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Th1 polarized response induced by intramuscular DNA-HSP65 immunization is preserved in experimental atherosclerosis

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Abstract

We previously reported that a DNA vaccine constructed with the heat shock protein (HSP65) gene from *Mycobacterium leprae* (DNA-HSP65) was protective and also therapeutic in experimental tuberculosis. By the intramuscular route, this vaccine elicited a predominant Th1 response that was consistent with its protective efficacy against tuberculosis. It has been suggested that the immune response to Hsp60/65 may be the link between exposure to microorganisms and increased cardiovascular risk. Additionally, the high cholesterol levels found in atherosclerosis could modulate host immunity. In this context, we evaluated if an atherogenic diet could modulate the immune response induced by the DNA-HSP65 vaccine. C57BL/6 mice (4-6 animals per group) were initially submitted to a protocol of atherosclerosis induction and then immunized by the intramuscular or intradermal route with 4 doses of 100 μg DNA-HSP65. On day 150 (15 days after the last immunization), the animals were sacrificed and antibodies and cytokines were determined. Vaccination by the intramuscular route induced high levels of anti-Hsp65 IgG2a antibodies, but not anti-Hsp65 IgG1 antibodies and a significant production of IL-6, IFN-γ and IL-10, but not IL-5, indicating a Th1 profile. Immunization by the intradermal route triggered a mixed pattern (Th1/Th2) characterized by synthesis of anti-Hsp65 IgG2a and IgG1 antibodies and production of high levels of IL-5, IL-6, IL-10, and IFN-γ. These results indicate that experimentally induced atherosclerosis did not affect the ability of DNA-HSP65 to induce a predominant Th1 response that is potentially protective against tuberculosis.

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- Tuberculosis
- Th1
- Cholesterol

Introduction

Approximately two billion people are infected with *Mycobacterium tuberculosis* (Mtb) and at least 14 million are co-infected with HIV (1). Among those carrying the pathogen, about 8.8 million persons come down with clinical disease every year and about 1.5 million die (2).

The only vaccine against tuberculosis

(TB) currently available is Calmette-Guérin Bacillus. However, its efficacy remains controversial, particularly against pulmonary TB in young adults (3). Development of a better vaccine is urgently needed to counter this global threat. A DNA vaccine containing the gene encoding the heat shock protein of 65 kDa (HSP65) from *Mycobacterium leprae* presented a prophylactic and therapeutic effect against experimental TB (4-7). These effects were associated with a cellular immune response against Hsp65, characterized by production of high IFN-γ levels and activation of cytotoxic lymphocytes (5,6). Administration of this DNA vaccine by the subcutaneous route using a gene gun induced a predominant humoral antigen-specific immune response, with no protective activity (7).

Since a higher incidence of TB is being observed in the adult population, it is expected that a new TB vaccine would be applied to this group in addition to newborns. The immunological background of this population could affect the ensuing immune response induced by DNA-HSP65. A previous immune response against Hsp65/60 would be highly expected in the adult population because Hsp65 is a ubiquitous antigen in the environment. Antibodies and T lymphocytes specific for Hsp65 have, indeed, been described in normal individuals (8).

In addition, it has been well established that immune mechanisms are involved in all stages of atherosclerotic disease, which is highly prevalent in the western world (9-12). Within the group of antigens that may be responsible for this immunological activation during atherosclerosis, Hsp65/60 became a strong candidate. Immunization of normocholesterolemic rabbits with Hsp65 led to the development of atherosclerotic lesions in the aortic intima and these primary inflammatory lesions were aggravated by a cholesterol-rich diet (13). Interestingly, these lesions completely resembled human fatty streaks and atherosclerotic plaques (13,14). Furthermore, T cells isolated from atherosclerotic lesions showed reactivity against mycobacterial Hsp65, suggesting that cell-mediated immune responses to Hsp60 are also involved in the pathogenesis of this disease (14). An increased cross-reactivity of anti-Hsp65 serum antibodies was also described in subjects with carotid atherosclerosis diagnosed by sonography (15,16). It is, therefore, expected that the adult population could be already primed for an anti-Hsp65 response that could affect an ensuing response to DNA-HSP65 vaccination. In addition, hypercholesterolemia has been associated with a modulatory activity on immune functions (17,18). A preferential differentiation towards a strong Th2 phenotype was previously described (19) and recently confirmed in the presence of an extreme hypercholesterolemic condition (20).

