The Importance of Adjuvant Formulation in the Development of a Tuberculosis Vaccine

Susan L. Baldwin, Sylvie Bertholet, Valerie A. Reese, Lance K. Ching, Steven G. Reed and Rhea N. Coler

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An effective protein-based vaccine for tuberculosis will require a safe and effective adjuvant. There are few adjuvants in approved human vaccines, including alum and the oil-in-water–based emulsions MF59 (Novartis Vaccines and Diagnostics), AS03 and AS04 (GlaxoSmithKline Biologics), AF03 (Sanofi), and liposomes (Crucell). When used with pure, defined proteins, both alum and emulsion adjuvants are effective at inducing primarily humoral responses. One of the newest adjuvants in approved products is AS04, which combines monophosphoryl lipid A, a TLR-4 agonist, with alum. In this study, we compared two adjuvants: a stable oil-in-water emulsion (SE) and a stable oil-in-water emulsion incorporating glucopyranosyl lipid adjuvant, a synthetic TLR-4 agonist (GLA-SE), each together with a recombinant protein, ID93. Both the emulsion SE and GLA-SE adjuvants induce potent cellular responses in combination with ID93 in mice. ID93/SE induced Th2-biased immune responses, whereas ID93/GLA-SE vaccines did not induce protection in mice and guinea pigs, whereas no protection was observed with ID93/SE, as assessed by reductions in bacterial burden, survival, and pathology. These results highlight the importance of properly formulating subunit vaccines with effective adjuvants for use against tuberculosis. The Journal of Immunology, 2012, 188: 000–000.

Approximately 1.3 million deaths per year are associated with Mycobacterium tuberculosis in HIV seropositive populations and an additional 0.38 million deaths occur in people coinfected with HIV (1). Efforts are currently under way to develop vaccines, such as subunit vaccines, that can boost the current bacille Calmette-Guérin (BCG) vaccine or that can be used as a stand-alone prophylactic vaccine in immunocompromised individuals where BCG immunization is not recommended. Vaccination with an Ag alone is frequently insufficient to elicit a protective immune response. Adjuvants are critical for use with subunit vaccines to increase the magnitude and duration of adaptive immunity. Emulsion adjuvants effectively induce humoral responses and are optimal for vaccines requiring dose-sparing of the Ag, such as pandemic influenza vaccines (2). Vaccines that effectively induce a potent Th1 cellular response need additional components in the adjuvant, such as monophosphoryl lipid A (MPL; a TLR-4 agonist). MPL is present in the GlaxoSmithKline Biologics adjuvants AS01 (liposomal formulation plus QS21; a saponin), AS02 (combined with a water-in-oil emulsion plus QS21), and AS04 (combined with alum) (3). Of the MPL-containing adjuvants, only AS04 is approved for use in humans for use with hepatitis B virus (Fendrix) and human papillomavirus (Cervarix) vaccines (GlaxoSmithKline) (3). AS02A and AS01B (GlaxoSmithKline) are currently being evaluated in human clinical trials with the tuberculosis (TB) subunit vaccine M72 (4). We hypothesized that our candidate TG Ag, ID93, would elicit protection against M. tuberculosis infection when combined with a synthetic TLR-4 agonist (glucopyranosyl lipid adjuvant) formulated in a stable oil-in-water emulsion (GLA-SE) because of the potent Th1-inducing properties afforded by the TLR-4 component (5, 6).

