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Synergistic Effect of Bacillus Calmette Guerin and a Tuberculosis Subunit Vaccine in Cationic Liposomes: Increased Immunogenicity and Protection

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In the present work, we evaluated a new TB vaccine approach based on a combination of the Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccine and a subunit vaccine consisting of the proteins Ag85B and ESAT-6. We demonstrate that in addition to its vaccine efficacy BCG is an immune modulator that can potentiate a Th1 immune response better than the well-known adjuvant mono phosphoryl lipid A, leading to enhanced recognition of the subunit vaccine Ag85B-ESAT-6. Importantly, adding a vehicle to the vaccine, such as the cationic liposome dimethyl dioctadecyl ammonium bromide (DDA), significantly increased the potentiating effect of BCG. This synergistic effect between BCG and Ag85B-ESAT-6/liposome required drainage to the same lymph node of all vaccine components but did not require direct mixing of the components and was therefore also observed when BCG and Ag85B-ESAT-6/liposome were given as separate injections at sites draining to the same lymph node. The resulting optimized vaccine protocol consisting of BCG and subunit in liposomes (injected side by side) followed by boosting with the subunit in conventional adjuvant resulted in an impressive increase in the protective efficacy of up to 7-fold compared with BCG alone and 3-fold compared with unaugmented BCG boosted by the subunit vaccine. Thus, these studies suggest an immunization strategy where a novel TB subunit vaccine is administered as part of the child vaccination program together with BCG in neonates and followed by subunit boosting. The Journal of Immunology, 2007, 178: 3721–3730.

Tuberculosis (TB) is a re-emerging disease that remains one of the leading causes of morbidity and mortality in humans and it represents a major public health problem in many countries. The current vaccine against Mycobacterium tuberculosis (M.tb) is Mycobacterium bovis bacillus Calmette-Guérin (BCG). Since 1921 when BCG was introduced, >3 billion people have received BCG, which makes this vaccine the most widely used in the world. It is cheap and safe and protects children efficiently against the early manifestations of TB (1–4). However, estimates of protection against adult pulmonary TB have ranged from 0 to 80%, and the vaccine has limited activity in individuals sensitized by environmental mycobacteria, latent TB, or prior BCG vaccination. Thus, an improved second-generation TB vaccine is urgently needed.

Current attempts to improve BCG center around either influencing the type of immune response induced by BCG or by increasing the immune response against selected mycobacterial Ags. Thus, one approach to increase the efficacy of BCG (and the CD8 response) is the urease C-deficient Hly-secreting BCG-vaccine strain (ΔureC hly"rBCG) that secretes listeriolysin (Hly) of Listeria monocytogenes with the goal of improving Ag release from the lysosome for cross-priming (5, 6). Another approach to improve BCG involves the addition of genes coding for proteins known to be protective when used as subunit vaccines. Compared with M.tb, ~129 open reading frames are absent from various strains of BCG. These proteins may contain important immunodominant protective T cell targets in M.tb that are not provided by BCG vaccination. One such region (designated RD1), containing 9 open reading frames, has been studied to greater extent than the rest. RD1 contains several genes of interest, some of which have been shown to induce protection against M.tb (7–10). Interestingly, BCG expressing the RD1 region did in fact induce increased protection compared with BCG (11), indicating that overexpressing some of the RD1-encoded proteins may have a beneficial effect on the protection against M.tb. However, overexpression of the RD1 encoded protein ESAT-6 produced mixed results, ranging from minor to no increase in protection against M.tb compared with BCG, even though ESAT-6, administered as a subunit vaccine, has been shown to induce protection against M.tb (7, 11, 12). Overexpression of immunodominant proteins shared between BCG and M.tb has also been tested. Thus, BCG expressing and secreting the Ag85B protein (rBCG30) induced stronger protective immunity against aerosol challenge with M.tb than conventional BCG vaccine in mice, guinea pigs, and cattle (13–16). In addition, mice vaccinated with BCG overexpressing the 38-kDa protein contained half the amount of M.tb bacteria in the lung 120 days postinfection as BCG-vaccinated mice, whereas at earlier time points no difference in bacterial numbers were observed (17). Finally, supplementing BCG with the M.tb 72-kDa fusion protein Mt72F increased protection against infection with M.tb in guinea pigs (18). None of these studies, however, have addressed the question of how BCG affects the immune response against these target Ags.
Several previous studies have reported that BCG can act as an activator of innate immunity and thus as an adjuvant when coadministered with vaccine Ags. This BCG-induced adjuvant effect, or stimulation of innate immunity, has been exploited by generating recombinant BCG-expressing vaccine Ags against other diseases, such as malaria or diphtheria-pertussis (19, 20), or by co-injecting BCG with vaccines against infectious agents such as *schistosoma japonicum* (21) or *Leishmania spp.* (22). In addition, BCG is commonly used in immunotherapy of solid tumors (23, 24). In particular, intravesical immunotherapy with BCG is the most effective treatment for preventing recurrence of superficial bladder cancer (23, 25). Taken together, these studies indicate that BCG has the ability to act as an adjuvant and/or as a potentiator of an already on-going immune response.

