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Enhanced Immunogenicity and Protective Efficacy with the Use of Interleukin-12-Encapsulated Microspheres plus AS01B in Tuberculosis Subunit Vaccination§

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Tuberculosis subunit vaccines codelivered with interleukin-12 (IL-12)-encapsulated microspheres (IL-12EM) are designed for a sustained release of IL-12 and could induce strong Th1 immune responses specific to Ag85A and ESAT-6. The adjuvant combination of IL-12EM plus AS01B was a more efficient way to induce a sustained Th1 immunity and protection against *Mycobacterium tuberculosis*.

Since the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine has shown little effectiveness against tuberculosis (TB) in adults (4), the development of an effective TB vaccine to prevent *M. tuberculosis* infection is urgently needed. Subunit vaccines against tuberculosis show some promise but require coadministration of adjuvants to stimulate a Th1 immune response, which plays a key role in protection against *M. tuberculosis* infection. Although interleukin-12 (IL-12) has been reported to induce a strong Th1 immune response, the in vivo use of recombinant IL-12 (rIL-12) protein as an adjuvant of subunit vaccines was not sufficient to improve both Th1 immune response and protection, due to its rapid in vivo clearance and inactivation. The use of IL-12-encapsulated microspheres (IL-12EM) could be a solution that overcomes the rapid in vivo clearance of IL-12 protein, because cytokine-encapsulated biodegradable polymer microspheres have been known to achieve local and sustained expression of therapeutic agents, including cytokines (6, 8, 11, 12, 18, 20, 30). We evaluated IL-12EM as an adjuvant in a TB subunit vaccine model. In addition, we examined the combined adjuvant effect of IL-12EM plus AS01B, another Th1-inducing adjuvant, which is composed of monophosphoryl lipid A (MPL) and saponin molecule (QS21) (2, 21).

rIL-12 was encapsulated into poly($_{DL}$-lactic-co-glycolic acid) (PLGA) microspheres by use of a water-in-oil-in-water double-emulsion solvent evaporation technique (5, 13, 14). An aqueous solution of rIL-12 (50 μg) and bovine serum albumin (12.5 mg) was emulsified in dichloromethane containing 500 mg of PLGA. The amount of IL-12 incorporated into microspheres was determined using an enzyme-linked immunosorbent assay (ELISA) after dissolving IL-12EM in dimethyl sulfoxide. Figure 1 shows in vivo release of IL-12 from IL-12EM at different time points. The burst release of IL-12 in mice injected with rIL-12 was observed within 30 min, but the levels declined rapidly. In contrast, the release of IL-12 from IL-12EM was persistent after 9 days, indicating that encapsulation of rIL-12 using PLGA was effective, and a slow release of IL-12 could be achieved in vivo using IL-12EM.

To test the adjuvant effect of IL-12EM in the TB subunit vaccine, we used Ag85A and ESAT-6 (A+E) as subunit vaccine components because they are known to be protective antigens that induce a strong Th1 immune response (17, 28). Each subunit of vaccine contained 20 μg of Ag85A protein and 15 μg of ESAT-6 protein (Standardia Diagnostics, Inc., Suwon, Korea) and was emulsified in either alum (Pierce, Rockford, IL) or AS01B (SmithKline Beecham Biologicals S.A., Belgium) with or without IL-12EM containing 0.1 μg of IL-12. Mice were immunized with the experimental vaccines by a dorsal, subcutaneous route at 0 and 8 weeks. End-point titers of antibodies specific to Ag85A and ESAT-6 were determined using serum ELISA at 4 weeks after the primary immunization (Table 1). The alum-immunized (A+E/alum) group showed higher levels of total immunoglobulin G (IgG) (fourfold) and IgG1 (eightfold) specific to Ag85A than the AS01B-immunized (A+E/AS01B) group. However, the IgG2a level was fourfold lower in the alum-immunized group than in the

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§ Supplemental material for this article may be found at http://iai.asm.org/.