Previously, we published work showing that our lead TB vaccine candidate, ID93 combined with GLA-SE, boosts the effects of BCG and protects mice against a low-dose aerosol (LDA) infection with M. tuberculosis (7). ID93 is a fusion of four M. tuberculosis proteins, including Rv2608, Rv3620, Rv1813, and Rv3619. Each protein is segregated into different M. tuberculosis protein categories: Rv2608 falls within the PE/PPE family of proteins, Rv3619 and Rv3620 are in the EsX family of virulence factors, and Rv1813 is associated with latent growth of M. tuberculosis and is expressed during hypoxia (8). ID93 was developed after rigorous screening of a large panel of M. tuberculosis recombinant proteins (8). Proteins were preselected based on their ability to induce IFN-γ from healthy human tuberculin purified protein derivative-positive [PPD(+)] PBMCs. A subset of these proteins was tested further, each individually combined with Cpg in the M. tuberculosis aerosol mouse model, to determine whether they could reduce lung bacterial load in infected mice (8). In the current study, the mouse and guinea pig models were selected to test the prophylactic efficacy of ID93 by measuring bacterial burden within the lungs of mice and by monitoring survival and lung pathology after M. tuberculosis challenge in guinea pigs (9). Guinea pigs develop lung pathology during pulmonary TB that...
resembles some aspects of pathology observed in infected humans, including necrotic centers within the granulomatous lesions (9). In this study, we report that a Th1 immune response is generated in ID93/GLA-SE immunized mice and bacterial burden is decreased in the lungs of mice aerogenically infected with M. tuberculosis. In addition, we show that ID93/GLA-SE induces Ag-specific T cell proliferative responses and is capable of extending survival and preventing severe lung pathology in guinea pigs after challenge. When ID93 is combined with an emulsion in the absence of the TLR-4 agonist, Ag-specific T cell proliferative responses are also induced. However, this adjuvant formulation fails to increase survival and actually worsens pathology in the lungs compared with untreated animals.

This work demonstrates that our candidate subunit vaccine Ag, ID93, combined with two adjuvant formulations can lead to two different outcomes. ID93 combined with the Th1 adjuvant, GLA-SE (5), enhances the quality of the immune response, leading to protection (functional reduction of bacterial burden in the lungs of M. tuberculosis-infected mice, increased survival, and decreased lung pathology in guinea pigs); whereas ID93 combined with a Th2 adjuvant, stable oil-in-water emulsion (SE) alone, does not induce protection (no significant reduction of bacterial burden in the lungs of M. tuberculosis-infected mice, accelerated death, and failure to protect against immunopathology in the lungs of guinea pigs).

Materials and Methods

ID93

ID93 is a fusion protein that incorporates the three proteins that comprise ID93 (Rv1813, Rv2620, and Rv2608) (10) plus an additional M. tuberculosis protein, Rv3619, produced as previously described (7).

Immunization (mice)

Female C57BL/6 mice, 5–7 wk old, were purchased from Charles River Laboratories (Wilmington, MA) and were housed in the Infectious Disease Research Institute (Seattle, WA) animal care facility under specific pathogen-free conditions. Ten mice per group were immunized three times, 3 wk apart. Injections were administered i.m. with saline or ID93 (0.5 µg) plus either SE (at 2%) or GLA-SE (5 µg glucopyranosyl lipid adjuvant) formulated in the oil-in-water emulsion (SE) alone, does not induce protection (no significant reduction of bacterial burden in the lungs of M. tuberculosis-infected mice, accelerated death, and failure to protect against immunopathology in the lungs of guinea pigs).

Ab endpoint titers (mice)

Mice were bled at days 0, 14, and 56, and ID93-specific endpoint titers for IgG1, IgG2c, and total IgG were performed. Briefly, Nunc Polyorb plates were coated with 2 µg/ml recombinant protein (ID93) in 0.1 M bicarbonate and blocked overnight at 4°C with 0.05% PBS–Tween 20/1% BSA. Plates were washed and developed using SureBlue tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg MD). The enzymatic reaction was stopped with 1 N H2SO4. Plates were read within 30 min at 450 nm with a reference filter set at 650 nm using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA) and Soft Max Pro5 software (San Diego, CA) with a cutoff of 0.1 absorbance unit.

Cytokine ELISPOT assays (mice)

One week after the last immunization, mice (n = 3/group) were euthanized to perform IFN-γ and IL-5 ELISPOT assays. A MultiScreen 96-well filtration plate (Millipore, Bedford, MA) was coated with 10 µg/ml rat anti-mouse IFN-γ or IL-5 capture Abs (eBioscience) and incubated overnight at 4°C. Plates were washed with PBS, blocked with RPMI 1640 and 10% FBS for at least 1 h at room temperature, and washed again. Splenocytes were plated in duplicate at 2 x 10^5 cells/well and stimulated with media, Con A (3 µg/ml), or ID93 (10 µg/ml) for 48 h at 37°C. The plates were then washed with 0.1% PBS–Tween 20 and incubated overnight with a biotin-conjugated rat anti-mouse IFN-γ secondary Ab (eBioscience) or rat anti-mouse IL-5 secondary Ab (eBioscience) diluted 1:250 in 0.1% PBS–Tween 20/0.5% BSA. The filters were developed using the VectaS-tain ABC avidin peroxidase conjugate and Vectastain AEC substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. The reaction was stopped by washing the plates with deionized water. Plates were dried in the dark, and spots were counted on an automated ELISPOT reader (C.T.L. Seri3A Analyzer; Cellular Technology, Cleveland, OH) and analyzed with ImmunoSpot software (CTL Analyzer).

