immunization, mice ($n = 9$–12) were challenged with *M. tuberculosis* H37Rv (~250 CFU deposited into the lung). After 6 wk, lungs and spleens were cultured on Middlebrook agar plates containing TCH (5 μg/ml). Results are shown for lung and spleens in *A* and *B*, respectively. Results are expressed as the mean ($\log_{10}$CFU) ± SEM. *, Significant differences ($p < 0.05$) by Student's *t* tests in bacterial load between the various groups clustered by arrows. The origin of the arrow (circle) designates the reference group with which statistical comparisons were made.

Only the 2× i.n. BCG and i.n. BCG/i.n. M.85A groups had detectable T cell responses to the 85A $CD4^+$ T cell epitope in their LLN (Fig. 6*A*). These groups showed the highest levels of protection in both lungs and spleen following challenge, which indicates that Ag85A-specific T cell responses in the LLN correlate better with protection in the lung than splenic immune responses. No T cell response was detected in the LLN to the 85A $CD8^+$ P11 peptide in any group (Fig. 6*A*), most likely because the BCG dose used in the challenge experiment was too low to induce a specific $CD8^+$ T cell response (Fig. 4).

Some differences were observed between groups in the splenic assays. While all groups produced comparable responses in ELISPOT to PPD (data not shown), the Ag85A $CD4^+$ T cell response to P15 in the i.n. BCG/i.n. M.85A and i.n. BCG/p. M.85A groups were increased 2- and 3-fold, respectively, over that in the 1× i.n. BCG group (Fig. 6*B*). Responses to the P11 $CD8^+$ T cell epitope were detectable at low levels in the i.n. BCG/p. M.85A group in ELISPOT (data not shown). In ^{51}Cr release assays performed on cultured splenocytes, only this group produced significant levels of specific lysis (30%) of P11-pulsed targets (Fig. 6*C*). Again, because of the lower BCG dose used in the challenge experiment, the detection of 85A-specific $CD8^+$ T cells in the spleen was most likely induced by M.85A vaccination alone.

Discussion

In this study the BALB/c mouse model was used to examine two strategies to increase BCG-mediated protection against *M. tuberculosis* infection: 1) boosting BCG-induced T cell responses with either M.85A or a second dose of BCG, and 2) eliciting Ag85A-specific mucosal T cell responses by i.n. immunization.

Recombinant MVA effectively induces both $CD4^+$ and $CD8^+$ IFN-γ-secreting T cells to foreign Ags, including Ag85A, and is particularly effective as a boosting vector (18, 20, 27). Nonrecombinant MVA has been administered, without significant side effects, to >100,000 people as part of the smallpox eradication program (28). A recombinant MVA encoding malaria Ags has been administered safely to >200 volunteers (A. V. S. Hill et al., unpublished observations). In this study M.85A immunization boosted Ag85A-specific $CD4^+$ and $CD8^+$ T cell pools induced by BCG, demonstrating that a recombinant virus can also boost T cell responses induced by a bacterial prime. $CD4^+$ T cells have a clear role in controlling progression to tuberculosis disease in man (29). While Ag85A-specific $CD8^+$ T cell lines have been derived from BCG vaccinees and healthy *M. tuberculosis*-exposed individuals, the exact role of $CD8^+$ T cells in human tuberculosis remains to be defined. (30). A recent report suggests that in mice, $CD8^+$ T cells protect more against reactivation tuberculosis than $CD4^+$ T cells (31). This implies that vaccination regimens that can effectively boost low level *M. tuberculosis*-specific $CD8^+$ T as well as $CD4^+$ T cell responses, such as MVA, may be particularly effective in maintaining long term protection from disease.

The protective effects of one vs two doses of BCG given either p. or i.n. were compared. In BALB/c mice, regardless of the route of delivery, two immunizations were more protective than one in lungs and spleens. In humans, repeated intradermal BCG immunization did not provide any greater protection against pulmonary tuberculosis regardless of age at time of boost or time between BCG immunizations in Malawi (32). However, in the region examined, a single BCG immunization conferred no protection against pulmonary tuberculosis. Multiple BCG doses or indeed boosting with M.85A may still provide some benefit in a population in which a single BCG immunization is partially protective.