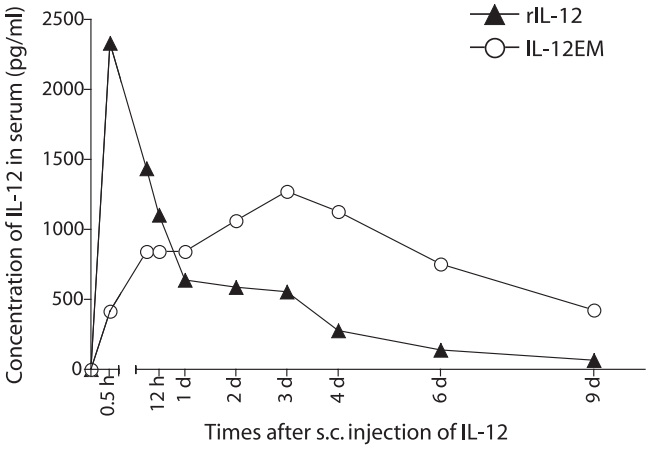

FIG. 1. In vivo release of IL-12 from IL-12EM. Mice were injected subcutaneously (s.c.) with 0.5 μg of rIL-12 or IL-12EM containing the same amount of IL-12. At the indicated time points after injection (d, days), IL-12 concentration in serum was measured by ELISA.

AS01B-immunized group, indicating that alum induces a Th2-type antibody response rather than a Th1-type response. Coinjection of IL-12EM with alum (alum+IL-12EM) or AS01B (AS01B+IL-12EM) increased IgG2a preferentially over IgG1. While the levels of Ag85A-specific IgG2a in groups with IL-12EM were 128- to 256-fold higher than the levels in groups without IL-12EM, the IgG1 levels were increased two- to eightfold. The AS01B+IL-12EM-immunized group showed a 1,024-fold-higher level of IgG2a than but the same level of IgG1 as the alum-immunized group. After secondary immunization, alum-injected mice still showed a lower IgG2a response than AS01B-injected mice. The AS01B+IL-12EM-immunized group showed the highest ratio of IgG2a to IgG1 specific to Ag85A (125 and 1,000 for primary and secondary responses, respectively), suggesting that a strong Th1 immunity would be established in this group (Table 1). A similar pattern of IgG subtype distribution was observed with the ESAT-6-specific antibody responses. Although little is known about the role of antibody subtypes in the control of tuberculosis, there are some reports showing that the IgG2a isotype is associated with a Th1 cytokine response (22, 23). Furthermore, the induction of Th1 cellular immunity strongly correlates with protective immunity against *M. tuberculosis* (25). Interestingly, the BCG-immunized group showed low levels of Ag85A-specific total IgG and IgG1 responses and no IgG2a production, indicating that BCG immunization itself could not effectively induce the antibody responses specific to Ag85A, a major protein of culture filtrate protein (CFP). Also, the ESAT-6-specific response could not be induced after BCG immunization because BCG lacks the ESAT-6-encoding gene.

To investigate the adjuvant effect of IL-12EM on the induction of the Th1 immune response, a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay was performed using lymphocytes from spleens (Fig. 2A) and draining lymph nodes (Fig. 2B). BCG-immunized mice had a threefold-higher level of IFN-γ specific to CFP than to Ag85A. This is unlike other subunit vaccine-immunized mice, which indicates that CFP-specific T-cell responses induced by BCG vaccination may be comprised of responses specific to TB antigens other than Ag85A. The pattern observed in the IgG2a responses of alum- and AS01B-injected groups was also found in the IFN-γ response. AS01B, but not alum, induced Ag85A-, ESAT-6-, and CFP-specific Th1 immune responses. The IL-12EM combination with alum or AS01B also led to the enhancement of IFN-γ production. It is worthwhile to note that overall IFN-γ responses are observed to be higher in spleens than in draining lymph nodes. This suggests that, as shown in the previous reports, effector memory cells during a secondary response are preferentially enriched in nonlymphoid tissues and spleens

TABLE 1. Antibody end-point titers and IgG2a/IgG1 ratios for the immunized mice[a]