It is generally accepted that an efficient adjuvant involves a depot effect (mediated by a vehicle), and an immune-modulating effect, the latter involving receptors that stimulate innate immunity (26, 27). BCG is known to stimulate several TLRs, but would not be expected to act as a depot for coadministered Ags (28, 29). In this study, we examined the ability of BCG to act as a potentiatior, or adjuvant, on a subunit vaccine against TB. As the vaccine Ag we choose the fusion protein consisting of Ag85B and ESAT-6 which has been shown to be highly protective against TB in several animal models (10, 30, 31). The potentiating effect of BCG was analyzed in the absence or presence of the vehicle dimethyl dioctadecyl ammonium bromide (DDA) used for the subunit vaccine. Detailed immunological analysis showed that BCG acted as a moderate adjuvant on its own. However, in the presence of DDA, BCG acted as a powerful Th1 coadjuvant on Ag85B-ESAT-6. Of importance for a potential clinical application of our observation the synergy between the subunit vaccine and BCG did not require mixing of the vaccine components before administration, since simultaneous but separate injections of the two vaccines had the same effect as long as the injection sites drained to the same lymph node. By this new approach a strong Th1 response was induced which correlated significantly with an 86% reduction in *M. tuberculosis* bacterial numbers after infection compared with that observed in BCG-vaccinated mice, and a 98% reduction compared with non-vaccinated mice. Potential future application of these results are discussed.

Materials and Methods

**Animals**

Studies were performed with 8- to 12-wk-old BALB/c × C57BL/6 F1 female mice, purchased from Harlan Scandinavia. Infected animals were housed in cages contained within laminar flow safety enclosures in a BSL-3 facility. The use of mice was in accordance with both the regulations set forward by the Danish Ministry of Justice and Animal Protection Committees and in compliance with EC Directive 86/609 and the U.S. American Laboratory Animal Care recommendations for the care and use of laboratory animals.

**Bacteria**

*M. tuberculosis Erdman* was grown at 37°C in suspension in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose.

**Immunization**

Mice were immunized three times at 2-wk intervals with experimental vaccines containing 5 μg of Ag85B-ESAT-6, 250 μg of DDA, 25 μg of monophosphoryl lipid A (MPL), and 5 × 10^6 CFU BCG Danish 1331, or as indicated in the figure. At the same time, as the first subunit vaccination, control mice received a single dose of BCG Danish 1331 (5 × 10^6 CFU) injected s.c. at the base of the tail. Mice were challenged 10 wk after the first vaccination.

Experimental infections

The animals were infected by the aerosol route with ~50 CFU of *M. tuberculosis* Erdman/mouse. Mice were sacrificed 6 wk after challenge. Numbers of bacteria in the lung were determined by serial 3-fold dilutions of individual whole-organ homogenates in duplicate on 7H11 medium. Organs from the BCG-vaccinated animals were grown on medium supplemented with 2 μg of 2-thiophene-carboxylic acid hydrazide (TCH) to selectively inhibit the growth of the residual BCG bacteria in the test organs. Colonies were counted after 2–3 wk of incubation at 37°C.

Lymphocyte cultures

All immunological analyses were performed on four to nine mice, in at least four independent experiments. Lymphocytes from spleens or lymph nodes (from individual mice) were obtained as described previously (30). BCG and subunit vaccines (PBMCs) were purified on a density gradient. Cells pooled from five mice in each experiment were cultured in microtiter wells (96-well plates; Nunc) containing 2 × 10^5 cells in a volume of 200 μl of RPMI 1640 supplemented with 5 × 10^−5 M 2-ME, 1% penicillin-streptomycin, 1 mM glutamine, and 5% (v/v) FCS. Based on previous dose–response investigations, the mycobacterial Ags were all used at 5 μg/ml. All preparations were tested in cell cultures and found to be nontoxic to the concentrations used in the present study. Supernatants were harvested from cultures after 72 h of incubation for the investigation of IFN-γ.

**IFN-γ ELISA**

Microtiter plates (96 wells; Maxisorb; Nunc) were coated with monoclonal hamster anti-murine IFN-γ (Genzyme) in PBS at 4°C. Free binding sites were blocked with 1% (v/v) BSA. 0.05% Tween 20. Culture supernatants were tested in triplicate, and IFN-γ was detected with a biotin-labeled rat anti-murine mAb (clone XMG1.2; BD Pharmingen). Recombinant IFN-γ (BD Pharmingen) was used as a standard.

**IFN-γ ELISPOT**

Ninety-six-well microtiter plates (Maxisorb; Nunc) were coated with 2.5 μg of monoclonal hamster anti-murine IFN-γ (Genzyme). Free binding sites were blocked with BSA followed by washing with PBS-0.05% Tween 20. Analyses were always conducted on cells pooled from three to five mice. Cells were stimulated with 5 μg of ESAT-6, Ag85B-ESAT-6, or Ag85B for 24–42 h directly in the ELISPOT plates. The cells were removed by washing, and the site of cytokine secretion was detected with a biotin-labeled rat anti-murine mAb (clone XMG1.2; BD Pharmingen) and phosphate-conjugated streptavidin (Jackson ImmunoResearch Laboratories). The enzyme reaction was developed with 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich). ELISPOT plates were counted using an AID plate reader (Autoimmun Diagnostika). The correlation between the number of cells per well and the number of spots was linear at concentrations of 2 × 10^5–2.5 × 10^5 cells/well. Wells with fewer than 10 spots were not used for calculations.

**FACS analysis of lymphocytes**

Cells were isolated, as described above, from the blood and spleen of mice. 2 × 10^5 cells were stimulated 1 h with 2 μg/ml Ag and subsequently incubated for 6 h with 10 μg/ml brefeldin A (Sigma-Aldrich) and Ag. Thereafter, cells were incubated with Fc block (BD Pharmingen), washed in FACS buffer, and stained for the indicated surface markers. Cells were then washed in PBS, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and stained intracellularly with PE-labeled anti-IFN-γ mAb. After washing, cells were finally resuspended in PBS containing 0.1% sodium azide, and analyzed by FACS (BD Immunocytometry Systems).