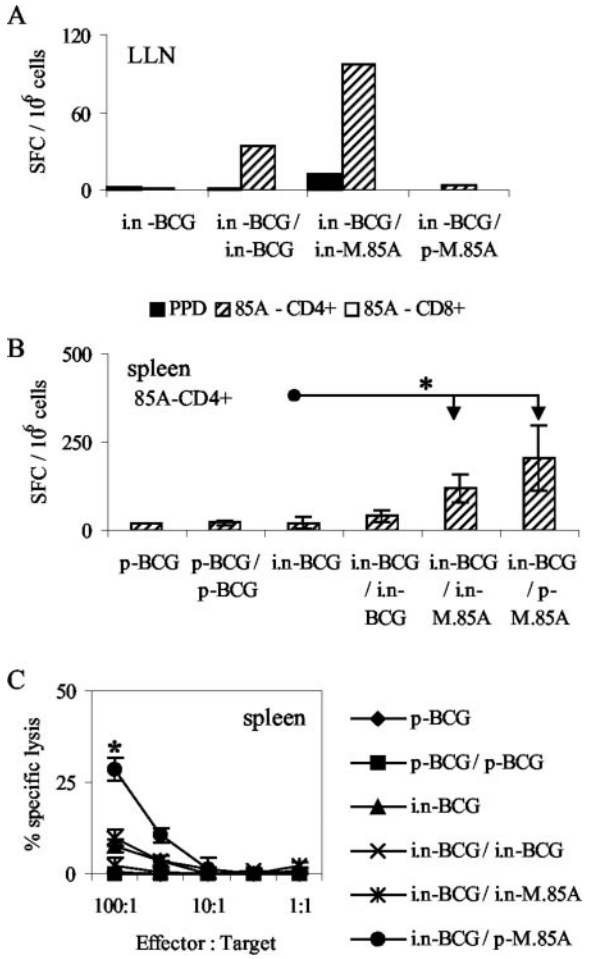

FIGURE 6. Protection against aerosol *M. tuberculosis* correlates with the presence of specific IFN-γ-secreting $CD4^+$ T cells in the LLN. Additional mice ($n = 4$) were sacrificed at the time of challenge, and pooled LLN and spleens were assayed for T cell responses to PPD, 85A-$CD4^+$ and 85A-$CD8^+$ peptides in IFN-γ ELISPOT (*A* and *B*). The results are expressed as SFC per 10^6 cells. In *B*, ∗ indicates a significant differences ($p < 0.05$) by Student's *t* test in SFC frequency between the groups clustered by arrows. The origin of the arrow (circle) designates the reference group with which statistical comparisons were made. Splenocytes from each mouse were cultured with 85A-$CD8^+$ peptide and assayed in a ^{51}Cr release assay (*C*). Results are expressed as the percentage of specific lysis ± SEM. ∗, $p < 0.05$, as determined by linear regression analysis comparison of all groups.

We investigated whether protection from pulmonary tuberculosis could be improved through eliciting lung-localized T cell populations following mucosal delivery of tuberculosis vaccines. We showed that while i.n. BCG was as effective as p. BCG in inducing peripheral T cell responses, the induction of responses in lymph nodes is highly dependent on the route of immunization. Intranasal delivery of BCG elicited robust responses in the LLN that could be further boosted by i.n. immunization with M.85A. When these vaccination regimens were tested in an aerosol *M. tuberculosis* challenge, a single dose of i.n. BCG gave the same level of protection as p. BCG. In mice that were immunized intranasally with BCG and again boosted i.n. with either a second BCG dose or M.85A, the levels of protection in the lungs and spleens were both significantly higher than those with a single BCG immunization. To our knowledge this level of protection (2.5 logs) has not previously been reported in murine vaccination studies.