Antibody	Vaccine group[b]	Primary antibody response (end-point titer)[c]			Ratio of IgG2a to IgG1[d]	Secondary antibody response (end-point titer)[c]			Ratio of IgG2a to IgG1[d]
		Total IgG	IgG1	IgG2a		Total IgG	IgG1	IgG2a	
Anti-Ag85A	PBS	<8[e]	<8	<8		<8	<8	<8	
	BCG	128	128	<8		128	128	<8	
	A+E/alum	32,768	1,048,576	128	0.1	524,288	8,388,608	4,096	0.5
	A+E/AS01B	8,192	131,072	512	3.9	1,048,576	8,388,608	131,072	15.6
	A+E/alum+IL-12EM	262,144	2,097,152	16,384	7.8	2,097,152	16,777,216	262,144	15.6
	A+E/AS01B+IL-12EM	1,048,576	1,048,576	131,072	125.0	4,194,304	2,097,152	2,097,152	1,000.0
Anti-ESAT-6	PBS	<8	<8	<8		<8	<8	<8	
	BCG	<8	<8	<8		<8	<8	<8	
	A+E/alum	131,072	1,048,576	4,096	3.9	1,048,576	16,777,216	32,768	2.0
	A+E/AS01B	16,384	16,384	4,096	249.9	524,288	1,048,576	131,072	125.0
	A+E/alum+IL-12EM	1,048,576	2,097,152	32,768	15.6	4,194,304	16,777,216	262,144	15.6
	A+E/AS01B+IL-12EM	2,097,152	524,288	524,288	1,000.0	4,194,304	4,194,304	1,048,576	250.0

[a] Serum samples were obtained from peripheral blood of the mice in each group (20 to 28 mice per group). Sera from mice in the same group were pooled with the same volume of individual sera.

[b] C57BL/6 mice were subcutaneously immunized once with BCG (10^7 CFU) at 0 weeks or injected twice at 8-week intervals with the experimental subunit vaccines in different adjuvant combinations. PBS, phosphate-buffered saline.

[c] Total IgG, IgG1, and IgG2a end-point titers obtained using the serum pool from each group were determined by ELISA and are represented as serum dilutions (*n*-fold). Primary antibody responses were measured at 4 weeks after priming with the indicated vaccines. Secondary antibody responses were measured at 4 weeks after boosting with the indicated vaccines.

[d] For the calculation of the ratio of IgG2a to IgG1, the end-point titer of IgG2a was divided by that of IgG1 and then multiplied by 1,000.

[e] Undetectable antibody level in serum diluted eightfold.