Flow cytometry (intracellular cytokine staining, mice)

One week after the last immunization, splenocytes from three individual mice per group were plated at 1 x 10^6 to 2 x 10^6 cells/well in 96-well V-bottom plates and were stimulated for 12 h with anti-CD28/CD49d (eBioscience), each at 1 ng/ml, and either media, ID93 (10 µg/ml), or PMA/ionomycin (1 µg/ml; included as a positive control) in the presence of GolgiStop (eBioscience). The cells were fixed for 10 min with Cytofix (BD Biosciences, San Jose, CA), washed in PBS BSA 0.1%, and incubated with FC Block (anti-CD16/CD32; eBioscience) for 15 min at 4°C. Cells were stimulated with fluorochrome-conjugated mAbs against CD3, CD4, CD44, IFN-γ, TNF, IL-2 (eBioscience), and CD4 mAb (Invitrogen) in Perm/Wash buffer 1× (BD Biosciences) for 30 min at 4°C, washed twice in Perm/Wash buffer, suspended in PBS, and analyzed on a modified three-laser LSRII flow cytometer (BD Biosciences). Viable lymphocytes were gated by forward and side scatter, and 20,000 CD3^+ CD4^+ events were acquired for each sample and analyzed with BD FACSDiva software v.5.0.1 (BD Biosciences).

M. tuberculosis infection (mice)

Six weeks after the last immunization, mice (n = 7/group) were aerogenically infected with an LDA of M. tuberculosis H37Rv (ATCC No. 35718; American Type Culture Collection, Manassas, VA) using a Glass-Col aerosol generator (Terre Haute, IN) to deliver 50–100 CFU at the base of the tail. Protection was determined 6 wk after challenge by harvesting the lungs from the infected mice, homogenizing

FIGURE 1. Increased ID93-specific IgG2c endpoint titers are induced after mouse immunization with ID93/GLA-SE; increased IgG1 titers are induced with ID93/SE. Serum was collected 2 wk after the first immunization (day 14) or 2 wk after the last immunization (day 56). Mean reciprocal dilutions are represented as the endpoint titer (log10) ± SD. This is one representative study of at least two independent studies. A two-tailed Mann–Whitney t test was used to determine significance. *p < 0.05 (comparing ID93/SE with ID93/GLA-SE).
the tissue in 0.05% PBS–Tween 80, and plating 5-fold serial dilutions on
7H10 agar plates (Molecular Toxicology, Boone, NC) for bacterial growth.
Bacterial colonies were counted 2–3 wk later after incubation at 37°C.
Reductions in bacterial burden in the lungs were calculated as follows:
mean log_{10} CFU_{saline} – mean log_{10} CFU_{vaccine}.

Immunization (guinea pig)

Female Hartley guinea pigs (400–450 g; Charles River Laboratories) were
housed in the Infectious Disease Research Institute animal care facility
under specific pathogen-free conditions. All animals were treated in ac-
CORDance with the regulations and guidelines of the Infectious Disease
Research Institute Animal Care and Use Committee.

Guinea pigs (n = 5 to 7 per group) were immunized i.m. three times, 3
wk apart. For these immunizations, ID93 (10 μg) was combined with ei-
ther saline, SE (2%), or GLA-SE (5 μg). Animals in the BCG group were
immunized intradermally with a single dose (5 × 10^4) of live BCG Pasteur
(Sanofi Pasteur). A University of Wisconsin-Madison aerosol exposure
Chamber was then calibrated to deliver 20–100 bacteria (M. tuberculosis
H37Rv strain; ATCC No. 35718) 6 wk after the last immunization. Ani-
mals were inoculated with 50–100 bacteria in an aerosol, as determined by
24-h CFU. Weight loss and survival was monitored for greater than
200 d postchallenge until all saline-treated animals died.