At the time of challenge only the i.n. prime/i.n. boost vaccination regimens produced detectable responses in LLN and the highest levels of protection. The level of splenic T responses did not correlate with the strong protection observed in these groups. These data suggest that the frequency of Ag-specific, IFN-γ-secreting T cells in the LLN may constitute a better indicator of protection in the lung than systemic responses against pulmonary tuberculosis. We suggest two explanations for this observation. Firstly, in mice that have been infected aerogenically with *M. tuberculosis*, bacilli disseminate first to the LLN, possibly via dendritic cells, and then to the periphery (33, 34). The induction of *M. tuberculosis*-specific T cells follows dissemination of bacilli. The responses are induced first in the LLN (but not in other LN compartments) and later in the spleen (33). The strong protection observed in both lungs and spleens in the i.n. primed/i.n. boosted groups may have resulted from the microbicidal actions of resident *M. tuberculosis*-specific T cells in the LLN limiting the dissemination of bacilli back to the lungs and periphery. Whether *M. tuberculosis* dissemination from the lung parenchyma to the LLN also occurs in humans is unknown, but in both symptomatic and asymptomatic individuals pathology is often found in the hilar lymph nodes. Alternatively, we showed a correlation between the frequency of 85A-specific T cells in the lung and LLN, implying that Ag-specific T cells in LLN may reflect lymphatic drainage of protective T cell populations resident in the lung (35, 36). Other murine studies have shown a correlation between an influx of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells into the lung during acute infection and protection from tuberculosis (37–39). In humans, Condos et al. (40) showed that the broncheo-alveolar lavage of patients with less clinically advanced pulmonary tuberculosis contained higher levels of IFN-γ-secreting lymphocytes than patients with advanced tuberculosis who conversely had increased numbers of neutrophils in their broncheo-alveolar lavage.

The acute *M. tuberculosis* challenge model used here did not in itself distinguish between the utility of BCG and M.85A as boosting agents after BCG priming. Previous studies have shown that BCG is still recoverable from the lung, albeit in very low numbers, 1 mo after lung immunization (41). At this time point, histological analysis showed an ongoing inflammatory response in the lungs, and the numbers of macrophages, neutrophils, NK cells, and antimicrobial mediators such as IL-12 and TNF-α in lung lavage were elevated (41, 42). The level of protection observed following challenge of the 2× i.n. BCG group could be due in part to ongoing innate immune reactions induced by the residual BCG in the lungs. In contrast, it is unlikely that innate immune mechanisms contributed to the protection observed in the i.n. BCG/i.n. M.85A group. Unlike BCG, the MVA virus does not persist in rodent lungs (43). In cotton rats that are highly susceptible to vaccinia virus infection, MVA could not be detected in lungs 5 days after i.n. immunization, and no pathology was observed. Similarly, i.n. immunization of BALB/c mice with 5×10^7 PFU (1.5 logs greater that the vaccination dose in this study) of nonrecombinant MVA did not provide any protection from challenge with the lung pathogens, respiratory syncytial virus or parainfluenza virus (44, 45). In those studies, groups of mice vaccinated with recombinant MVA expressing respiratory syncytial virus or parainfluenza virus proteins were protected following i.n. challenge 1 mo after vaccination.

While the i.n. prime/i.n. boost vaccination regimen was highly protective in this study, was i.n. delivery of the vaccines necessary for both the prime and the boost? A single i.n. BCG vaccination induced significantly higher T cell responses in the lung than p. BCG. Even so, the frequency of T cells in the lung elicited by p. BCG vaccination was sufficient to afford protection equivalent to that of i.n. BCG in the lungs. A similar observation was recently