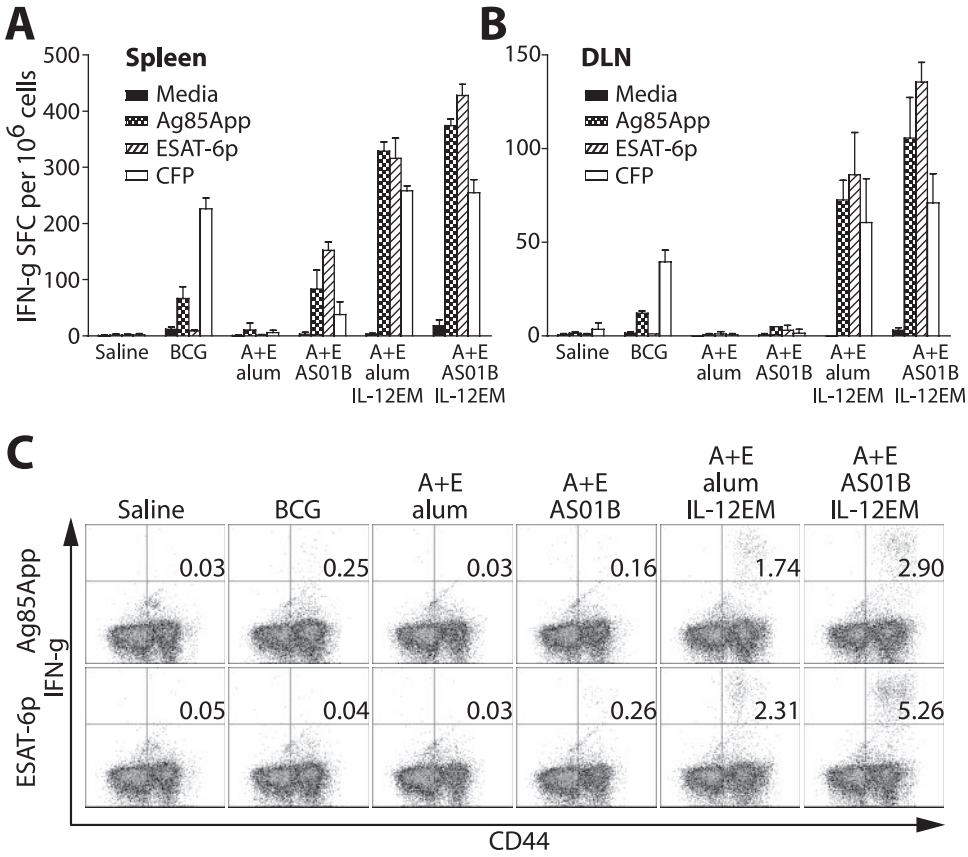

FIG. 2. Effect of experimental TB subunit vaccines on IFN-γ immune responses. Mice were subcutaneously immunized at 0 and 8 weeks with recombinant Ag85A and ESAT-6 proteins in different adjuvant combinations, or they received a BCG vaccination. Eight weeks after the last immunization, spleens (A) and draining lymph nodes (DLN) from abdominal and mediastinal sites (B) from three mice were pooled and used in an ELISPOT assay to determine the numbers of cells secreting IFN-γ in response to media, Ag85A peptide pool (Ag85App, 20-mer peptides overlapping by 10 amino acids; 1 μg/ml for each peptide), ESAT-6 peptide (3, 29) (ESAT-6p, CD4 epitope; 10 μg/ml), or CFP (10 μg/ml). The numbers of IFN-γ-producing cells per 10^6 splenocytes are represented as the average spot-forming cells (SFC) (±standard deviations) in triplicate wells. These results were reproduced in two independent experiments. (C) Lung lymphocytes from the same mice were pooled and stimulated in vitro for 5 h with media, Ag85App, or ESAT-6p in the presence of syngeneic spleen antigen-presenting cells. After being stained with anti-CD4, anti-CD44, and anti-IFN-γ, live $CD4^+$ T cells were gated and analyzed for intracellular IFN-γ expression (*y* axis) and CD44 activation marker (*x* axis) by flow cytometry. The percentages of both IFN-γ- and CD44-positive $CD4^+$ T cells were calculated and are shown within each plot. The data represent two independent experiments with similar results.

rather than in lymph nodes (16, 19, 26, 27). To evaluate the peripheral immune response of the lungs as a primary infection site of *M. tuberculosis*, we performed intracellular IFN-γ staining for $CD4^+$ T cells isolated from lungs (Fig. 2C). Similarly to the results observed with the ELISPOT assay, the highest numbers of IFN-γ-secreting $CD4^+$ T cells (2.90% and 5.26% of $CD4^+$ T cells after stimulation with Ag85App and ESAT-6p, respectively) were detected in the AS01B+IL-12EM group. This indicates that this combination is effective in generating peripheral Th1 immune responses as well as systemic Th1 immune responses.

To investigate whether the immune response enhanced by IL-12EM can lead to increased protection against tuberculosis, the mice were challenged aerogenically with two different doses of *M. tuberculosis* at 16 weeks after the first immunization. The subsequent course of infection was monitored in the lung, the primary site of infection (Fig. 3). BCG-immunized mice had threefold fewer bacteria than saline-immunized mice after a high-dose challenge (Fig. 3A) (P of <0.001 and P of <0.01 at 4 and 9 weeks postchallenge, respectively). While alum did not reduce the bacterial load, AS01B slightly reduced the bacterial number by 1.5-fold on average at 4 weeks compared with saline, although the difference was not statistically significant. In contrast, the two groups containing IL-12EM exhibited significantly reduced bacterial numbers compared to the saline group at 4 weeks (1.7-fold [$P < 0.05$] and 5.4-fold [$P < 0.001$] for alum+IL-12EM- and AS01B+IL-12EM-immunized groups, respectively). The growth control in the AS01B+IL-12EM-immunized group was better than that in the BCG-immunized group (1.7-fold, $P < 0.05$). The reduction of bacterial loads in saline control mice at 9 weeks compared to that at 4 weeks might be due to acquired immunity induced by *M. tuberculosis* infection. At 9 weeks postchallenge, the mice immunized with BCG or subunit vaccines showed a statistically significant decrease in bacterial numbers compared with saline-immunized mice ($P < 0.05$ for all groups). This suggests that the vaccine-induced memory immune response