Ab endpoint titers (guinea pig)

ID93-specific IgG1, IgG2, or total IgG were measured from immunized
guinea pigs at 42 d (2 wk after the second immunization) or 77 d (3 wk after
the third immunization). ELISA plates (Costar ELIA/RIA 96-well plates,
Corning Life Sciences) were coated with ID93 at 2.0 μg/ml in 0.1 M bi-
carbonate coating buffer. Plates were incubated overnight at 4°C and
blocked with 0.1% PBS–Tween 20 and 5% milk (Carnation milk powder)
for 2 h at room temperature. Plates were washed in PBS–Tween 0.1% fol-
lowed by a single wash in 1× PBS. Sera were serially diluted 5-fold starting
at either a 1:10 or 1:100 dilution and then added to the plates for 2 h at room
temperature. Plates were washed, and secondary Ab (1:2000 dilution),
either goat anti-guinea pig IgG (H+L), IgG1-, or IgG2-conjugated to HRP
(Southern Biotech), was added to the plates for 1 h at room temperature.
Plates were washed, and SureBlue tetramethylbenzidine substrate solution
(100 μl) was added to the plates for 1–2 min. The reaction was stopped with
H₂SO₄, and plates were read within 30 min on a microplate ELISA reader
(Molecular Devices) at 450 nm with a reference filter set at 650 nm using
Soft Max Pro5 software. Reciprocal dilutions corresponding to endpoint
titers were determined with GraphPad Prism 4 (GraphPad Software, San
Diego, CA) with an 0.1 absorbance cutoff.

Histology

A single lobe of the lung was fixed for at least 7 d in 10% normal buffered
formalin. The tissues were examined grossly by a pathologist and trimmed
into cassettes and paraffin processed. Tissues were arranged such that any
visible gross lesions were on the tissue face to be sectioned. Each tissue
block had three microscopic sections cut 4 μm thick, which were then
mounted on glass microscopic slides and stained with H&E, Fite’s acid-
fast stain, and a trichrome stain by the BioGenetics Research Laboratories
(Greenbank, WA). Qualitative analyses were performed by a board-
certified veterinary pathologist (Dr. Lawrence L. Kunz) in a blinded fash-
tion. Two to three sections were evaluated per animal. Histological analysis
of the lung included the organization of cellular infiltrate, number of
granulomas, visual quantification of acid-fast bacilli (AFB), and pulmo-
nary fibrosis, and the severity was graded and scored by the pathologist
(Supplemental Table I). The severity of the lesions was graded according
to the referenced standardized grading scheme for non-neoplastic lesions
(11).

Lesion grades are defined as follows: 0, normal tissue morphology with
no lesion present; 1, lesions involving <10% of the tissue and minimal
infiltration of fibroblasts, mononuclear, or polymorphonuclear inflamma-
tory cells; 2, lesions affecting 10–20% of the tissue and mild cellular in-
filtration; 3, lesions covering 21–40% of the tissue and moderate cellular
infiltration; 4, lesions covering 41–100% of the tissue and marked cellular
infiltration. The relative number of organisms between treatment groups

![FIGURE 2. ID93/GLA-SE increases IFN-γ responses, whereas ID93/SE increases IL-5 responses. Mice were immunized three times, 3 wk apart, and spleens were harvested from individual mice (n = 3/group) 1 wk after the last immunization. The frequency of (A) IFN-γ- or (B) IL-5-secreting T cells was determined by ELISPOT assay. Results are represented as spot-forming units/million cells ± SD. This is one representative study of at least two inde-
pendent studies. *p < 0.05 (compared with saline).](http://www.jimmunol.org/)
was determined by counting the number of AFB in five high-power fields (HPFs; ×600) of the granuloma zones containing the highest concentration of bacilli. A total of five HPF counts were averaged. Slides were viewed on a Nikon Labophot-2 microscope. High-resolution digital microscopic images (×16 to ×1000 magnification) were obtained using a Nikon D5000 digital microscopic camera and a Nikon Super CoolScan 5000 microscopic scanner (×1 magnification). The results are summarized in Supplemental Table I.