reported by Palendira et al. (46). These results conflict with earlier studies in other animal models in which aerosolized BCG afforded better protection than parenterally delivered BCG (10, 11). In these early studies the time between vaccination and challenge was relatively short, and as described above, any ongoing inflammatory reaction in the lung may have contributed to increased levels of protection. In our study and that by Palendira et al. (46), BCG was eliminated from the lung by resting the animals after BCG vaccination (22 wk) or by antibiotic treatment. Palendira et al. (46) also showed that regardless of the route of BCG vaccination, the recruitment of IFN-γ-secreting $CD4^+$ T cells to the lung following aerogenic *M. tuberculosis* challenge was similar. This suggests, in this model at least, that following a single immunization of BCG, protection is independent of route of vaccination. Studies in viral systems have similarly shown that following a single i.n. immunization, T cell responses first develop in the LLN and then extravasate stochastically from the blood to organs such as spleens and lungs (9, 36, 47). When the animals were challenged i.n., the presence of Ag drew T cells back to the lung (9, 36, 46, 48). Applying these observations to the prime-boost regimen in this study, an i.n. M.85A boost of BCG-immunized mice will express Ag85A in the lung and not only expand Ag85A-specific T cells resident in the respiratory compartment, but also pull in memory T cells from the periphery (49). In support of this, we have observed that i.n. boosting with M.85A of p. BCG-immunized mice strongly increases Ag85A $CD4^+$ T cell responses in the LLN (9). This view is not in conflict with the wealth of vaccinology publications showing that mucosal vaccination affords longer lasting and greater protection than parenteral vaccination from mucosal pathogens (reviewed in Ref. 50). In the majority of these studies vaccination regimens have involved repeated mucosal vaccination, each time not only boosting, but also targeting, memory T cells to the mucosal tissue.

These data suggest that the induction of T cell responses in the respiratory compartment is important in protection from pulmonary disease. Even so, strong peripheral T cell responses remain central to protection in the spleen. Parenteral M.85A boosting of mice immunized with i.n. BCG increased the level of protection in the spleen, but not the lung. No Ag85A-specific immune responses were detected in the i.n. BCG/p. M.85A group in the LLN. However, splenic immune responses in this group were significantly higher than those in other groups. Parenteral immunization of recombinant MVA has been previously reported to elicit strong lytic T cells responses in the periphery, but not the mucosa (9). Together this suggests that p. boosting of BCG-immunized mice with M.85A expanded peripheral $CD4^+$ T cell populations that, following challenge, better controlled the levels of *M. tuberculosis* bacilli that had disseminated from the LLN. In humans, p. BCG vaccination is protective against systemic forms of disease, and p. BCG vaccination in rhesus macaques appears to protect from hemogenous spread of bacilli (2, 51). In summary, while LLN T cell responses may correlate with protection in the lung, specific T cell responses in the spleen may constitute a good indicator of protection from systemic forms of disease.

In the clinic, regardless of the route of delivery, M.85A boosting may be of more benefit than multiple BCG vaccination for several reasons. Increasing evidence suggests that exposure to environmental mycobacteria has a negative effect on BCG-induced protection (4). Unlike BCG and as outlined above, the strong immunogenicity and protective capacity of M.85A occurs without any viral replication and therefore is very unlikely to be compromised by exposure to environmental mycobacteria (52). In addition, MVA has been given without adverse effects to severely immunocompromised primates, suggesting that it would carry less risk in HIV-positive individuals than subsequent vaccinations with live BCG (53). We show here that following a BCG immunization M.85A expands the low level, Ag85A-specific $CD8^+$ T cell response as well as $CD4^+$ T cell responses. We are investigating vaccination regimens in mice and macaques to further augment the induction of 85A-specific $CD8^+$ T cells following vaccination at lower BCG doses. The ability to boost $CD8^+$ T cell responses with recombinant viruses should provide an advantage, particularly for long term protection, over multiple BCG immunizations and possibly over other subunit vaccination regimens that induce T cell responses that are predominately $CD4^+$ restricted. We have now begun clinical studies to investigate the potential of a BCG/M.85A prime-boost immunization to improve protection against pulmonary tuberculosis.

