Tuberculosis remains a major health problem affecting millions of people worldwide (5). Combination chemotherapy is very effective in curing this disease but, unfortunately, the treatment is long and expensive and requires stringent compliance to avoid the development of multi-drug-resistant forms of *Mycobacterium tuberculosis*. The only tuberculosis vaccine currently available is an attenuated strain of *M. bovis*, termed bacillus Calmette-Guérin (BCG). BCG continues to be widely administered to children in developing countries, yet its efficacy remains controversial, particularly against pulmonary tuberculosis in young adults (4). Clearly, the development of a better vaccine could be an effective solution to the global threat of tuberculosis.

The protective antigens for tuberculosis are still not precisely defined, and this seriously hampers every effort to improve or replace the existing tuberculosis vaccine. It has been hypothesized for more than a decade that extracellular (secreted or cell-wall-associated) proteins rather than intracellular, cytoplasmic proteins are the key antigens recognized by the protective immune response (27). Immunization with whole culture filtrate, which is a rich source of these exported proteins, has been described to protect mice and guinea pigs to some extent against subsequent challenge with the tubercle bacillus (1, 17, 28, 29). A major portion of the secreted proteins in *M. tuberculosis* and BCG culture filtrate is formed by the Ag85 complex, a 30- to 32-kDa family of proteins (Ag85A, Ag85B, and Ag85C) (39). Ag85 complex induces strong T-cell proliferation and gamma interferon (IFN-γ) production in most healthy individuals infected with *M. tuberculosis* or *M. leprae* and in BCG-vaccinated mice and humans (19, 24, 30, 31), making it a promising candidate as a protective antigen. We have previously shown that intramuscular (i.m.) vaccination with plasmid DNA encoding Ag85A induced strong humoral and cell-mediated immune responses and conferred significant protection in C57BL/6 mice challenged by aerosol with live *M. tuberculosis* H37Rv (20).

Administration of plasmid DNA expression vectors seems broadly applicable for generating protective immune responses against infectious pathogens without the need for live organisms, replicating vectors, or adjuvants (12, 35). Two major inoculation routes have been used so far for DNA vaccination: i.m. needle injection of DNA in saline (40) and epidermal gene gun (gg) bombardment with DNA-coated gold particles (32). For i.m. injections, routine doses of DNA in the mouse range between 10 and 100 µg. gg injections use considerably less DNA, with standard doses of between 0.1 and 1 µg. Because of the low plasmid doses used in gg immunizations, this technique has the potential of lower vaccine cost. Furthermore, mixing of a number of plasmids is possible in gg vaccinations, and pools of plasmids can be screened by expression library immunization (3). Finally, gg immunization does not require the use of needles, which makes it an ideal method for use in children and human immunodeficiency virus-infected populations; also, this technique is easier to apply to a large-scale immunization.

In order to analyze whether gg immunization with plasmid DNA would be applicable to tuberculosis, we have compared the two current DNA immunization protocols, i.e., i.m. needle injection and gg bombardment with plasmid DNA encoding Ag85A from *M. tuberculosis*. Since we have previously shown that C57BL/6 mice demonstrate a stronger Th1-type immune response toward Ag85 following *M. bovis* BCG vaccination than BALB/c mice (in which this response is partly counter-balanced by Th2 cells) (19), comparative analysis of the gg and
i.m. routes was performed on both strains. Whereas gg immunization induced strong antibody and CTL responses, TH-type cytokine production was disappointingly low compared to i.m. immunization. Furthermore and unexpectedly, gg immunization was effective only in BALB/c mice and not in C57BL mice.

MATERIALS AND METHODS

Plasmid construction. Plasmid DNA encoding Ag85A was prepared as described previously. Briefly, the 85A gene of M. tuberculosis was amplified without its mycobacterial signal sequence from plasmid p85A.Tub (7) by PCR and ligated to the dephosphorylated VR1020 (Vical, Inc., San Diego, Calif.) vector. Recombinant plasmid DNA was amplified in Escherichia coli DH5 and purified on two cesium chloride-ethidium bromide gradients. Plasmid DNA was adjusted to a final concentration of 1 mg/ml in saline and stored at -20°C. In this plasmid, the Ag85A gene is expressed under control of the promoter and intron A of the first immediate-early antigen IE1 from cytomegalovirus and followed by a polyadenylation site of the bovine growth hormone. In the VR1020 vector a leader sequence of human tissue plasminogen activator is cloned upstream of the mature Ag85A gene, resulting in increased transcription and translation efficacy and increased immunogenicity (2).