**FIGURE 3.** ID93/GLA-SE induces polyfunctional CD4+ T cells (IFN-γ, TNF-α, IL-2). (A) Cytokine production from ID93-specific CD4+ T cells in immunized mice (n = 3 individual mice per group) was measured by flow cytometry. Splenocytes from vaccinated mice were stimulated with ID93 for 12 h in the presence of GolgiStop. ID93-stimulated splenocytes were identified by intracellular cytokine staining based on CD3 and CD4 expression and were further gated on CD44high cells. The percent frequency of cells expressing triple cytokines (IFN-γ, TNF-α, and/or IL-2), double cytokines, or single cytokines ± SD for each of the groups are depicted in (B) (pie charts) and (C) (bar graphs). This is one representative study of at least two independent studies. *p < 0.05 (ID93/SE compared with ID93/GLA-SE; Student t test), #p < 0.05 (compared with saline; Student t test).
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**PBMC isolation (guinea pig)**

Cardiac bleeds were performed on anesthetized guinea pigs for isolation of PBMCs (4 ml). Heparinized blood was obtained from guinea pigs and diluted 1:1 with PBS. Diluted blood was placed over a Ficoll-Paque Premium 1.084 (GE Healthcare, Piscataway, NJ) gradient and spun for 25 min at 2000 rpm. Cells at the interface were collected using a 2-ml pipette and washed several times with PBS. Cells were counted using a Guava ViaCount (Guava Technologies, Hayward, CA) and frozen in 90% heat-inactivated FBS/10% DMSO (Sigma, St. Louis, MO).

**eFluor670 cell labeling for proliferation (guinea pig)**

Frozen guinea pig PBMCs were thawed, washed twice with PBS, and resuspended in 0.5 ml PBS. Cell Proliferation Dye eFluor670 (4 μM in 0.5 ml; eBioscience) was added, mixed, and incubated at 37°C for 10 min in the dark. Cold RPMI 1640 plus 10% FBS, 1% penicillin/streptomycin, and 0.1% 2-mercaptoethanol (cRPMI) (in 12 ml) was added to cells and incubated on ice for 5 min to stop the reaction. Cells were spun down and washed twice with cRPMI.

**In vitro cell stimulation (guinea pig)**

eFluor670-labeled PBMCs were cultured for 5 d at 37°C with 5% CO$_2$ in round-bottom 96-well plates at up to 5 x 10$^5$ cells per well for each condition. The following culture conditions were tested: a) cRPMI only, b) BSA-coated beads (3 μl/well), c) ID93-coated beads (3 μl/well), d) ID93 peptide pool (ID93pp; 0.5 μg/ml), e) 0.4% DMSO (Sigma Hybri-Max) control for ID93pp (selected samples only) or 1 μg/ml Con A (Sigma-Aldrich). Beads were 3-μm latex Polybeads (Polysciences, Warrington, PA). Fifteen microliters of beads (21 x 10$^6$) in a sterile Eppendorf tube was washed three times with sterile dH$_2$O, resuspended in 50 μl 0.33 mg/ml BSA (Sigma-Aldrich) or ID93 in PBS, and incubated on a rotator at 4°C overnight. Two hundred fifty microliters of cRPMI was added to the bead suspensions before dispensing into cultures.

**Results**

**ID93/GLA-SE induces Th1-biased responses whereas ID93/SE induces Th2-biased responses in mice**

Mice were immunized with ID93 adjuvanted with either SE or GLA-SE. The two vaccine formulations (SE and GLA-SE) had distinct effects on the Ab isotype response, as determined by Ab ELISA (Fig. 1). ID93/SE induced significant levels of ID93-specific IgG1 Ab titers after immunization, indicative of a Th2-biased response. In contrast, ID93/GLA-SE immunized mice induced greater ID93-specific IgG2c responses, indicative of a Th1-biased response. The cytokine profiles in the mouse model, as determined by ELISPOT assay, are also reflective of the adjuvant-induced biases. Mice immunized with ID93/SE induced a significantly higher frequency of IL-5-secreting T cells, whereas ID93/GLA-SE induced a greater frequency of IFN-γ-secreting T cells (Fig. 2). Immunization with ID93/GLA-SE but not ID93/SE also led to the generation of polyfunctional CD4$^+$ T cells expressing IFN-γ, TNF-α, and IL-2 (Fig. 3).
ID93/GLA-SE reduces bacterial burden in the lungs of mice

A Th1-mediated cellular response is an important factor in the generation of protective immune responses against *M. tuberculosis*. In this study, we show that the same Ag, ID93, given with either a Th1-inducing adjuvant (GLA-SE) or a Th2-inducing adjuvant (SE) leads to either a significant reduction in lung bacterial burden (as seen with ID93/GLA-SE) or to no significant reduction in lung bacterial load (as observed with ID93/SE) (Table I). These different outcomes demonstrate the importance of adjuvant formulation with a TB vaccine protein, such as ID93.