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References

1. Calmette, A., and C. Guérin. 1924. Vaccination des bovides contre la tuberculose et methode nouvelle de prophylaxie de la tuberculose bovine. *Ann. Inst. Pasteur. 38:371.*
2. Colditz, G. A., T. F. Brewer, C. S. Berkey, M. E. Wilson, E. Burdick, H. V. Fineberg, and F. Mosteller. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis: meta-analysis of the published literature. *JAMA 271:698.*
3. Sterne, J. A., L. C. Rodrigues, and I. N. Guedes. 1998. Does the efficacy of BCG decline with time since vaccination? *Int. J. Tuberc. Lung Dis. 2:200.*
4. Fine, P. E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet 346:1339.*
5. Dupuis, S., R. Doffinger, C. Picard, C. Fieschi, F. Altare, E. Jouanguy, L. Abel, and J. L. Casanova. 2000. Human interferon-γ-mediated immunity is a genetically controlled continuous trait that determines the outcome of mycobacterial invasion. *Immunol. Rev. 178:129.*
6. Flynn, J. L., and J. Chan. 2001. Immunology of tuberculosis. *Annu. Rev. Immunol. 19:93.*
7. Lalvani, A., R. Brookes, R. J. Wilkinson, A. S. Malin, A. A. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and A. V. Hill. 1998. Human cytolytic and interferon γ-secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA 95:270.*
8. Gallichan, W. S., and K. L. Rosenthal. 1996. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. *J. Exp. Med. 184:1879.*
9. Belyakov, I. M., B. Moss, W. Strober, and J. A. Berzofsky. 1999. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. *Proc. Natl. Acad. Sci. USA 96:4512.*
10. Barclay, W. R., W. M. Busey, D. W. Dalgard, R. C. Good, B. W. Janicki, J. E. Kasik, E. Ribi, C. E. Ulrich, and E. Wolinsky. 1973. Protection of monkeys against airborne tuberculosis by aerosol vaccination with bacillus Calmette-Guérin. *Am. Rev. Respir. Dis. 107:351.*
11. Gheorghiu, M. 1994. BCG-induced mucosal immune responses. *Int. J. Immunopharmacol. 16:435.*
12. Rosenthal, S. R., J. T. McEnery, and N. Raisys. 1968. Aerogenic BCG vaccination against tuberculosis in animal and human subjects. *J. Asthma Res. 5:309.*
13. Cusumano, C. L., J. A. Jernigan, and R. H. Waldman. 1975. Aerosolized BCG (Tice strain) treatment of bronchogenic carcinoma: phase I study. *J. Natl. Cancer Inst. 55:275.*
14. Garner, F. B., C. A. Meyer, D. S. White, and A. Lipton. 1975. Aerosol BCG treatment of carcinoma metastatic to the lung: a phase I study. *Cancer 35:1088.*
15. Harth, G., B. Y. Lee, J. Wang, D. L. Clemens, and M. A. Horwitz. 1996. Novel insights into the genetics, biochemistry, and immunocytochemistry of the 30-kilodalton major extracellular protein of *Mycobacterium tuberculosis*. *Infect. Immun. 64:3038.*
16. Lee, B. Y., and M. A. Horwitz. 1999. T-cell epitope mapping of the three most abundant extracellular proteins of *Mycobacterium tuberculosis* in outbred guinea pigs. *Infect. Immun. 67:2665.*
17. Huygen, K., J. Content, O. Denis, D. L. Montgomery, A. M. Yawman, R. R. Deck, C. M. DeWitt, I. M. Orme, S. Baldwin, C. D'Souza, et al. 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat. Med. 2:893.*
18. McShane, H., S. Behboudi, N. Goonetilleke, S. Gilbert, R. Brookes, and A. Hill. 2002. Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cvells pulsed with both $CD8^+$- and $CD4^+$-T-cell epitopes from antigen 85A. *Infect. Immun. 70:1623.*
19. Horwitz, M. A., G. Harth, B. J. Dillon, and S. Maslesa-Galic. 2000. Recombinant bacillus calmette-guerin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity

against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc. Natl. Acad. Sci. USA 97:13853.*
20. Schneider, J., S. C. Gilbert, T. J. Blanchard, T. Hanke, K. J. Robson, C. M. Hannan, M. Becker, R. Sinden, G. L. Smith, and A. V. Hill. 1998. Enhanced immunogenicity for $CD8^+$ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat. Med. 4:397.*
21. Gonzalez-Juarrero, M., O. C. Turner, J. Turner, P. Marietta, J. V. Brooks, and I. M. Orme. 2001. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *Mycobacterium tuberculosis*. *Infect. Immun. 69:1722.*
22. De Bruyn, J., K. Huygen, R. Bosmans, M. Fauville, R. Lippens, J. P. Van Vooren, P. Falmagne, M. Weckx, H. G. Wiker, M. Harboe, et al. 1987. Purification, characterization and identification of a 32 kDa protein antigen of *Mycobacterium bovis* BCG. *Microb. Pathog. 2:351.*
23. Phillpotts, R. J., T. J. Brooks, and C. S. Cox. 1997. A simple device for the exposure of animals to infectious microorganisms by the airborne route. *Epidemiol. Infect. 118:71.*
24. Grange, J., M. Yates, and I. de Kantor. 1996. Guidelines for speciation within the *Mycobacterium tuberculosis* complex. WHO, Geneva, pp. 1–23.
25. Denis, O., A. Tanghe, K. Palfliet, F. Jurion, T. P. van den Berg, A. Vanonckelen, J. Ooms, E. Saman, J. B. Ulmer, J. Content, et al. 1998. Vaccination with plasmid DNA encoding mycobacterial antigen 85.A stimulates a $CD4^+$ and $CD8^+$ T-cell epitopic repertoire broader than that stimulated by *Mycobacterium tuberculosis* H37Rv infection. *Infect. Immun. 66:1527.*
26. Brooks, J. V., A. A. Frank, M. A. Keen, J. T. Bellisle, and I. M. Orme. 2001. Boosting vaccine for tuberculosis. *Infect. Immun. 69:2714.*
27. Hanke, T., T. J. Blanchard, J. Schneider, C. M. Hannan, M. Becker, S. C. Gilbert, A. V. Hill, G. L. Smith, and A. McMichael. 1998. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine 16:439.*
28. Stickl, H. A. 1974. Smallpox vaccination and its consequences: first experiences with the highly attenuated smallpox vaccine "MVA." *Prev. Med. 3:97.*
29. Rieder, H. L., G. M. Cauthen, G. W. Comstock, and D. E. Snider, Jr. 1989. Epidemiology of tuberculosis in the United States. *Epidemiol. Rev. 11:79.*
30. Smith, S. M., M. R. Klein, A. S. Malin, J. Sillah, K. Huygen, P. Andersen, K. P. McAdam, and H. M. Dockrell. 2000. Human $CD8^+$ T cells specific for *Mycobacterium tuberculosis* secreted antigens in tuberculosis patients and healthy BCG-vaccinated controls in The Gambia. *Infect. Immun. 68:7144.*
31. van Pinxteren, L. A., J. P. Cassidy, B. H. Smedegaard, E. M. Agger, and P. Andersen. 2000. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur. J. Immunol. 30:3689.*
32. Karonga Prevention Trial Group. 1996. Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed *Mycobacterium leprae* vaccine for prevention of leprosy and tuberculosis in Malawi: Karonga Prevention Trial Group. *Lancet 348:17.*
33. Chackerian, A. A., J. M. Alt, T. V. Perera, C. C. Dascher, and S. M. Behar. 2002. Dissemination of *Mycobacterium tuberculosis* is influenced by host factors and precedes the initiation of T-cell immunity. *Infect. Immun. 70:4501.*
34. Jiao, X., R. Lo-Man, P. Guermonprez, L. Fiette, E. Deriaud, S. Burgaud, B. Gicquel, N. Winter, and C. Leclerc. 2002. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. *J. Immunol. 168:1294.*
35. Lehmann, C., A. Wilkening, D. Leiber, A. Markus, N. Krug, R. Pabst, and T. Tschernig. 2001. Lymphocytes in the bronchoalveolar space reenter the lung tissue by means of the alveolar epithelium, migrate to regional lymph nodes, and subsequently rejoin the systemic immune system. *Anat. Rec. 264:229.*
36. Topham, D. J., M. R. Castrucci, F. S. Wingo, G. T. Belz, and P. C. Doherty. 2001. The role of antigen in the localization of naive, acutely activated, and memory $CD8^+$ T cells to the lung during influenza pneumonia. *J. Immunol. 167:6983.*