Mice. BALB/c (H-2 7), C57BL/6 (H-2 1), and C57BL/10 (H-2 2) mice were bred in the Animal Facilities of the Pasteur Institute of Brussels. Only female mice, 6 to 8 weeks old at the start of vaccination, were used.

Coating of gold beads. Gold beads were coated with plasmid DNA according to the manufacturer’s recommendations. In short, 25 mg of gold powder was mixed with 100 µl of spermidine (0.05 M, Sigma) and sonicated. Next, 100 µg (first preparation) or 50 µg (all other experiments) of plasmid DNA encoding Ag85A in a 100-µl volume was added. Finally, 200 µl of 1 M CaCl2 was added dropwise to the mixture with gentle vortexing. After a 10-min precipitation step at room temperature, the pellets were washed three times and then resuspended in 100% ethanol. Finally, the pellets were resuspended in an ethanol solution containing 0.01 mg polyvinylpyrrolidone (Bio-Rad) per ml and coated on special tubing (Gold-Coat tubing; Bio-Rad). Cut cartridges containing nitrogen-dried m gold beads coated with 20°C.

gg immunization. Mice were vaccinated on the shaved ventral skin using the Helios Gene Gun System (Bio-Rad) at a helium discharge pressure of 400 lb/in². In the first experiment, three gg immunizations were performed at 3-week intervals consisting of two nonoverlapping spots of 0.5 µg of either 0.6, 1. or 1.6-µm-diameter gold beads coated with 5 µg of plasmid DNA. In the other experiments, mice were vaccinated three times at 3-week intervals with two nonoverlapping spots of 1-µm gold beads coated with 1 µg of plasmid DNA. i.m. DNA vaccination. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine (100 and 10 mg/kg, respectively) and injected i.m. in the abdomen using DNA-coated gold particles 0.6, 1, and 1.6 µm in diameter (5-10 µg/ml) at various effector/ target ratios. Data are expressed as the percent specific lysis. Spontaneous release was generally 10 to 15% of the total release.

M. tuberculosis challenge. BALB/c and C57BL/6 mice were vaccinated three times at 3-week intervals with control plasmid or Ag85A DNA either by gg bombardment (two shots, 1 µg/shot) or by i.m. injection (two injections, 50 µg/injection). Mice were rested for 2 months after the third DNA immunization and challenged intravenously in a tail vein with 109 CFU of M. tuberculosis H37Rv grown as a surface pellicle for 2 weeks on synthetic Sauton medium and stored as a stock solution at -70°C in glycerol. Three weeks after challenge, mice were sacrificed, and serial threefold spleen and lung homogenate dilutions were plated on 7H11 Middlebrook agar supplemented with OADC (33). Petri dishes were incubated for 4 weeks in sealed plastic bags at 37°C, and colonies were counted visually. For statistical analysis (Student’s t test), data obtained from two or three experiments were used to calculate the mean log 10 CFU values per spleen or lung. Data are expressed as mean log 10 values per experimental group (each consisting of four to six animals).

RESULTS

Antibody production in mice vaccinated with 10 µg of plasmid DNA encoding Ag85A from M. tuberculosis administered i.m. by needle or epidermally with gold particles of three different sizes. In a preliminary experiment, we compared gg and i.m. administration of a same dose of plasmid DNA, i.e., 10 µg/injection. Although this dose was probably not optimal for another route (too high for gg method and too low for the i.m. method), the idea was to have the same level of immunostimulatory effects linked to the CpG ODN content (23) of the vector backbone for both immunization routes. Ag85A DNA was administered three times at 3-week intervals to BALB/c or C57BL/10 mice, either by i.m. injection in saline in both quadriceps muscles (5 µg/hind leg) or epidermally by two gg injections in the abdomen using DNA-coated gold particles 0.6, 1, and 1.6 µm in diameter (5 µg/bead). Mice were bled 3 weeks after the last DNA inoculation. As shown in Fig. 1, significant Ag85-specific antibody production could be detected following gg vaccination in BALB/c mice, whereas antibody levels in gg-injected B10 mice were only slightly above values obtained in mice vaccinated by gg with empty VR1020 vector. A bead size of 1 µm was found to give the best antibody response, i.m. vaccination with 10 µg of plasmid DNA induced elevated antibody levels in B10 mice, but this route was too immunineffective in BALB/c mice. Mean Ag85-specific antibody levels in gg-injected BALB/c mice were ca. 40,000 ng/ml, and mean antibody levels in i.m. vaccinated B10 mice were ca. 75,000 ng/ml.