In the present study, we determined if a protocol of atherosclerosis induction that included immunization with Mtb associated with a high cholesterol diet (HCD) could affect the immune response pattern normally induced by the DNA-HSP65 vaccine administered by the intramuscular (*im*) and intradermal (*id*) routes.

Material and Methods

Animals

Female specific pathogen-free C57BL/6 mice were bred in the Animal Facility of the School of Medicine of Ribeirão Preto, University of São Paulo, and used at 4 to 6 weeks of age.

Experimental design

Atherosclerosis was induced by an HCD (1.25%) containing 0.5% cholic acid administered for 105 days (15 weeks) in combination with 3 doses of 250 µg heat-killed Mtb

(H37Ra) emulsified in incomplete Freund's adjuvant on days 0, 15, and 30. On day 105, HCD was replaced with a chow diet and the mice were immunized with DNA (4 doses of 100 µg pVAX-HSP65 or 100 µg pVAX administered by the *im* route in the presence of 25% saccharose, or with 4 doses of 1 µg pVAX-HSP65 or 1 µg pVAX administered with a gene gun by the *id* route) on days 105, 115, 125, and 135. The following control groups (4-6 mice per group) were included: Mtb (animals fed the HCD and immunized with Mtb); pVAX *im* and pVAX *id* (animals fed the HCD, immunized with Mtb and injected with the empty vector by the *im* or *id* route); normal mice (animals fed a chow diet). Weights were recorded weekly and blood samples were obtained twice: by the end of the HCD and just before animal sacrifice. On day 150 (15 days after the last DNA immunization), mice were sacrificed, anti-Hsp65 antibodies were determined in individual sera and cytokines (IL-5, IL-6, IL-10, and IFN-γ) were quantified in spleen cell culture supernatants.

Determination of cholesterol levels

Venous blood was collected on day 105 (end of the HCD) and on day 150 (15 days after the last DNA immunization). Plasma cholesterol levels were measured by an enzyme-based method (Colestat enzimático, Weiner Lab SAIC, Rioambra, Rosario, Argentina) according to manufacturer instructions. Data are reported as g/L.

DNA-HSP65 vaccine construction

The pVAX-HSP65 vaccine was derived from the pVAX vector (Invitrogen®, Carlsbad, CA, USA), previously digested with *Bam*HI and *Not*I (Gibco BRL, Gaithersburg, MD, USA) by inserting a 3.3-kb fragment corresponding to the *M. leprae* HSP65 gene and the cytomegalovirus intron A. The empty pVAX vector was used as a control. DH5α *E. coli* transformed with plasmid pVAX or the plasmid containing the HSP65 gene (pVAX-HSP65) was cultured in LB liquid medium (Gibco BRL) containing kanamycin (100 µg/mL). The plasmids were purified using the Concert High Purity Maxiprep System (Gibco BRL). Plasmid concentrations were determined by spectrophotometry at $\lambda = 260$ and 280 nm using the Gene Quant II apparatus (Pharmacia Biotech, Buckinghamshire, UK).

Coating of gold microparticles with DNA

Gold microparticles (1.6 µm in diameter) were coated with plasmid DNA according to manufacturer instructions. Briefly, 50 mg gold powder was mixed with 100 µL 50 mM spermidine (Bio-Rad, Hercules, CA, USA) and sonicated. One hundred micrograms plasmid DNA encoding HSP65 or control vector pVAX in a 100-µL volume was added to the mixture. Then, 100 µL 1 M $CaCl_2$ was added drop by drop to the mixture with gentle vortexing. After a 10-min precipitation at room temperature, the pellets were washed three times and then re-suspended in 200 µL 100% ethanol solution containing 0.1 mg/mL polyvinylpyrrolidone (Bio-Rad) and loaded into cartridges of the Tubing Prep Station (Bio-Rad). Cartridges containing nitrogen-dried DNA-coated gold microparticles were stored at 4ºC.

Immunization procedure

For *im* immunization, mice were injected with 100 µg DNA (pVAX or pVAX-HSP65) in combination with 25% saccharose. For *id* immunization, mice were shot with 1 µg DNA (pVAX or pVAX-HSP65) into previously shaved abdominal skin using the Helius Gene Gun System (Bio-Rad) with a discharge pressure of 1792 kPa. Four gene gun immunizations were executed at 10-day intervals, corresponding to a total of 4 µg DNA. Control animals received only the

pVAX vector by the *im* or *id* route. Four *im* doses were administered at 10-day intervals for a total of 400 µg DNA.