Derivation and stimulation of dendritic cells

Mice (6- to 8-wk-old female C57BL/6J (H-2b); Bomholtegaard) were killed by cervical dislocation, and femurs and tibias were aseptically removed and dissected free of adherent tissue. The bone ends were cut and marrow flushed out with complete RPMI 1640 using a 23-gauge needle. A single-cell suspension was prepared by gently forcing suspensions through an 18-gauge needle. Cells were pelleted by centrifugation (350 × g, 5 min) and resuspended in complete RPMI 1640 supplemented with 10% FCS (2.5 × 10^6 cells/ml). This cell suspension was distributed in 5-cm petri dishes (Nunc) and incubated (5% CO₂, 37°C, and 90% humidity) for 4 h, after which it was washed to remove nonadherent cells and fresh complete RPMI 1640 with recombinant murine GM-CSF (40 ng/ml) plus IL-4 (100 U/ml) addition. The medium was changed every second day. On day 7, cells were collected, washed twice with ice-cold PBS, and adjusted to 2.5 × 10^5 cells/ml in complete RPMI 1640 with 10% FCS and incubated in 24-well
plates with the adjuvants being tested. Supernatants were collected on days 1, 3, and 7 and stored at −20°C for later analysis of IL-12 and NO.

**NO detection by the Griess reaction**

Dendritic cell production of NO was assayed by transfer of culture supernatants (50 µl/well) to 96-well flat-bottom plates (Nunc), followed by the addition of 50 µl of 1% (w/v) sulfanilamide (Sigma-Aldrich) in 2.5% phosphoric acid (H₃PO₄) and then 50 µl of 0.1% (w/v) naphthylethylenediamine dihydrochloride (Sigma-Aldrich) in 2.5% H₃PO₄. The absorbance was measured at 540 nm after 10 min of incubation at room temperature in the dark, and values were quantified using a NaNO₂ standard curve. The results were generated from triplicate cultures and presented in pg/ml as mean of triplicate ± SD.

**IL-12 detection by ELISA**

The amounts of IL-12 in the supernatants were quantified by the same procedure as for IFN-γ (see above). The Abs used were as follows: IL-12 Capture (catalog no. 20171; BD Pharmingen) and IL-12 Detection (catalog no. 18482D; BD Pharmingen). Concentration of IL-12 was calculated using standard curves generated from recombinant mouse IL-12 (catalog no. 19361V; BD Pharmingen). Results are expressed in pg/ml ± SD.

**Statistical methods**

The data obtained were tested by ANOVA. Differences between means were assessed for statistical significance by Tukey’s test. A P value of <0.05 was considered significant.

**Results**

**The effect of BCG on the Ag85B-ESAT-6 subunit vaccine**

We first studied the effect of BCG on Ag85B-ESAT-6 with or without a vehicle (DDA-based cationic liposomes (7)). The vaccines were given s.c. three times at 2-wk intervals. The immune response at different time points was investigated by culturing splenocytes, blood cells, or cells isolated from the draining lymph nodes with different concentrations of the purified fusion protein Ag85B-ESAT-6. IFN-γ release (assessed by ELISA) following stimulation with Ag85B-ESAT-6 showed that vaccination with BCG/Ag85B-ESAT-6/DDA induced a substantial response (6300–7700 pg/ml IFN-γ) already 1 wk after the first vaccination in lymph nodes and spleen (Fig. 1, A and B). Two weeks after the first vaccination the Ag85B-ESAT-6 response had increased to 12,300 ± 2,644 pg/ml IFN-γ (Fig. 1C), and 3 wk after the final vaccination we still observed a significant recognition of Ag85B-ESAT-6, as measured by ELISA (data not shown) or ELISPOT (272 ± 3.5 per 10⁵ cells) on Ag-stimulated PBMCs (Fig. 1D). In contrast, vaccinating with Ag85B-ESAT-6, Ag85B-ESAT-6/DDA, BCG, or BCG/DDA induced only a limited recognition of Ag85B-ESAT-6 (in blood, lymph nodes, and spleen) (Fig. 1 and data not shown). Taken together, our results indicated that the response against Ag85B-ESAT-6 was due to a synergistic effect of BCG and Ag85B-ESAT-6/DDA and that the BCG induced potentiation of Ag85B-ESAT-6 recognition increased in the presence of DDA.