As shown in Fig. 2, antibody levels in gg-vaccinated BALB/c mice started to increase after the second shot of DNA and increased further after the third gg administration. After i.m. immunization of B10 mice, immunoglobulin antibodies started to increase already after one injection, and concentrations increased after the second and third immunization. In gg-
Antibody production in mice vaccinated with plasmid DNA encoding Ag85A from *M. tuberculosis*. Antibody responses in gg-vaccinated BALB/c and B10 mice were preferentially of the IgG1 isotype with little IgG2a, a finding indicative of Th2-type helper-T-cell activation (37). In contrast, antibody isotypes in i.m.-vaccinated C57BL/10 mice were strongly indicative of Th1 activation, with IgG2a and IgG2b titers 10- to 20-fold higher than IgG1 titers both at low and high DNA doses. Isotypes in i.m.-vaccinated BALB/c mice were of a mixed phenotype, even at the high DNA dose used, indicating that it is not only the dose of DNA that determines the isotype profile in DNA vaccination, but the genetic background of the mouse strain as well. Antibody isotypes in i.m.-vaccinated B6 mice were also strongly biased towards IgG2 (20).

IL-2 and IFN-γ production in mice vaccinated by the gg or the i.m. route with plasmid DNA encoding Ag85A from *M. tuberculosis*. As shown in Fig. 3A, spleen cells from BALB/c mice vaccinated with plasmid DNA encoding Ag85A produced weak IL-2 levels in response to purified Ag85A following gg or low-dose i.m. injection. High-dose i.m. DNA induced significant IL-2 production in BALB/c mice. In B10 mice, elevated IL-2 levels could be induced following i.m. vaccination with both low and high doses of DNA, whereas gg vaccination was completely ineffective for IL-2 induction in B10 mice. As for IL-2, IFN-γ production in spleen cell cultures from DNA-vaccinated mice restimulated in vitro with purified Ag85A was highest in B10 mice injected i.m. (about 6 log₂ U, corresponding to ca. 3,200 pg of IFN-γ per ml (Fig. 3B). Spleen cell cultures from BALB/c mice produced considerably lower titers (between 400 and 800 pg/ml) following either gg or low-dose i.m. injection, whereas significantly better IFN-γ production, albeit still at least twofold lower than in B10 mice, was observed upon high-dose i.m. immunization. As for IL-2, gg vaccination elicited only a marginal Ag85-specific IFN-γ response (ca. 100 pg/ml) in spleen cell cultures from B10 (or B6 [data not shown]) mice.

CTL responses in BALB/c mice vaccinated by the gg or the i.m. route with plasmid DNA encoding Ag85A from *M. tuberculosis*. So far we have only been able to detect Ag85A-specific CD8⁺-mediated CTL activity (as measured in a ⁵¹Cr release assay) following i.m. immunization in BALB/c mice but not in B10 or B6 mice (11). Whether this lack of detectable CTL activity in *H-2b* mice vaccinated with Ag85A DNA is a technical problem or related to an absence of immunodominant
MHC-I-restricted epitopes for the H-2Kb and H-2Db alleles on the protein is not yet clear. Therefore, we could only analyze BALB/c mice for the generation of CTL activity. Both gg and i.m. immunization were capable of generating strong CTL responses to the MHC-I H-2Kd-restricted peptide from Ag85A (amino acids 144 to 152), which we have defined previously (11) (Fig. 4). As for antibody responses, gg immunization was effective for the induction of CTL responses at plasmid doses 50-fold lower than those required for optimal CTL induction by i.m. immunization. I.m. immunization with doses of 2 or 10 μg of plasmid DNA induced only suboptimal CTL responses (data not shown).