Immunological responses to vaccination with ID93 vaccines in guinea pigs

Ag-specific Ab responses were measured, and, unlike the mouse data, the IgG1 and IgG2 responses were not found to be different in guinea pigs immunized with either ID93/SE or ID93/GLA-SE after the boost immunization (Fig. 4). Significant IgG1 and IgG2 Ag-specific Ab responses were observed to ID93 in both vaccine groups compared with the saline-injected guinea pigs, representing an IgG1/IgG2 mixed Ab response.

To characterize immune responses further, proliferative responses to ID93 were measured after stimulation of PBMCs from immunized guinea pigs. PBMCs were isolated by Ficoll-Paque gradient as previously described (12) and labeled with eFluor670 (a cell proliferation dye). Labeled PBMCs were cultured for 5 d with either saline, BSA beads (used as a negative control), ID93 beads, or a peptide pool encompassing the entire ID93 protein (Rv2608, Rv1813, Rv3620, Rv3619). Cells were stained with anti-CD4 and anti-CD8 and gated as shown in Fig. 5A. Dead cells were excluded by propidium iodide fluorescence. As expected, CD4+ or CD8+ T cell proliferative responses were not observed from guinea pigs injected with saline only (Fig. 5B). CD4+ T cell proliferative responses were enhanced in both the ID93/SE and ID93/GLA-SE groups compared with either media or BSA beads; however, the results were not considered statistically significant (Fig. 5B). Statistically significant CD8+ T cell

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**FIGURE 5.** ID93-specific proliferative responses in guinea pig PBMCs after immunization with either ID93/SE or ID93/GLA-SE. PBMCs were isolated 3 wk after the third immunization and frozen. Thawed PBMCs were labeled with cell proliferation dye eFluor670, cultured for 5 d with the indicated stimulators, stained with anti-CD4–RPE and anti-CD8–FITC Abs, and dead cells labeled with propidium iodide (PI). Proliferation of CD4+ or CD8+ lymphocytes was defined as a decrease in eFluor670 fluorescence intensity in live cells and expressed as either percent of live CD4+ cells or percent of live CD8+ cells ± SEM. (A) Gating strategy. (B) Percent proliferation of CD4+ or CD8+ T cells from stimulated PBMCs. An additional study was performed in guinea pigs using the same adjuvants with an ID83 subunit vaccine, which resulted in similar results. *p < 0.05 compared to saline group.
proliferative responses to ID93 were observed in the group given ID93/GLA-SE (Fig. 5B).

**ID93 plus GLA-SE improves survival in guinea pigs after aerosol challenge with *M. tuberculosis* whereas ID93 plus SE worsens survival**

To test the protective efficacy of ID93, weekly weight measurements were performed (data not shown), and long-term survival to day 210 after challenge with *M. tuberculosis* was included as a readout in guinea pigs. ID93/GLA-SE significantly improved survival in guinea pigs compared with unimmunized guinea pigs (*p* < 0.05) (Fig. 6) with a mean survival time of 210 d (Supplemental Table I). ID93 combined with SE, however, was not protective (Fig. 6, Supplemental Table I). ID93/SE appeared to exacerbate infection, where the animals died slightly earlier (mean survival of 143 d) than animals given saline alone (mean survival of 170 d); however, these responses were not statistically different. BCG, similar to ID93/GLA-SE, significantly protected guinea pigs after *M. tuberculosis* challenge (Fig. 6).