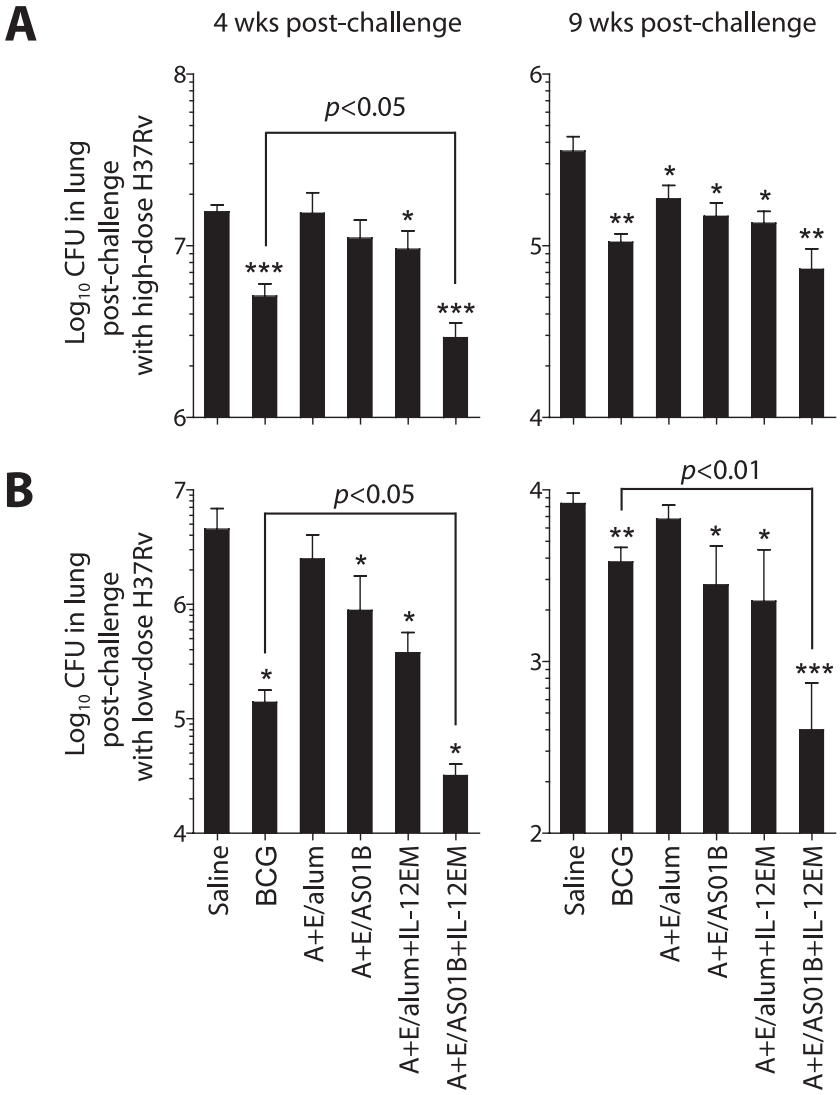

FIG. 3. Protection against *M. tuberculosis* by immunization with experimental TB vaccines. Mice were subcutaneously immunized at 0 and 8 weeks with recombinant Ag85A and ESAT-6 proteins in different adjuvant combinations. Positive-control mice received a single subcutaneous injection of *M. bovis* BCG at the first immunization. Eight weeks after the last immunization, mice were aerogenically challenged with high-dose (240 CFU per lung) (A) or low-dose (20 CFU per lung) (B) *M. tuberculosis* H37Rv using a previously described method (10). The numbers of live bacilli in the lungs of four to seven mice per group were assessed at 4 and 9 weeks (wks) after the challenge. The data are presented as mean CFU (±standard errors of the means). The lower limit of detection was 10^2 per organ. Thus, some organs which had 0 CFU on plates seeded with undiluted samples in the low-dose-infection experiment were scored with a CFU of 10^2 for statistical purposes. The levels of statistical significance for differences between test groups and the saline control group were determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The levels of significance of the differences between the A+E/AS01B+IL-12EM group and BCG group are shown above the bars.

was boosted by the *M. tuberculosis* challenge and that the immune response could control bacterial growth. Also, within this time period, BCG- and AS01B+IL-12EM-immunized groups showed 3.4-fold- and 4.9-fold-lower numbers of bacteria, respectively, than the saline-treated group ($P < 0.01$ for both). In cases of low-dose infection (Fig. 3B), the numbers of bacteria were reduced in the AS01B-, alum+IL-12EM-, and AS01B+IL-12EM-immunized groups compared to numbers in the control group. Alum-immunized mice did not show a meaningful protective efficacy at either 4 or 9 weeks. The difference in adjuvant protection effects between alum versus AS01B was definitive in the low-dose-challenge experiment. AS01B+IL-12EM was superior to BCG at 4 and 9 weeks (4.4-fold, $P < 0.05$, and 9.5-fold, $P < 0.01$, respectively). Reviewing the histopathology, a substantial portion of the air spaces in the lungs was filled with monocytes at 4 weeks of infection in control mice, but no significant difference was found between experimental groups despite a significant difference in bacterial loads (data not shown). This might be due to the infiltration of monocytes into the area of *M. tuberculosis* infection regardless of the bacterial number in the lesions. Bacterial loads in the spleen were lower than those in the lung. The differences between groups were less significant in the lung data, because bacteria were administered via an aerosol