IL-4 and interleukin-10 production in mice vaccinated with plasmid DNA encoding Ag85A from M. tuberculosis, administered i.m. or by gg with 1-μm gold particles. As shown in Table 2 and as already described following i.m. immunization (20), IL-4 and IL-10 production in spleen cells from gg-vaccinated mice was very low following in vitro restimulation with the purified Ag85A. However, production of these two Th2-type cytokines following polyclonal PWM stimulation showed that the basal production level was about fivefold higher in BALB/c mice than in B10 mice and that i.m. but not gg immunization could reduce the PWM-induced stimulation of IL-4 more than 10-fold. However, PWM-induced IL-4 levels in i.m.-vaccinated BALB/c mice were still considerable (ca. 1,000 pg/ml). PWM-induced IL-10 levels did not seem to be affected by either immunization route and remained at least five times higher in BALB/c than in B10 mice.

Protection against M. tuberculosis H37Rv replication in the spleen and lungs of mice vaccinated with Ag85A DNA by the i.m. and not the gg route. As shown in Fig. 5, only i.m. vaccination and not gg vaccination with Ag85A DNA was capable of reducing significantly the number of CFU in the spleen and lungs compared to the number of CFU in animals vaccinated with empty vector. Furthermore, this protection was only observed in B6 mice and not in BALB/c mice. C57BL/10 mice

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**TABLE 2.** IL-4 and IL-10 production in BALB/c and B10 mice vaccinated with plasmid DNA encoding Ag85A by gg (2 μg) or i.m. (100 μg) injections

<table>
<thead>
<tr>
<th>Immunization (route)</th>
<th>IL-4 (pg/ml) stimulated with:</th>
<th>IL-10 (pg/ml) stimulated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ag85A</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>223</td>
<td>113</td>
</tr>
<tr>
<td>Ag85A (gg)</td>
<td>337</td>
<td>267</td>
</tr>
<tr>
<td>Ag85A (i.m.)</td>
<td>145</td>
<td>41</td>
</tr>
<tr>
<td>C57BL/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Ag85A (gg)</td>
<td>87</td>
<td>15</td>
</tr>
<tr>
<td>Ag85A (i.m.)</td>
<td>&lt;5</td>
<td>42</td>
</tr>
</tbody>
</table>

*IL-4 and IL-10 levels in 72-h spleen cell culture supernatants from control or DNA-vaccinated mice (four mice pooled/group) stimulated in vitro with purified Ag85A or PWM are shown.*
were also protected by i.m. Ag85A DNA vaccination (data not shown). BALB/c mice were more susceptible to a same intra-venous inoculum (10^6 CFU) of *M. tuberculosis* than B6 mice, as reflected by higher CFU counts, in spleen and lungs, in control DNA vaccinated animals.

**DISCUSSION**

Over the last 4 years, we and others have reported that vaccination of mice with plasmid DNA encoding the 65-kDa heat shock protein (34), the 38-kDa phosphate-binding PstS-1 homolog (41), the 40-kDa phosphate-binding PstS-3 homolog (33), and the 30- to 32-kDa trehalose-mycolyl-transferase antigens 85A (20) and 85B (22) from *M. tuberculosis* is a powerful means for inducing strong humoral and cell-mediated immune responses and protective immunity against tuberculosis challenge (18). All of these observations used the i.m. immunization route and, to our knowledge, no data on the immunogenicity or protective efficacy of tuberculosis DNA vaccines administered by gg have been published thus far.

Using plasmid DNA encoding the nucleoprotein and hemagglutinin from influenza, Feltquate et al. have reported that i.m. injection of BALB/c mice with saline solutions of DNA induces preferentially a Th1-type T-helper response, whereas epidermal gg injection biases the immune response toward a strong Th2-type profile (13, 36). In order to find out whether this dichotomy could also be observed with plasmid DNA encoding mycobacterial genes, which are all characterized by a high GC content (ca. 70%) and therefore probably a high inherent CpG linked Th1-type immunostimulatory activity (23), we vaccinated BALB/c and C57BL mice with various doses of plasmid DNA encoding Ag85A from *M. tuberculosis* by epidermal gg bombardment or i.m. needle injection.