Cytokine evaluation

On day 150 (15 days after the last DNA immunization), the animals were sacrificed and spleen cells were collected and adjusted to 5 x 10^6 cells/mL in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 5% fetal calf serum, 20 mM glutamine and 40 IU/L gentamicin and polymixin (30 µg/mL). The cells were cultured on 48-well flat-bottomed culture plates (Nunc, Life Technologies Inc., Maryland, MA, USA) in the presence of 10 µg/mL recombinant Hsp65 (r-Hsp65), 40 µg/mL concanavalin A or a 1/5000 final dilution of *Staphylococcus aureus* Cowan I. Cytokine levels in culture supernatants were determined 48 h later by enzyme-linked immunosorbent assay; the limit of detection was 39 pg/mL. Levels of IL-5, IL-6, IL-10, and IFN-γ were measured according to manufacturer instructions (BD Biosciences, PharMingen, San Diego, CA, USA). Purified monoclonal antibodies anti-IFN-γ (R4-6A2), IL-5 (TRKF5), IL-6 (MP5-20F3), and IL-10 (JES55-22A5) were used at 1 µg/mL as capture antibodies. The following biotinylated antibodies were used for detection: anti-IFN-γ (XMG1.2), IL-5 (TRFK4), IL-6 (MP5-32C11), and IL-10 (SXC-1) at 0.5 µg/mL.

Anti-Hsp65 antibody levels

Sera from vaccinated mice were collected by retro-orbital bleeding before atherosclerosis induction (pre-immune sera) and on day 150 (15 days after the last DNA immunization). To assess anti-Hsp65-specific antibody levels, 96-well plates (Maxisorp Nunc-Immuno plates, Life Technologies Inc.) were coated with 0.1 mL purified r-Hsp65 (5 µg/mL) in coating solution (14.3 mM Na_2CO_3, 10.3 mM $NaHCO_3$, pH 9.6), incubated at 4ºC overnight, and then blocked with 10% fetal calf serum in PBS for 60 min at 37ºC. Ten-fold dilutions of serum samples were tested. After incubation for 2 h at 37ºC, anti-mouse IgG1 and IgG2a biotinylated conjugates (A85-1 and R19-15, respectively; Pharmingen) were added for the detection of specific antibodies. After washing, plates were incubated at room temperature for 30 min with the StreptAB kit (Dako, Carpinteria, CA, USA) and developed by adding OPD + H_2O_2. Color development was stopped with H_2SO_4 and absorbance was measured at 490 nm.

Statistical analysis

Data are reported as means ± SEM. Statistical analysis was performed using analysis of variance (ANOVA), followed by the Tukey test, with the level of significance set at $P < 0.05$.

Results

Weight, cholesterol levels and liver alterations

Average animal weight was 17 g at the beginning of the experiments. A discrete increase in body weight (data not shown) was observed during administration of the HCD for 105 days. As the animals showed signs of toxicity and because this period of time was described in the literature as sufficient to induce atherosclerosis (21), after 105 days the HCD was replaced with a chow diet. By day 105, serum cholesterol levels were significantly higher in all groups fed the HCD compared to normal mice. By the end of the experiment, day 150, cholesterol levels were similar to control, as shown in Figure 1A. Steatosis (data not shown) and evident macroscopic alterations (Figure 1B) were observed by day 150, even though the HCD had been replaced with a chow diet 45 days before.

Anti-Hsp65 antibody levels

In a previous report, we observed that immunization with DNA-HSP65 by the *im* route preferentially induced anti-Hsp65 IgG2a antibodies whereas immunization by the *id* route led to higher IgG1 production (7). To determine if an atherogenic diet could interfere with or modulate antibody production induced by DNA-HSP65 immunization, mice submitted to the atherosclerosis-induction protocol were immunized with DNA-HSP65. Baseline levels of anti-Hsp65 antibodies (pre-immune serum) were detected before DNA immunization (Figure 2). A significant increase in antibody production was detected after pVAX-HSP65 immunization and the route of vaccine administration affected the profile of antibody production. The *id*, but not the *im*, route induced high levels of IgG1. Both routes were associated with significant and specific IgG2a production but the *id* route was associated with significantly higher levels comparing with the *im* route. As expected, empty vector injected by both routes did not induce antibody production.