**BCG acts as an adjuvant on Ag85B-ESAT-6**

As BCG also expresses Ag85B, and therefore contributes to the recognition of Ag85B-ESAT-6, these results did not reveal whether the increased Ag85B-ESAT-6 response observed following vaccination with a mix of BCG and Ag85B-ESAT-6/DDA was due to BCG acting as an adjuvant on Ag85B-ESAT-6 or merely due to Ag85B being present in both BCG and Ag85B-ESAT-6 (leading to an increased Ag85B Ag load, and therefore an increased Ag85B-ESAT-6, response). To differentiate between these two possibilities we next focused on the response against ESAT-6. As ESAT-6 (in contrast to Ag85B) is not expressed by BCG, the response against this Ag could be used to monitor the subunit-specific response. Splenic T cells were taken 1 wk after the first or second vaccination and IFN-γ release (as assessed by ELISA) following stimulation with ESAT-6 was measured. Only 1 wk after the first vaccination the adjuvant effect of BCG was already apparent (Fig. 2A), and 1 wk after the second vaccination we observed an ESAT-6 response (11,960 ± 1,336 pg/ml IFN-γ), that was significantly higher in BCG/Ag85B-ESAT-6/DDA-vaccinated mice than in mice vaccinated with Ag85B-ESAT-6 in DDA/MPL (or Ag85B-ESAT-6 in DDA; data not shown), which at both time points remained <500 pg/ml (Fig. 2, A and B). We also analyzed the number of splenic ESAT-6-specific IFN-γ-secreting T cells 3 wk after the third vaccination by in vitro ELISPOT after Ag stimulation with ESAT-6. The result showed that mice immunized...
with BCG/Ag85B-ESAT-6/DDA exhibited a strong increase in ESAT-6 specific splenic T cells (160 ± 30 per 10^5 cells), which was significantly higher than that induced in MPL/Ag85B-ESAT-6/DDA (70 ± 13 per 10^5 cells)- or Ag85B-ESAT-6/DDA-vaccinated mice (Fig. 2C and data not shown; p < 0.01). As expected, we also observed an increased number of Ag85B-specific cells following BCG/Ag85B-ESAT-6/DDA vaccination, in this case most likely also due to the direct priming of Ag85B responses by both the subunit vaccine and BCG (Fig. 2D). Thus, in addition to its vaccine activity, BCG acted as a powerful coadjuvant that even surpassed MPL, and as observed in Fig. 1, the adjuvant effect of BCG was highest in the presence of DDA.

To examine whether the differences in the ability of MPL/Ag85B-ESAT-6/DDA and BCG/Ag85B-ESAT-6/DDA to induce a Th1 T cell response was reflected in their ability to activate innate immunity, mice were vaccinated with either BCG/Ag85B-ESAT-6/DDA or MPL/Ag85B-ESAT-6/DDA. One week later, the draining lymph node (inguinal) was taken and analyzed by FACS for the presence of dendritic cells (CD11c-positive cells) and neutrophils (Ly6-G-positive cells). Compared with naive mice, an increased number of neutrophils was observed in both BCG/Ag85B-ESAT-6/DDA (6.14%)- and Ag85B-ESAT-6/DDA-vaccinated mice (Fig. 3A and data not shown; p < 0.001). Groups significantly different from the naive groups are indicated (*∗, p < 0.001, ANOVA and Tukey’s test). Cells were pooled from five mice per group. Values represent the means and SEM of triplicate values (results from one of four representative experiment are shown). H1 indicates Ag85B-ESAT-6.

**FIGURE 2.** Adjuvant effect of BCG on ESAT-6. A and B, In vitro IFN-γ responses of blood cells from mice vaccinated as indicated in the figure taken 1 wk after the first vaccination (A) or 1 wk after the second vaccination (B). Cells were stimulated with 0.5 μg/ml ESAT-6. C and D, In vitro ELISPOT analysis of ESAT-6- or Ag85B-stimulated splenic cells from mice vaccinated as indicated, taken 3 wk after the final vaccination. Groups significantly different from the naive groups are indicated (∗∗, p < 0.001, ANOVA and Tukey’s test). Cells were pooled from five mice per group. Values represent the means and SEM of triplicate values (results from one of four representative experiment are shown). H1 indicates Ag85B-ESAT-6.

In summary, the results showed that compared with MPL/Ag85B-ESAT-6/DDA-vaccinated mice, whereas in both vaccinated groups high recruitment of B cells was observed. In support of these results, BCG also induced a significantly higher secretion of both NO and IL-12 from bone marrow dendritic cells in vitro after 1, 3, and 7 days, suggesting that the difference observed in MPL/Ag85B-ESAT-6/DDA and BCG/Ag85B-ESAT-6/DDA groups could be attributed to a difference in the ability of BCG and MPL to activate innate immunity (Fig. 3, C and D).

**Protective efficacy of BCG/Ag85B-ESAT-6/DDA**

We next analyzed the protective efficacy of BCG/Ag85B-ESAT-6/DDA. In four independent experiments mice were vaccinated three times with BCG mixed with Ag85B-ESAT-6/DDA and 6 wk after the last vaccination, the mice were subjected to an aerosol challenge with virulent *M. tb*. Six weeks after challenge, the mice were killed and the bacterial numbers were determined in the lungs. Mice vaccinated with BCG/Ag85B-ESAT-6/DDA showed a bacterial (*M. tb*) burden of 4.197 log_{10} ± 0.08 CFU in the lung, and thus an impressive 1.67 (log_{10} CFU) decrease in the bacterial burden compared with none-vaccinated mice (5.872 log_{10} ± 0.23 CFU, p < 0.001) (Fig. 4A, which shows the results from one representative experiment). In bacterial numbers this constituted a reduction of 98% in the lung, compared with nonvaccinated mice, or a 77% reduction compared with BCG-vaccinated mice. Mice vaccinated with Ag85B-ESAT-6/DDA (data not shown), MPL/Ag85B-ESAT-6/DDA, BCG, or three times with BCG all showed a bacterial burden that was higher than in mice vaccinated with BCG/Ag85B-ESAT-6/DDA, and only BCG/Ag85B-ESAT-6/DDA-vaccinated mice showed a bacterial burden significantly different from BCG-vaccinated mice (p < 0.01) (Fig. 4A).