Confirming Feltquate’s data, the gg immunization was a very effective technique for inducing strong antibody responses, with an isotypic profile suggestive of Th2 activation. Plasmid doses needed for an optimal antibody response were at least 50-fold lower with the gg immunization than with i.m. immunization. A new finding, however, was that only BALB/c mice produced strong antibody responses following gg immunization, whereas C57BL mice were only very weakly stimulated by this immunization route. This discrepancy between the two mouse strains could not be attributed directly to MHC-linked differences, since MHC congenic BALB.B10 mice also demonstrated strong antibody responses following gg immunization (data not shown). BALB/c mice have been reported to be Th2 “prone” in a number of experimental situations, such as experimental leishmaniasis (16, 25), BCG vaccination (19), and tuberculosis infection (8). It is tempting to speculate that this Th2 “proneness” of the genetic BALB/c background is the major factor determining their strong antibody reactivity following gg immunizations. Furthermore, the capacity to produce the Th2-type cytokines IL-4 and IL-10 following polyclonal stimulation with PWM was clearly higher in BALB/c mice than in B10 mice. In line with these findings, Virelizier previously reported that BALB/c mice produce significantly

![Image of graphs showing CFU counts in spleen and lungs over time for different immunization routes and doses of plasmid DNA encoding Ag85A from *M. tuberculosis*.](http://iai.asm.org/Downloaded from http://iai.asm.org/ on September 21, 2017 by guest)
lower IFN-γ titers in response to PWM than did B10 mice (38). Finally, antibody isotypes were of a clearcut Th1 type in B10 mice vaccinated i.m. with DNA, but in BALB/c mice even the high i.m. dose of 100 µg of plasmid DNA induced a mixed isotype profile with elevated IgG1 and IgG2 titers, indicating that it is very difficult to overcome this Th2 proneness of BALB/c mice with the Ag85A DNA vaccine. An in vivo dominance of the Th2 over the Th1 response might explain why the Ag85A DNA vaccine only conferred protection against an intravenous M. tuberculosis challenge in C57BL mice and not in BALB/c mice, although i.m. immunization clearly induced a strong IL-2 and IFN-γ response in both mouse strains (although the B10 response was consistently higher than the BALB/c response). Interestingly, only i.m. vaccination of B10 mice completely blocked the PWM-induced IL-4 response, whereas in i.m.-vaccinated BALB/c mice this PWM-induced IL-4 response was still quite elevated, albeit somewhat reduced compared to the response of naive or gg-immunized BALB/c mice. With the exception of the 65-kDa heat shock protein, the new experimental tuberculosis vaccines, whether protein or DNA based, have all been tested in C57BL mice (1, 17, 20, 22, 26, 33, 41). Whether they are also effective in BALB/c mice remains an open question in the light of the results presented here.

A new and unexpected finding was that C57BL mice reacted only weakly to the gg immunization protocol, even at a plasmid dose of 10 µg, at which i.m. immunization induced elevated immune responses comparable to those induced by 100 µg given i.m. As DNA vaccines prime for immune response through the action of professional antigen-presenting cells (APC) (9), our findings could indicate that the Langerhans cells from the skin (the probable APC population involved in gg immunization) would be more effective in some mouse strains, such as BALB/c and BALB.B10, than in others, such as B10 and B6.

Protective immunity with the Ag85A DNA vaccine could only be induced in B6 (and B10) mice and not in BALB/c mice and only by i.m. and not by gg immunization, thus demonstrating that high Th1-type IFN-γ responses rather than strong CTL or antibody responses are critical immune parameters. Indeed, CD8+ cell-mediated CTL activity was very efficiently induced in gg-vaccinated BALB/c mice, but no protection against intravenous M. tuberculosis challenge could be observed. As CD8+-mediated CTL responses require endogenous processing of the antigen, this generation of strong CTL responses despite low Th1 cytokine titers was not completely blocked by the PWM-induced IL-4 response, whereas in i.m.-vaccinated BALB/c mice this PWM-induced IL-4 response was still quite elevated, albeit somewhat reduced compared to the response of naive or gg-immunized BALB/c mice. With the exception of the 65-kDa heat shock protein, the new experimental tuberculosis vaccines, whether protein or DNA based, have all been tested in C57BL mice (1, 17, 20, 22, 26, 33, 41). Whether they are also effective in BALB/c mice remains an open question in the light of the results presented here.

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lates a CD4^+ and CD8^+ T-cell epitopic repertoire broader than that stimulated by Mycobacterium tuberculosis H37Rv infection. Infect. Immun. 66: 1527–1533.


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