Cytokine induction

To further characterize the immunological profile induced by the two immunization routes, we quantified the cytokines initially induced by a specific stimulus. On day 150 (15 days after the last DNA immunization), mice were sacrificed and spleen cells cultured in the presence of r-Hsp65. Significant IFN-γ production was detected in cultures from animals vaccinated with pVAX-HSP65 by both routes, in comparison with the Mtb control group and the groups that received only the vector pVAX (Figure 3). Significant IL-5 levels were also detected in spleen cultures from *id*-, but not from *im*-immunized animals compared to other groups. Significant production of IL-6 and IL-10 was induced by r-Hsp65 in cultures from mice vaccinated by both routes. However, significantly higher levels were observed in the group immunized with pVAX-HSP65 by the *id* route.

The splenic cytokine profile was also evaluated after nonspecific stimulation with concanavalin A or *S. aureus.* Animals that received pVAX or pVAX-HSP65 by the *im* route produced similar amounts of IFN-γ, IL-5, IL-6, and IL-10 after nonspecific stimulation (data not shown). However, animals vaccinated by the *id* route produced more IFN-γ and IL-5, but not IL-6 or IL-10 after nonspecific stimulation compared to animals injected with the vector by the same route. In addition, *id* DNA-HSP65 inoculation was associated with higher production of these four cytokines compared to *im* immunization, even though this difference was significant only for IFN-γ production (data not shown).

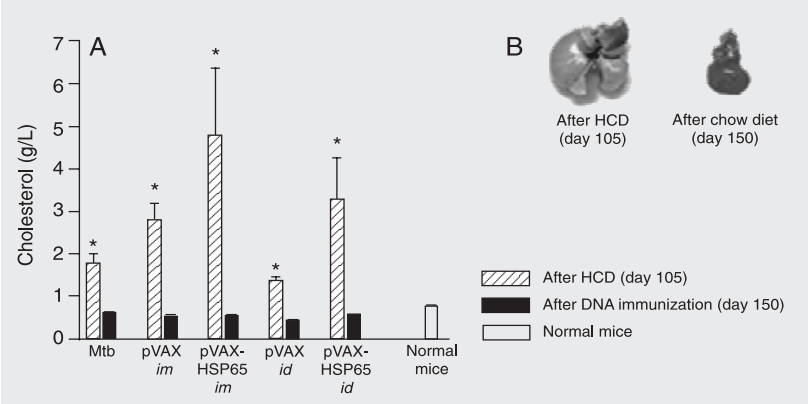

Figure 1. Serum cholesterol levels and macroscopic liver alterations in mice submitted to an atherosclerosis-induction protocol. Female C57BL/6 mice (4-6 mice per group) were fed an atherogenic diet (high cholesterol diet, HCD) for 105 days associated with 3 doses of 250 µg heat-killed *M. tuberculosis* (Mtb) on days 0, 15, and 30. On day 105, the animals started to receive a normal chow diet and were immunized with DNA (4 doses of 100 µg pVAX-HSP65 by the intramuscular (*im*) or intradermal (*id*) route (with a gene gun) on days 105, 115, 125, and 135. The following control groups (4-6 mice per group) were included: Mtb (animals fed the HCD and immunized with Mtb); pVAX *im* and pVAX *id* (animals fed the HCD, immunized with Mtb and injected with the empty vector by the *im* or *id* route); normal mice (animals fed a chow diet). *A*, Serum cholesterol levels were measured in samples collected on the 105th day (end of the HCD) and on the 150th day (end of the experiment, 15 days after the last DNA immunization). *B*, Macroscopic liver alterations observed on the 150th day included a change in color to yellowish and an increased size. *P < 0.05 compared with normal mice (ANOVA followed by Tukey test).