**ID93/GLA-SE but not ID93/SE vaccination reduces lung pathology**

Histology was performed on the lungs of immunized animals after infection with *M. tuberculosis* and extensively analyzed by a board-certified pathologist in a blinded fashion. Lesion scores are based on the percentage of lung comprising lesions, the nature of the cellular infiltration, and the extent of collagen deposition, as an indication of fibrosis. The presence of AFB present in the lung was also determined. Three of five ID93/GLA-SE immunized guinea pigs (which were protected after challenge) had moderate histopathological tissue response grades (grade 3); histology was not performed for one guinea pig that died on day 136 (Supplemental Table I). One guinea pig from this group had marked lung tissue involvement (grade 4, 41–100%), and a large number of bacilli were present within the lung tissue (21 bacilli/HPF) (Supplemental Table I). Animals immunized with BCG were assigned scores ranging from 1 to 3: two grade 2, and two grade 1. Grade 1 responses are considered minimal, where there is less than 10% lung involvement and minimal infiltration of fibroblast and mononuclear inflammatory cells into the tissue. Grade 2 lesions correspond to mild histological changes, where between 11 and 20% of the lung tissue was involved (Fig. 7, Supplemental Table I). Four of five animals in the saline control group and four of six animals in the ID93/SE group had grade 4, or marked histological changes, whereby 41–100% of the lung was involved; lung tissue was not available for one ID93/SE immunized guinea pig (Supplemental Table I). Marked diffuse or focal infiltration of fibroblast, mononuclear or polymorphonuclear inflammatory cells into the tissue was observed in the lungs (Fig. 7, Supplemental Table I). Notably, all lungs contained only 0–2 bacilli/HPF, suggesting that the extensive pathology rather than the lack of bacterial containment may account for mortality in the guinea pigs given saline or ID93/SE (Fig. 7, Supplemental Table I).

**Discussion**

The imminent threat of multiple drug-resistant strains of *M. tuberculosis* and the lack of protection afforded by BCG in adults has led to the development of many new experimental TB vaccines, some of which are currently in human clinical trials (13). These vaccines will likely be used (if approved) to boost the existing response elicited by BCG, which is given at birth. Even so, a vaccine that is effective without the need of BCG priming could also be of clinical use, particularly in immune-compromised populations (such as HIV-infected people) where the attenuated BCG vaccine could potentially replicate without containment from the host’s immune response. In the current study, we investigated the protective capacity of the fusion protein, ID93, against *M. tuberculosis* in both the mouse and guinea pig models. The
vaccine was combined with either a Th1-inducing adjuvant (GLA-SE) or an emulsion with Th2-inducing properties (SE) in non-BCG-primed guinea pigs. In this study, we demonstrate the importance of adjuvant formulation on protective efficacy.

Both ID93 in a heterologous BCG prime/boost guinea pig study and the closely related ID83 Ag and ID93 in a prophylactic mouse model have been shown to protect against *M. tuberculosis* in the presence of GLA-SE (7, 10). However, in this report, we show that ID93 combined with GLA-SE is protective against *M. tuberculosis* in guinea pigs, as a prophylactic vaccine, in the absence of a BCG prime. We also demonstrate the necessity to properly formulate such vaccines to elicit a desired immunological response. When ID93 is combined with SE alone, a Th2 cytokine IL-5 response is elicited that fails to protect against *M. tuberculosis* and is detrimental in both models. In contrast, the addition of glucopyranosyl lipid adjuvant (GLA) to the adjuvant formulation (GLA-SE) has immunostimulatory properties that enhance early innate immune responses such as enhanced IL-12 production, which promotes a Th1-mediated immune response, known to be protective in the host response against *M. tuberculosis*. Previously, we demonstrated that GLA stimulates cytokine production specifically through binding TLR-4 on both murine and human monocyte/macrophage-derived cell lines (10). In addition, GLA signals through MyD88 and/or Toll IL-1R (TIR) domain-containing adapter-inducing TRIF (TIR) domain-containing adapter-inducing TRIF-dependent signaling pathways (5). The stimulation of dendritic cells with GLA-SE leads to increased immunological functions such as dendritic cell maturation (increased HLA-DR, CD40, CD83, CD86, and CCR7) and upregulation of MHC class II on APCs, in addition to the production of many proinflammatory cytokines and chemokines (5). Recently, we also demonstrated that mRNA for IL-12 and TNF-α is upregulated after stimulation of guinea pig whole blood, PBMCs, or splenocytes with several different TLR agonists (12), including the TLR-4 agonist GLA. GLA-SE is also promising when combined with vaccines for influenza and malaria. We reported that the inclusion of GLA-SE with a seasonal influenza vaccine, Fluzone (Sanofi Pasteur), increases both the magnitude and breadth of the Ab response against antigenically drifted influenza virus strains (14). By using a massively parallel signature sequencing approach, we demonstrated that immunization with *Plasmodium vivax* Ag, PyRIL, combined with GLA-SE enhances the host’s Ab repertoire to include a much broader recognition of B cell V-region sequences (15). Furthermore, the *Leishmania* spp. subunit vaccine Ag, Leish-110f, which has been tested both preclinically and clinically, is protective in mice in the presence of GLA-SE, even though no efficacy against parasite challenge is observed in the presence of the emulsion without the TLR-4 ligand (16). Agger et al. (17) also showed in mice that combining an Ag85B-ESAT6 subunit vaccine with aluminum hydroxide, Al(OH)₃, results in a Th2 response, similar to the oil-in-water emulsion in our study, which provided no protection against *M. tuberculosis*, whereas a strong Th1-inducing adjuvant (cationic liposomes containing MPL) combined with the same subunit vaccine elicited a protective Th1 immune response (17).