Since lipid encapsulation has been reported to increase the efficacy of BCG (32), our results could be partially due to a direct influence of administering BCG in DDA liposomes. To examine...
this, mice were vaccinated with BCG or with BCG in liposomes and the bacterial numbers were determined in the lungs 6 wk after challenge with virulent M. tb. The results showed that the bacterial burden in mice vaccinated with BCG/DDA was not significantly different from that in mice vaccinated with BCG (Fig. 4B).

Taken together, vaccinating with BCG/Ag85B-ESAT-6/DDA not only increased the recognition of Ag85B-ESAT-6 (and the individual vaccine components), but also increased the protection against M. tb compared with BCG-vaccinated mice.

Boosting with MPL/Ag85B-ESAT-6/DDA instead of BCG/Ag85B-ESAT-6/DDA

The results so far demonstrated that BCG had superior coadjuvant activity in combination with the subunit vaccine and performed even better in that regard than MPL. In these experiments BCG was used not only for the prime but also for the booster injection. We therefore continued by investigating whether BCG was required as a coadjuvant for both priming and boosting to obtain maximal effect. To address this point we exchanged BCG for MPL in the two booster vaccinations (but not in the priming vaccination), and then analyzed the immune recognition of ESAT-6 and Ag85B.

Mice were vaccinated with BCG/Ag85B-ESAT-6/DDA and boosted with either BCG/Ag85B-ESAT-6/DDA or MPL/Ag85B-ESAT-6/DDA and the immune recognition of ESAT-6 and Ag85B was investigated 2 wk after the first booster vaccination (Fig. 5A) or the number of IFN-γ-secreting ESAT-6 or Ag85B-specific T

FIGURE 3. Effect of BCG/Ag85-ESAT-6/DDA vaccination on innate immunity. A and B, FACS analysis of cells isolated from draining (inguinal) lymph nodes 1 wk after vaccination with BCG/H1/ DDA or MPL/H1/DDA. Cells were stained as indicated, and the percentage of cells are present in the four quadrants. C and D, In vitro analysis of NO and IL-12 production by stimulated dendritic cells. Dendritic cells were cultured at 2.5 × 10^6 cells/ml in complete medium supplemented with BCG (2.5 × 10^6 CFU/ml) or MPL (100 ng/ml), and the culture supernatants were harvested at days 1, 3, and 7 for analysis. Results are the means and SDs of triplicate wells, and the results are representative of one of three different experiments. BCG-stimulated cells that were significantly different from the MPL-stimulated cells are indicated (***, p < 0.001; **, p < 0.01, paired t test). H1 indicates Ag85B-ESAT-6.

FIGURE 4. The protective efficacy of BCG mixed with Ag85B-ESAT-6/DDA. A and B, Bacterial burden in vaccinated mice (expressed as log_{10} CFU) compared with unvaccinated controls challenged by the aerosol route with virulent M. tb 10 wk after the first vaccination. Six weeks postchallenge, the mice were killed, and the bacterial burden (CFU) was measured in the lung (*, p < 0.05; **, p < 0.01, ANOVA and Tukey’s test). H1 indicates Ag85B-ESAT-6.
cells was examined by ELISPOT 2 wk after the last booster vaccination (Fig. 5B). Boosting with MPL/Ag85B-ESAT-6/DDA induced as high a response against ESAT-6 as boosting with BCG/Ag85B-ESAT-6/DDA (10,000–20,000 pg/ml IFN-γ) and the number of ESAT-6-specific IFN-γ-producing cells in MPL/Ag85B-ESAT-6/DDA boosted mice at week 2 after the second boost (140 ± 9 per 10⁵ cells) was not significantly different from that observed in BCG/Ag85B-ESAT-6/DDA-boosted mice (160 ± 30 per 10⁵ cells) (Fig. 5B). The response against the other component of the fusion protein, Ag85B, was also not significantly different between the two groups, measured by either the amount of secreted IFN-γ after one boost (Fig. 5C) or the number of IFN-γ secreting cells after two boosts (Fig. 5D). In contrast, exchanging BCG for MPL in the priming vaccination led to a significant decrease in both ESAT-6 and Ag85B specific T cells (p < 0.01 or 0.001, Fig. 5). To examine the phenotype of the IFN-γ-secreting Ag85B or ESAT-6 specific cells promoted by this optimized immunization protocol, we analyzed animals primed with BCG/Ag85B-ESAT-6/DDA (or BCG) and boosted with MPL/Ag85B-ESAT-6/DDA by intracellular cytokine FACS analysis following stimulation of splenocytes with Ag85B, ESAT-6 (or Ag85B-ESAT-6). The results showed that the majority of Ag-specific cells were found within the CD4⁺/CD44⁺ subset of T cells (Fig. 6A). In mice primed with BCG/Ag85B-ESAT-6/DDA, 0.5% of all CD4 T cells were specific for Ag85B-ESAT-6. In comparison mice primed with BCG alone showed a lower number of Ag specific cells in agreement with the results shown in Fig. 5 (Fig. 6B).