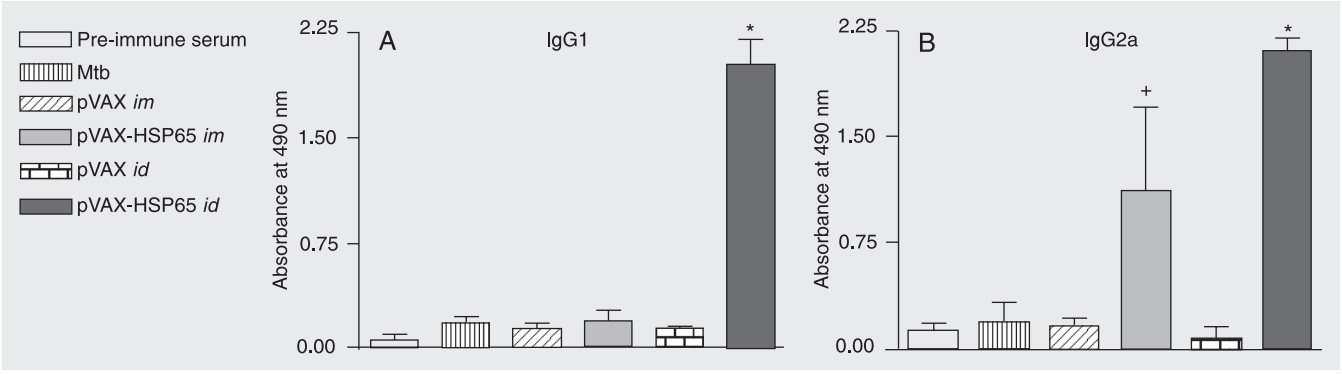

Figure 2. Serum antibody levels. Female C57BL/6 mice (4-6 mice per group) were fed for 105 days an atherogenic diet (high cholesterol diet, HCD) associated with 3 doses of 250 µg heat-killed *M. tuberculosis* (Mtb) on days 0, 15, and 30. On day 105, the animals started to receive a normal chow diet and were immunized with DNA (4 doses of 100 µg pVAX-HSP65) administered by the intramuscular (*im*) or intradermal (*id*) route on days 105, 115, 125, and 135. The following control groups (4-6 mice per group) were included: Mtb (animals fed the HCD and immunized with Mtb); pVAX *im* and pVAX *id* (animals fed the HCD, immunized with Mtb and injected with the empty vector by the *im* or *id* route). Pre-immune serum was collected before immunizations with DNA (105th day) and immune serum was collected 15 days after the last immunization (150th day) for the detection of IgG1 (A) or IgG2a (B) anti-Hsp65 antibodies by ELISA. Data are reported as mean ± SEM for 4-6 animals per group. *P < 0.05 compared with all other groups, $^{+}$P < 0.05 compared with the pre-immune, Mtb and pVAX-HSP65 *id* groups (ANOVA followed by Tukey test).