The results of these studies show that the ID93/GLA-SE vaccine protects against aerosol infection with *M. tuberculosis* in the mouse and guinea pig models. Demonstration of efficacy with ID93/GLA-SE in non-BCG-primed guinea pigs was observed by increased long-term survival after *M. tuberculosis* challenge. Histological analysis of lung tissue from animals vaccinated with ID93/GLA-SE suggests that the animals were protected against the severe pathology that was observed in the saline- and ID93/SE-treated groups. A fine balance exists between control of bacterial load and preservation of host tissue. Some evidence suggests that blocking immunopathology may be more important than inducing a strong Th1 response, especially when the Th1 response is mixed with a preexisting Th2 response (18). One hypothesis regarding the lack of effectiveness in BCG-vaccinated individuals in developing countries is the presence of Th2-type responses driven by helminth infection at the time of immunization (18).

Many of the proteins contained in ID93 are also produced in BCG. This observation is of interest, as vaccination with ID93 may allow for potential boosting in BCG-immunized individuals. Previously, we showed that ID93/GLA-SE is capable of boosting BCG-primed guinea pigs by extending survival after *M. tuberculosis* infection over that seen with BCG alone (7). Notably, BCG-immunized guinea pigs responded to the ID93 fusion protein with a delayed-type hypersensitivity (DTH) response (data not shown). Animals immunized with ID93 vaccines did not elicit a DTH response to PPD but were able to induce a DTH response to the vaccine Ag (data not shown). These data suggest that the ID93/GLA-SE vaccine is able to generate recall responses to BCG, and phenotypic differences in the responding T cells are likely responsible for the protection elicited by the ID93/GLA-SE formulated vaccine. We are currently investigating both innate and adaptive responses to ID93/GLA-SE in guinea pigs that could correlate with the observed protective responses.

In conclusion, we demonstrate the efficacy of ID93 when combined with the Th1-inducing adjuvant GLA-SE in both the mouse and guinea pig models. We demonstrate that immunization with this vaccine Ag combined with GLA-SE leads to Th1-biased CD4⁺ T cell responses and decreases bacterial burden within the lungs of mice infected with *M. tuberculosis*. ID93/GLA-SE also induces Ag-specific T cell proliferative responses, increases survival against *M. tuberculosis*, and preserves the lung tissue in infected guinea pigs. In contrast, ID93 combined with a Th2-biased adjuvant fails to protect against *M. tuberculosis* in either the mouse or guinea pig and may worsen the outcome of disease in guinea pigs after challenge. These results suggest that careful attention to the formulation of adjuvants should be considered in the development of subunit TB vaccines.

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**Disclosures**

S.G.R. is a founder of, and holds an equity interest in, Immune Design Corp., a licensee of certain rights associated with GLA. The other authors have no financial conflicts of interest.

**References**

Supplemental Table 1: Histological Data Summary

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<sup>a</sup>Animal identification (n=5-6 animals/group).

<sup>b</sup>Lesion Score (1-9) defined in materials and methods.

<sup>c</sup>Lesion Grade (1-4) defined in materials and methods.

Lung tissue was not available for histology on two of the guinea pigs (indicated by dashes).