Thus, including BCG in the priming is critically important for inducing a maximal response. In contrast, a conventional subunit vaccine based on the MPL-containing liposomal adjuvant can be used as booster vaccine, thereby avoiding repeated injections of BCG. The immune response promoted by this prime boost

**FIGURE 5.** Exchanging BCG/Ag85B-ESAT-6/DDA for MPL/Ag85B-ESAT-6/DDA in the booster vaccinations. A, In vitro IFN-γ responses of ESAT-6 (0.5 μg/ml)-stimulated blood cells from mice vaccinated as indicated in the figure taken 1 wk after the first booster vaccination. B, In vitro ELISPOT analysis of ESAT-6-stimulated splenic cells from mice vaccinated as indicated taken 3 wk after the final vaccination. C, In vitro IFN-γ responses of Ag85B (0.5 μg/ml)-stimulated blood cells from mice vaccinated as indicated in the figure taken 1 wk after the first booster vaccination. D, In vitro ELISPOT analysis of Ag85B-stimulated splenic cells from mice vaccinated as indicated taken 3 wk after the final vaccination. Groups significantly different from the naive groups are indicated (**, p < 0.01; ***, p < 0.001, ANOVA and Tukey’s test). Cells were pooled from five mice per group. Values represent the means and SEM of triplicate values (results from one of four representative experiments are shown).

**FIGURE 6.** Boosting BCG/Ag85B-ESAT-6/DDA with MPL/Ag85B-ESAT-6/DDA induces a CD4 T cell-mediated Ag85B-ESAT-6 response. A, FACS analysis of IFN-γ expression by CD4/CD8 spleen cells from BCG/Ag85B-ESAT-6/DDA-vaccinated mice boosted with MPL/Ag85B-ESAT-6/DDA and stimulated in vitro with Ag85B-ESAT-6, Ag85B, or ESAT-6. The figure shows the percentage of IFN-γ positives among the CD44-positive CD4 or CD8 cells. B, Histogram showing the percentage of IFN-γ-positive cells among the CD44-positive CD4 cells from mice vaccinated with BCG or BCG/Ag85B-ESAT-6/DDA and boosted with MPL/Ag85B-ESAT-6/DDA. Cells were stimulated in vitro with Ag85B-ESAT-6, Ag85B, or ESAT-6 as indicated. H1 indicates Ag85B-ESAT-6.
We continued by examining whether the increased number of Ag-components at the site of injection or due to events occurring after draining to the regional lymph node. We therefore investigated whether the observed synergistic effect between BCG and Ag85B-ESAT-6/DDA required mixing of the components before vaccine administration, or merely coddrainage of BCG and Ag85B-ESAT-6/DDA to the same lymph node.

Mice were vaccinated with a mixture of BCG, Ag85B-ESAT-6, and DDA or separately with BCG and Ag85B-ESAT-6/DDA, administered close enough to allow for drainage to the same inguinal lymph node ("BCG + Ag85B-ESAT-6/DDAxs"; ss, side by side). It should be noted that although the vaccine components BCG and Ag85B-ESAT-6/DDA were given at close proximity, they did not mix beneath the skin (two distinct nodules were observed immediately after vaccination). Alternatively, mice were vaccinated such that BCG and Ag85B-ESAT-6/DDA drained to different lymph nodes (left and right inguinal lymph nodes) ("BCG + Ag85B-ESAT-6/DDAds"; ds, different sites). Thereafter, mice were boosted with MPL/Ag85B-ESAT-6/DDA and the immune response was investigated by culturing blood cells with the purified fusion protein ESAT-6 or Ag85B-ESAT-6. Mice primed with BCG plus Ag85B-ESAT-6/DDAxs showed a strong recognition of Ag85B-ESAT-6 (27,886 ± 1,254 pg/ml IFN-γ) and ESAT-6 (10,780 ± 1,289 pg/ml IFN-γ) and, as expected, mice primed with BCG/Ag85B-ESAT-6/DDA also recognized Ag85B-ESAT-6 (20,135 ± 1,553 pg/ml IFN-γ) and ESAT-6 (4,976 ± 1,339 pg/ml IFN-γ) (Fig. 8, B and C). Mice primed with BCG plus DDAxs did not show an increased recognition of Ag85B-ESAT-6, compared with BCG-vaccinated mice (data not shown). Importantly, mice primed with BCG plus Ag85B-ESAT-6/DDAxs showed a significantly reduced recognition of Ag85B-ESAT-6 (6,999 ± 3,394 pg/ml IFN-γ) and ESAT-6 (1,569 ± 2,253 pg/ml IFN-γ) compared with mice vaccinated with BCG plus Ag85B-ESAT-6/ DDAAxs (p < 0.05). This indicated that the vaccine components did not have to be mixed but merely had to drain to the same lymph node in order for BCG to act as a coadjuvant on Ag85B-ESAT-6 and thereby contribute to the positive synergistic effect between the vaccine components (Fig. 8, B and C). Moreover, in agreement with the results shown in Fig. 2, the adjuvant effect of BCG injected next to the subunit vaccine surpassed that of MPL (in DDA liposomes).

Protective efficacy of BCG given side-by-side with Ag85B-ESAT-6/DDA

Having demonstrated that mice primed with BCG plus Ag85B-ESAT-6/DDAxs showed as strong a recognition of Ag85B-ESAT-6 as mice primed with a mixture of BCG, Ag85B-ESAT-6, and DDA, we evaluated the protective efficacy of the two vaccine strategies. Six weeks after the last vaccination, mice were infected by the aerosol route and the bacterial numbers were determined in the lungs 6 wk after challenge. BCG plus Ag85B-ESAT-6/DDAxs-vaccinated mice showed a bacterial burden of 3.92 log10 ± 0.23, which equaled a 97% reduction in bacterial numbers compared with unvaccinated mice (5.41 log10 ± 0.20), and a 76% reduction compared with BCG-vaccinated mice (4.76 log10 ± 0.37, p < 0.001). The mice vaccinated with a mixture of the three components BCG/Ag85B-ESAT-6/DDA, had a bacterial burden of 4.01 log10 ± 0.17 (slightly higher than in Figs. 4 and 7), whereas BCG plus Ag85B-ESAT-6/DDAxs-vaccinated mice showed a bacterial burden of 4.31 log10 ± 0.21 (Fig. 8). Only the groups vaccinated with BCG plus Ag85B-ESAT-6/DDAxs or BCG/Ag85B-ESAT-6/DDA had bacterial numbers significantly different from those observed in BCG control mice or BCG-vaccinated mice boosted with MPL/Ag85B-ESAT-6/DDA.