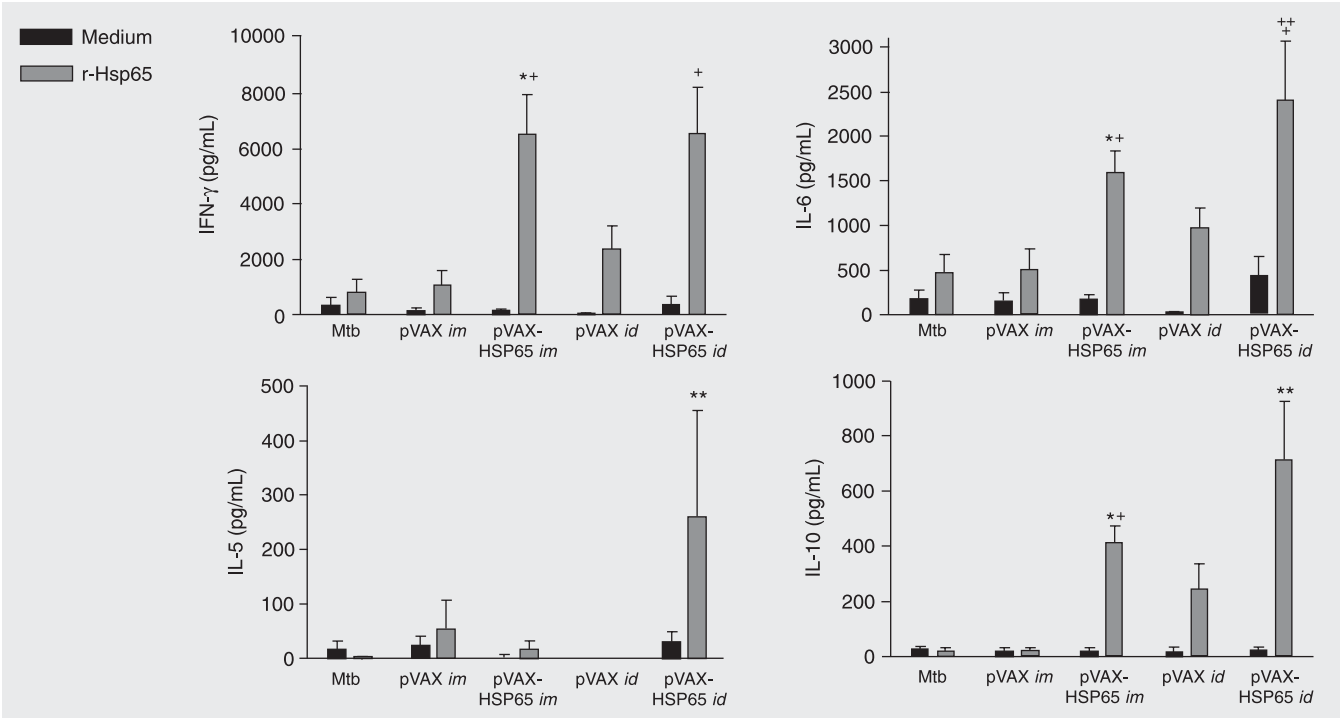

Figure 3. Cytokine production after specific stimulation with recombinant Hsp65 (r-Hsp65). Female C57BL/6 mice (4-6 mice per group) were fed for 105 days an atherogenic diet (high cholesterol diet, HCD) associated with 3 doses of 250 µg heat-killed *M. tuberculosis* (Mtb) on days 0, 15, and 30. On day 105, the animals started to receive a normal chow diet and were immunized with DNA (4 doses of 100 µg pVAX-HSP65) administered by the intramuscular (*im*) or intradermal (*id*) route on days 105, 115, 125, and 135. The following control groups (4-6 mice per group) were included: Mtb (animals fed the HCD and immunized with Mtb); pVAX *im* and pVAX *id* (animals fed the HCD, immunized with Mtb and injected with the empty vector by the *im* or *id* route). Fifteen days after the last DNA immunization (150th day), mice were sacrificed and spleen cell cultures were stimulated with r-Hsp65 for 48 h. Cytokines were measured in the culture supernatants by ELISA. Data are reported as mean ± SEM for 4-6 animals per group. $^{+}$P < 0.05 compared with the Mtb group; $^{++}$P < 0.05 compared with the pVAX *id* group; *P < 0.05 compared with the pVAX *im* group; **P < 0.05 compared with all other groups (ANOVA followed by Tukey test).

Discussion

In the current study, we evaluated the effect of an atherogenic diet on the immune response induced by a genetic vaccine against TB. This approach is relevant because atherosclerosis immunopathogenesis involves immunity against Hsp65 and this anti-TB vaccine is constructed with the HSP65 gene from *M. leprae*. In addition, this vaccine is designed to be also applied to the adult population, which has a very high incidence of atherosclerosis.

The experimental model of atherosclerosis used here consisted of C57BL/6 female mice submitted to an HCD (1.25%) and concomitantly immunized with three doses of heat-killed Mtb (H37Ra). Atherosclerosis induction ended after 105 days (15 weeks) when blood samples were collected for the quantitation of cholesterol and anti-Hsp65 basal antibody levels. Cholesterol levels were significantly elevated and livers showed clear steatosis. These results strongly suggest that the animals developed atherosclerosis, as described in the literature (21).

After atherosclerosis induction, the HCD was replaced with a standard chow diet and the animals were immunized with 4 doses of pVAX-HSP65 by the *im* or *id* route. On day 150 (15 days after the last DNA immunization), all animals gained weight and showed normal cholesterol levels but their livers were still enlarged and steatotic.

Mice immunized with pVAX-HSP65 by the *im* route produced significant amounts of anti-Hsp65 IgG2a antibodies but not specific IgG1. This finding indicates a Th1 pattern, which is usually the profile induced by this TB vaccine (6) and also by other genetic vaccines injected by the *im* route (22,23). Mice immunized by the *id* route produced significant levels of both anti-Hsp65 IgG1 and IgG2a antibodies, characterizing a Th1/Th2 response pattern. Even though the immunological mechanisms of atherosclerosis are not completely known, many reports suggest a contribution of both humoral and cellular responses against Hsp65, with a predominant role of Th1 cells (24-26). Therefore, it is important to stress that the immune response induced by the pVAX-HSP65 vaccine could contribute to the development of atherosclerosis. Hsp and specific anti-Hsp antibodies have been described in human atherosclerotic lesions (15,16,27). Also in human studies, higher anti-Hsp65 titers have been associated with progression and severity of lesions (15,16). In addition, these antibodies, that show cross-reactivity with human Hsp60 and bacterial Hsp65, mediate cytotoxicity against endothelial cells (28-30).