route (supplemental Fig. 1). After aerosol administration of *M. tuberculosis*, the mice given subunit vaccines together with different adjuvants displayed a pattern of IFN-γ response similar to that observed before the challenge. However, saline-treated mice also showed a substantial IFN-γ response. This suggests that the antigen-specific IFN-γ response observed postchallenge might reflect not only the recall response but also the response elicited by the remaining bacteria (supplemental Fig. 2).

Our study is the first report demonstrating that the use of IL-12EM as an adjuvant in a TB subunit vaccine model is effective in the establishment of an antigen-specific Th1 immune response and protection against *M. tuberculosis*. Protection induced by the subunit vaccine combined with AS01B+IL-12EM appeared to be superior to that achieved by conventional BCG, suggesting that this adjuvant combination has clinical potential. Although the importance of IL-12 in the induction of Th1 immune response and the control of *M. tuberculosis* has been widely accepted, the adjuvant effect of rIL-12 in subunit vaccines appears to be transient (9, 15, 24). Recent reports clearly demonstrate that continuous IL-12 production is necessary for maintenance of the pulmonary Th1 cells required for the control of persistent *M. tuberculosis* infection (7). We also found that codelivery of IL-12EM could induce higher Th1 and $CD8^+$ T-cell responses than that of rIL-12 in hepatitis B virus and influenza subunit vaccine models (unpublished data). A precondition for the successful implementation of TB subunit vaccines is the generation of long-term T-cell memory response. In contrast to previous TB subunit vaccines (1), our subunit vaccine combined with AS01B+IL-12EM could sustain antigen-specific Th1 responses 8 weeks after the last immunization. This may be caused by IL-12EM since it is known that long-term expression of IL-12 can maintain the memory T-cell response (9, 24). Our promising results may lay the groundwork for introducing such vaccines as a practical alternative to BCG in the near future.