Thus, together with the immune response data shown in Fig. 7, these results indicated that the strong coadjuvant activity that can be obtained by mixing BCG with the liposome adjuvanted subunit

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**FIGURE 7.** Boosting BCG/Ag85B-ESAT-6/DDA with MPL/Ag85B-ESAT-6/DDA increases protection against M. tb compared with BCG. Bacterial burden in vaccinated mice (expressed as log10 in CFU) as indicated in the figure compared with unvaccinated controls challenged by the aerosol route with virulent M. tb 10 wk after the first vaccination. Six weeks postchallenge, the mice were killed, and the bacterial burden (CFU) was measured in the lung. "+MPL/H1/DDA×2" indicated that the mice were boosted twice with MPL/H1/DDA or H1, where H1 indicates Ag85B-ESAT-6 (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ANOVA and Tukey’s test).

Combination was directed to both ESAT-6 and Ag85B and mediated by CD4+CD44+ IFN-γ+ T cells.

**Protective efficacy of BCG/Ag85B-ESAT-6/DDA boosted with MPL/Ag85B-ESAT-6/DDA**

We continued by examining whether the increased number of Ag-specific CD4+CD44+ IFN-γ+ T cells in the mice vaccinated with BCG/Ag85B-ESAT-6/DDA and boosted twice with MPL/Ag85B-ESAT-6/DDA correlated with an increased protection against M. tb. Vaccinated animals received an aerosol challenge with virulent M. tb and the bacterial numbers were determined in the lungs 6 wk after the challenge. Mice vaccinated with BCG/Ag85B-ESAT-6/DDA and boosted with MPL/Ag85B-ESAT-6/DDA showed a bacterial burden of 3.9 log10 ± 0.24 CFU in the lung which equaled a 98% reduction in bacterial numbers compared with nonvaccinated mice and a 76% reduction compared with BCG vaccinated mice, whereas mice boosted with BCG/Ag85B-ESAT-6/DDA showed a bacterial burden (M. tb) burden of 4.0 log10 ± 0.28 CFU (Fig. 7A). Only these two groups had a bacterial burden significantly different from that observed in mice vaccinated with BCG alone (p < 0.001). Thus, priming with either MPL/Ag85B-ESAT-6/DDA or BCG alone followed by boosting with MPL/ Ag85B-ESAT-6/DDA did not significantly reduce the bacterial burden in the lungs, compared with BCG-vaccinated mice.

Taken together, the results indicated that the BCG subunit/liposome vaccine combination provided the best possible and most efficient priming, whereas booster vaccinations with DDA/MPL-adjuvanted vaccines were fully sufficient for maximal responses.

Draining of BCG and Ag85B-ESAT-6 to the same lymph node is required for an increased recognition of Ag85B-ESAT-6

The results so far showed that BCG clearly had a coadjuvant activity when mixed with Ag85B-ESAT-6 in DDA liposomes and that to achieve the highest protection and immune recognition of both the vaccine components, the presence of all three components, BCG, Ag85B-ESAT-6, and DDA was required in the priming vaccination. However, the experiments so far did not reveal whether this was due to a favorable interaction between the components at the site of injection or due to events occurring after....
vaccine is independent of a physical interaction between BCG and the subunit vaccine components and only require that BCG and Ag85B-ESAT-6/DDA drain to the same lymph node.