The profile of a Th1-driven immune response was also confirmed by the specific induction of significantly high levels of IFN-γ, but not IL-5, by r-Hsp65 stimulation of spleen cells from *im*-vaccinated animals in comparison to the Mtb and pVAX control groups. The mixed pattern of humoral immune response found in mice immunized by the *id* route was also confirmed by the splenic cytokine profile after *in vitro* stimulation with r-Hsp65 that induced significant levels of both IFN-γ and IL-5. These findings differ from the strong Th2 response normally associated with gene gun (*id*) immunization (7). The fact that these animals could be already primed for a Th1 response to Hsp65 by the previous immunization with Mtb is an attractive hypothesis for this mixed pattern of immune response.

Taken together, these results show that this vaccine was able to induce a strong immune response by both immunization routes in mice previously submitted to an atherosclerosis-induction protocol, showing that the atherosclerosis status did not interfere with the immunogenicity of the vaccine. These results are certainly promising in the context of new tuberculosis vaccination strategies.

Due to their immunomodulatory action on atherosclerosis, IL-6 and IL-10 were also

quantified in splenic cell culture supernatants. Vaccination by both routes determined a higher production of IL-6 compared to the groups injected with vector or to the Mtb group. Different cellular sources may contribute to this synthesis, mainly macrophages and Th2 cells which have been described as the main source of this cytokine. This increase is consistent with the high level of specific antibody production observed by both immunization routes and also with the description of high IL-6 levels in animals injected with other genetic vaccines (31,32). Interestingly, a contribution of this cytokine to atherogenesis in both human and experimental models has been described. For example, high IL-6 levels were associated with an increased risk of myocardial infarction (33). Also, administration of supraphysiological concentrations of exogenous IL-6 in the murine apolipoprotein E-deficient model dramatically enhanced the development of atherosclerosis, suggesting a pivotal role for IL-6 in plaque progression (34). A point to be carefully considered, therefore, is the possibility that DNA-HSP65 immunization could trigger or worsen an atherosclerosis condition by increasing IL-6 production. An experimental approach would be necessary to test this hypothesis. However, recent data suggest the opposite possibility, i.e., IL-6 and genetic vaccines could have a protective effect against the development of atherosclerosis. Madan et al. (35) observed that the genetic deficiency of IL-6 enhanced the formation of diet- and/or pathogen-associated atherosclerotic plaques, suggesting that IL-6 may play an atheroprotective role. Also Mao et al. (36) recently observed that CpG DNA, which is frequently found in vectors used in genetic vaccine constructs, decreased the average percentage of aortic lesions in a rabbit model of atherosclerosis.

Concerning IL-10 production, significant levels were induced by Hsp65 in mice immunized by both routes compared to control (empty vector and Mtb) groups. Also, this increase was much more pronounced in the group vaccinated by the *id* route with a gene gun. Even though IL-10 was originally described as a Th2 cytokine in mice, more recently its immunomodulatory action associated with other T cell subsets has been emphasized. Recent publications in this field have pointed out the requirements of a self-regulatory immune response to avoid a deleterious effect on the host (37). In addition, IL-10 has been described as an important mediator of the control of autoimmune diseases (38). Mucosal immunization with Hsp65 by the oral or nasal route resulted in protection against atherosclerosis. It is believed that this effect was related to IL-10 production (39). These results lead us to hypothesize that this DNA vaccine, due to its stimulation of IL-10-producing cells, could be even prophylactic and/or therapeutic in atherosclerosis. Interestingly and in support of this hypothesis, induction of oral tolerance to Hsp65 attenuated Mtb-induced and high-fat diet-driven atherosclerotic lesions (40).

We showed that immunization with a DNA vaccine containing the mycobacterial HSP65 gene was able to induce a strongly polarized Th1 response in mice previously submitted to a protocol of atherosclerosis induction. Conversely, a possible deleterious or even protective effect of the immune response induced by the pVAX-HSP65 vaccine on the course of atherosclerosis should be investigated in the future.

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