37. Chackerian, A. A., T. V. Perera, and S. M. Behar. 2001. γ interferon-producing $CD4^+$ T lymphocytes in the lung correlate with resistance to infection with *Mycobacterium tuberculosis*. *Infect. Immun. 69:2666.*
38. Serbina, N. V., and J. L. Flynn. 1999. Early emergence of $CD8^+$ T cells primed for production of type 1 cytokines in the lungs of *Mycobacterium tuberculosis*-infected mice. *Infect. Immun. 67:3980.*
39. Lyadova, I. V., E. B. Eruslanov, S. V. Khaidukov, V. V. Yeremeev, K. B. Majorov, A. V. Pichugin, B. V. Nikonenko, T. K. Kondratieva, and A. S. Apt. 2000. Comparative analysis of T lymphocytes recovered from the lungs of mice genetically susceptible, resistant, and hyperresistant to *Mycobacterium tuberculosis*-triggered disease. *J. Immunol. 165:5921.*
40. Condos, R., W. N. Rom, Y. M. Liu, and N. W. Schluger. 1998. Local immune responses correlate with presentation and outcome in tuberculosis. *Am. J. Respir. Crit. Care Med. 157:729.*
41. Wakeham, J., J. Wang, and Z. Xing. 2000. Genetically determined disparate innate and adaptive cell-mediated immune responses to pulmonary *Mycobacterium bovis* BCG infection in C57BL/6 and BALB/c mice. *Infect. Immun. 68:6946.*
42. Wang, J., J. Wakeham, R. Harkness, and Z. Xing. 1999. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J. Clin. Invest. 103:1023.*
43. Weidinger, G., M. Ohlmann, B. Schlereth, G. Sutter, and S. Niewiesk. 2001. Vaccination with recombinant modified vaccinia virus Ankara protects against measles virus infection in the mouse and cotton rat model. *Vaccine 19:2764.*
44. Wyatt, L. S., S. S. Whitehead, K. A. Venanzi, B. R. Murphy, and B. Moss. 1999. Priming and boosting immunity to respiratory syncytial virus by recombinant replication-defective vaccinia virus MVA. *Vaccine 18:392.*
45. Wyatt, L. S., S. T. Shors, B. R. Murphy, and B. Moss. 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine 14:1451.*
46. Palendira, U., A. G. Bean, C. G. Feng, and W. J. Britton. 2002. Lymphocyte recruitment and protective efficacy against pulmonary mycobacterial infection are independent of the route of prior *Mycobacterium bovis* BCG immunization. *Infect. Immun. 70:1410.*
47. Belz, G. T., J. D. Altman, and P. C. Doherty. 1998. Characteristics of virus-specific $CD8^+$ T cells in the liver during the control and resolution phases of influenza pneumonia. *Proc. Natl. Acad. Sci. USA 95:13812.*
48. Walzl, G., S. Tafuro, P. Moss, P. J. Openshaw, and T. Hussell. 2000. Influenza virus lung infection protects from respiratory syncytial virus-induced immunopathology. *J. Exp. Med. 192:1317.*
49. Eo, S. K., M. Gierynska, A. A. Kamar, and B. T. Rouse. 2001. Prime-boost immunization with DNA vaccine: mucosal route of administration changes the rules. *J. Immunol. 166:5473.*
50. Berzofsky, J. A., J. D. Ahlers, and I. M. Belyakov. 2001. Strategies for designing and optimizing new generation vaccines. *Nat. Rev. Immunol. 1:209.*
51. Langermans, J. A., P. Andersen, D. van Soolingen, R. A. Vervenne, P. A. Frost, T. van der Laan, L. A. van Pinxteren, J. van den Hombergh, S. Kroon, I. Peekel, et al. 2001. Divergent effect of bacillus Calmette-Guérin (BCG) vaccination on *Mycobacterium tuberculosis* infection in highly related macaque species: implications for primate models in tuberculosis vaccine research. *Proc. Natl. Acad. Sci. USA 98:11497.*
52. Ramirez, J. C., M. M. Gherardi, and M. Esteban. 2000. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. *J. Virol. 74:923.*
53. Stittelaar, K. J., T. Kuiken, R. L. de Swart, G. van Amerongen, H. W. Vos, H. G. Niesters, P. van Schalkwijk, T. van der Kwast, L. S. Wyatt, B. Moss, et al. 2001. Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. *Vaccine 19:3700.*