Discussion

In this study, we evaluated a new vaccine approach, and variations thereof, with special focus on the ability of BCG to act as an adjuvant. Vaccinating with BCG/Ag85B-ESAT-6/DDA lead to a strong response against Ag85B-ESAT-6 and showed that the presence of BCG in the vaccine had an adjuvant effect on the response against ESAT-6 (Figs. 1 and 2). Only 1 wk after the second vaccination the ESAT-6 response reached an impressive level of >10,000 pg/ml IFN-γ secretion upon in vitro stimulation with ESAT-6. Mice immunized with BCG/Ag85B-ESAT-6 (e.g., without DDA) also showed an increased recognition of ESAT-6 compared with Ag85B-ESAT-6-vaccinated mice, although less than if DDA had been included in the vaccine (Fig. 2). This indicates that BCG is not a strong adjuvant on its own but is a strong coadjuvant in the presence of a vehicle. The requirement for a vehicle to achieve maximal adjuvant effect is in agreement with adjuvant research over the last decade, which have revealed that an adjuvant should ideally be composed of both a vehicle and an immunomodulator (26, 27). Thus, considering the presumed inability of BCG to act as a vehicle, our finding that BCG is only a moderate adjuvant on its own is not surprising, and the same dependency on a vehicle was also recently observed with the other immunomodulator used in this study, MPL (K. Korsholm, P. Andersen, and E. M. Agger, unpublished results). However, there are situations where BCG may be appropriate to use as a standalone “adjuvant,” e.g., where a strong stimulation of innate immunity is required to induce the effector cells of the innate immune system, such as NK cells and neutrophils, to exert their effector mechanisms, or when an activation of APCs (e.g., via TLRs) is needed to stimulate already ongoing immune responses. Indeed, this may explain its success as a therapeutic agent against superficial bladder carcinoma recurrences, where the immune response to some degree is ongoing, and where increased NK cell cytotoxicity against tumor cells seems important (23, 24). Although the mechanisms behind BCG’s immune-modulating activity are not fully known they do involve binding of TLRs on professional APCs (28, 29), as well as activation and recruitment of NK cells and neutrophil granulocytes (23, 33–35). In agreement with this, we showed that vaccinating with BCG/Ag85B-ESAT-6/DDA led to recruitment of neutrophils and dendritic cells to the draining lymph node and that BCG induces both NO and IL-12 production in bone marrow-derived dendritic cells, in vitro (Fig. 3). The strong recognition of ESAT-6 observed due to BCGs coadjuvant effect is in contrast to previous studies using recombinant BCG expressing Ag85B-ESAT-6 or ESAT-6. Mice immunized with these constructs did not show elevated recognition of ESAT-6 and experienced a reduction in M.tb bacterial burden ranging from minor (BCG-ESAT-6) (11, 36) to ~3 (BCG-Ag85B-ESAT-6) (11, 36), the latter probably due to an increased Ag85B response, which in other studies has been shown to induce a 0.5 log_{10} CFU reduction (~3-fold reduction in M.tb bacterial burden in the lung) compared with BCG vaccinated controls (11, 16, 36). The lack of ESAT-6 recognition could be due to this Ag being dominated by other Ags expressed by BCG and due to the lack of a vehicle.

Following priming with BCG/Ag85B-ESAT-6/DDA a strong induction of both Ag85B and ESAT-6-specific IFN-γ-producing cells was observed. This strong priming of the immune response was absolutely dependent on the presence of both BCG and DDA. However, although the adjuvant effect of MPL was significantly less than BCG, MPL could nevertheless substitute for BCG in the booster vaccinations without decreasing the recognition of Ag85B and ESAT-6 (Fig. 5). The boosted T cells were found to be
These mice with MPL/Ag85B-ESAT-6/DDA led to a strong recognition of Ag85B-ESAT-6 as the when all the com-
to allow for drainage to the same lymph node, we observed as BCG and Ag85B-ESAT-6/DDA at different sites, but close enough need to be part of the same vaccine formulation. By administering DDA) in the priming vaccine, the vaccine components did not of MPL was clearly stronger in the booster vaccinations, where it in
that this population is one of the main responding T cell population against TB (Fig. 7), in agreement with previous studies showing a significant correlation between their numbers and the protection
ing with BCG/Ag85B-ESAT-6/DDA (Fig. 6 and data not shown). They could be grouped in two groups according to their im-
different approaches used in the present work are compared. The major difference between the two groups was whether all three components were used in the priming vaccination.

Another important point when generating a recombinant BCG variant that overexpress an Ag shared by BCG, or when coinjecting BCG with such an Ag, is to examine how the im-
mune response against this Ag affects BCG proliferation and dissemination. It is known that a pre-existing immunity against BCG, or cross-reacting to BCG, can inhibit BCG (39). Therefore, if the Ag (e.g., Ag85B) is shared by BCG, it is relevant to examine its effect on BCG. Preliminary results from our labor-
atory indicate that coinjecting BCG with Ag85B-ESAT-6/DDA does influence BCG proliferation and dissemination.

Whether the influence is on the kinetics or the strength of BCG proliferation and dissemination is not known. We are presently performing these analyses.

In conclusion, by using the immune-modulatory effect of BCG, in the presence of a vehicle, we have generated a vaccine that induced a strong activation of innate immunity leading to increased T cell response and protection against infection with M.tb. The vaccine was superior to BCG, and could be boosted by the subunit vaccine in a conventional adjuvant. In particular, our work led to three important observations: 1) BCG is a strong co-
adjuvant (immune modulator) when a cationic vehicle is present, whereas on its own BCG is only a moderate adjuvant, 2) Whereas BCG is absolutely crucial in the priming vaccine, BCG can be exchanged for MPL in a booster vaccine, and 3) BCG and subunit/ liposome do not have to be mixed and can be injected separately and still give rise to a strong synergistic effect between the vehicle and the immunomodulatory activity of BCG. Considering previous studies showing that overexpressing additional M.tb Ags by BCG led to an increased protection (11, 16, 36), our results indicate that this can also be achieved by simply injecting the Ag next to BCG. Moreover, including a vehicle will enhance recognition of the Ag. The practical implication of this is important since there will be no interference with the established BCG vaccination procedure, and no need to address the regulatory issues related to new live (re-
combinant) mycobacterial vaccines (39).

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Disclosures
The authors have no financial conflict of interest.

References

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Log$_{10}$ CFU (BCG)</th>
<th>Bacterial No.</th>
<th>Fold Reduction in Bacterial No. Compared with BCG</th>
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<td>Non vacc.</td>
<td>5.62 ± 0.33 (4.68 ± 0.49)</td>
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*Protective efficacy of different vaccine strategies compared with BCG. In the “Log$_{10}$ CFU (BCG)” column, all the bacterial numbers are followed by the M.tb bacterial numbers in the BCG control group from the same experiment in brackets.
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