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REFERENCES

1. **Anacker, R. L., W. R. Barclay, W. Brehmer, C. L. Larson, and E. Ribi.** 1967. Duration of immunity to tuberculosis in mice vaccinated intravenously with oil-treated cell walls of Mycobacterium bovis strain BCG. J. Immunol. **98:** 1265–1273.
2. **Bojang, K. A., P. J. Milligan, M. Pinder, L. Vigneron, A. Allouecheb, K. E. Kester, W. R. Ballou, D. J. Conway, W. H. Reece, P. Gothard, L. Yamuah, M. Delchambre, G. Voss, B. M. Greenwood, A. Hill, K. P. McAdam, N. Tornieporth, J. D. Cohen, and T. Doherty.** 2001. Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet **358:**1927–1934.
3. **Brandt, L., T. Oettinger, A. Holm, A. B. Andersen, and P. Andersen.** 1996. Key epitopes on the ESAT-6 antigen recognized in mice during the recall of protective immunity to Mycobacterium tuberculosis. J. Immunol. **157:**3527–3533.
4. **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:**698–702.
5. **Crotts, G., and T. G. Park.** 1998. Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. J. Microencapsul. **15:**699–713.
6. **Egilmez, N. K., Y. S. Jong, M. S. Sabel, J. S. Jacob, E. Mathiowitz, and R. B. Bankert.** 2000. In situ tumor vaccination with interleukin-12-encapsulated biodegradable microspheres: induction of tumor regression and potent antitumor immunity. Cancer Res. **60:**3832–3837.
7. **Feng, C. G., D. Jankovic, M. Kullberg, A. Cheever, C. A. Scanga, S. Hieny, P. Caspar, G. S. Yap, and A. Sher.** 2005. Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. J. Immunol. **174:**4185–4192.
8. **Golumbek, P. T., R. Azhari, E. M. Jaffee, H. I. Levitsky, A. Lazenby, K. Leong, and D. M. Pardoll.** 1993. Controlled release, biodegradable cytokine depots: a new approach in cancer vaccine design. Cancer Res. **53:**5841–5844.
9. **Gurunathan, S., C. Prussin, D. L. Sacks, and R. A. Seder.** 1998. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nat. Med. **4:**1409–1415.
10. **Ha, S. J., B. Y. Jeon, S. C. Kim, D. J. Kim, M. K. Song, Y. C. Sung, and S. N. Cho.** 2003. Therapeutic effect of DNA vaccines combined with chemotherapy in a latent infection model after aerosol infection of mice with Mycobacterium tuberculosis. Gene Ther. **10:**1592–1599.
11. **Hill, H. C., T. F. Conway, Jr., M. S. Sabel, Y. S. Jong, E. Mathiowitz, R. B. Bankert, and N. K. Egilmez.** 2002. Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. Cancer Res. **62:**7254–7263.
12. **Iwata, M., Y. Nakamura, and J. W. McGinity.** 1999. In vitro and in vivo release properties of brilliant blue and tumour necrosis factor-alpha (TNF-alpha) from poly(D,L-lactic-co-glycolic acid) multiphase microspheres. J. Microencapsul. **16:**777–792.
13. **Kim, H. K., and T. G. Park.** 1999. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism. Biotechnol. Bioeng. **65:**659–667.
14. **Kim, T. H., and T. G. Park.** 2004. Critical effect of freezing/freeze-drying on sustained release of FITC-dextran encapsulated within PLGA microspheres. Int. J. Pharm. **271:**207–214.
15. **Lindblad, E. B., M. J. Elhay, R. Silva, R. Appelberg, and P. Andersen.** 1997. Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infect. Immun. **65:**623–629.
16. **Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois.** 2001. Preferential localization of effector memory cells in nonlymphoid tissue. Science **291:** 2413–2417.
17. **McMurray, D. N.** 2003. Recent progress in the development and testing of vaccines against human tuberculosis. Int. J. Parasitol. **33:**547–554.
18. **Pettit, D. K., J. R. Lawter, W. J. Huang, S. C. Pankey, N. S. Nightlinger, D. H. Lynch, J. A. Schuh, P. J. Morrissey, and W. R. Gombotz.** 1997. Characterization of poly(glycolide-co-D,L-lactide)/poly(D,L-lactide) microspheres for controlled release of GM-CSF. Pharm. Res. **14:**1422–1430.
19. **Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia.** 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature **401:**708–712.
20. **Sanchez, A., M. Tobio, L. Gonzalez, A. Fabra, and M. J. Alonso.** 2003. Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha. Eur. J. Pharm. Sci. **18:**221–229.
21. **Skeiky, Y. A., M. R. Alderson, P. J. Ovendale, J. A. Guderian, L. Brandt, D. C. Dillon, A. Campos-Neto, Y. Lobet, W. Dalemans, I. M. Orme, and S. G. Reed.** 2004. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J. Immunol. **172:**7618–7628.
22. **Snapper, C. M., and W. E. Paul.** 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science **236:**944–947.
23. **Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta.** 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature **334:**255–258.
24. **Stobie, L., S. Gurunathan, C. Prussin, D. L. Sacks, N. Glaichenhaus, C. Y. Wu, and R. A. Seder.** 2000. The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc. Natl. Acad. Sci. USA **97:**8427–8432.
25. **Tanghe, A., S. D'Souza, V. Rosseels, O. Denis, T. H. Ottenhoff, W. Dalemans, C. Wheeler, and K. Huygen.** 2001. Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. Infect. Immun. **69:**3041–3047.
26. **Tebo, A. E., M. J. Fuller, D. E. Gaddis, K. Kojima, K. Rehani, and A. J. Zajac.** 2005. Rapid recruitment of virus-specific CD8 T cells restructures immunodominance during protective secondary responses. J. Virol. **79:**12703–12713.

27. **Unsoeld, H., and H. Pircher.** 2005. Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7. J. Virol. **79:**4510–4513.
28. **Weinrich Olsen, A., L. A. van Pinxteren, L. Meng Okkels, P. Birk Rasmussen, and P. Andersen.** 2001. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. Infect. Immun. **69:**2773–2778.
29. **Winslow, G. M., A. D. Roberts, M. A. Blackman, and D. L. Woodland.** 2003. Persistence and turnover of antigen-specific CD4 T cells during chronic tuberculosis infection in the mouse. J. Immunol. **170:**2046–2052.
30. **Yang, J., and J. L. Cleland.** 1997. Factors affecting the in vitro release of recombinant human interferon-gamma (rhIFN-gamma) from PLGA microspheres. J. Pharm. Sci. **86:**908–914.

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