Streptococcus pneumoniae fructose-1,6-bisphosphate aldolase, a protein vaccine candidate, elicits Th1/Th2/Th17-type cytokine responses in mice

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Abstract. *Streptococcus pneumoniae* (*S. pneumoniae*) is a major pathogen worldwide. The currently available polysaccharide-based vaccines significantly reduce morbidity and mortality. However, the inherent disadvantages of the currently available polysaccharide-based vaccines have motivated the search for other bacterial immunogens capable of eliciting a protective immune response against *S. pneumoniae*. Fructose-1,6-bisphosphate aldolase (FBA) is a glycolytic enzyme, which was found to localize to the bacterial surface, where it functions as an adhesin. Previously, immunizing mice with recombinant FBA (rFBA) in the presence of alum elicited a protective immune response against a lethal challenge with *S. pneumoniae*. Thus, the aim of the present study was to determine the cytokine responses that are indicative of protective immunity following immunization with rFBA. The protective effects against pneumococcal challenge in mice immunized with rFBA with complete Freund's adjuvant (CFA) in the initial immunization and with incomplete Freund's adjuvant (IFA) in booster immunizations surpassed the protective effects observed following immunization with either rFBA + alum or pVAC^{fba}. CD4$^+$ T-cells obtained from the rFBA/CFA/IFA/IFA-immunized mice co-cultured with rFBA-pulsed antigen-presenting cells (APCs), exhibited a significantly greater proliferative ability than CD4$^+$ T-cells obtained from the adjuvant-immunized mice co-cultured with rFBA-pulsed APCs. The levels of the Th1-type cytokines, interferon (IFN)-γ, interleukin (IL)-2, tumor necrosis factor (TNF)-α and IL-12, the Th2-type cytokines, IL-4, IL-5 and IL-10, and the Th17-type cytokine, IL-17A, significantly increased within 72 h of the initiation of co-culture with CD4$^+$ T-cells obtained from the rFBA-immunized mice, in comparison with the co-cultures with CD4$^+$ T-cells obtained from the adjuvant-immunized mice. Immunizing mice with rFBA resulted in an IgG1/IgG2 ratio of 41, indicating a Th2 response with substantial Th1 involvement. In addition, rabbit and mouse anti-rFBA antisera significantly protected the mice against a lethal *S. pneumoniae* challenge in comparison with preimmune sera. Our results emphasize the mixed involvement of the Th1, Th2 and Th17 arms of the immune system in response to immunization with pneumococcal rFBA, a potential vaccine candidate.

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Introduction

Streptococcus pneumoniae (S. pneumoniae), which is a major pathogen found in infants, the elderly and the immunocompromised, is responsible for more than one third of the two million global annual deaths of children following acute respiratory infections. The currently available polysaccharide-based vaccines provide an antibody-dependent protection (1) and significantly reduce morbidity and mortality (2). The pneumococcal polysaccharide vaccine Pneumovax 23 (PPV23), consists of 23 pneumococcal polysaccharides from the most common disease-causing serotypes. However, PPV23 does not provide long-lasting protection and is ineffective in infants. The polysaccharide-protein conjugate vaccine, which includes up to 13 pneumococcal polysaccharides, elicits long-term protection, but provides only a limited coverage, as the elicited immune

response is serotype-specific (1). These inherent disadvantages have motivated the search for other bacterial immunogens, such as bacterial proteins, capable of eliciting a protective immune response against *S. pneumoniae* (3,4).

Probably the most important concern today in the field of vaccine development is our inadequate understanding (3) of how to induce a specific, potent and durable protective immune response (5). The majority of available vaccines confer protection through antibodies, the levels of which are defined as correlates of protection. Although the correlates of immune response to *S. pneumoniae* proteins are emerging, studies have found that the protection elicited by carriage or immunization with whole-cell vaccines is $CD4^+$ T-cell dependent and antibody-independent (6,7), and has been suggested to involve mainly the Th17 arm (8). However, other studies have shown that both the Th17 and the Th1 arms are necessary for the development of the protective immune response, whereas the Th2 response is negligible (9,10).

A variety of proteins displayed on the cell wall of *S. pneumoniae* have been found to confer protection against pneumococcal infection. Some of these proteins are adhesins, e.g., choline binding protein A (CbpA) (11,12), pneumococcal choline binding protein A (PcpA) (13), pneumococcal histidine triad protein D (PhtD) (3), pneumococcal surface adhesin A (PsaA) (14) and pneumococcal serine-rich repeat protein (PsrP) (15), while others are involved in various other bacterial functions e.g., pneumococcal surface protein A (PspA), which reduces complement deposition on the bacterium (16), protein required for cell wall separation of group B streptococcus (PcsB), which is involved in bacterial cell division; or serine/threonine protein kinase (StkP), which is involved in bacterial morphogenesis (10,17).

Fructose-1,6-bisphosphate aldolase (FBA) is a glycolytic enzyme that is highly conserved (99%) among *S. pneumoniae* strains. FBA has also been found to localize to the surface of different pathogens, including Gram-positive bacteria, Gram-negative bacteria and parasites (18-21). In *S. pneumoniae*, FBA surface localization was demonstrated by biochemical fractionation of the cell wall and by the flow cytometry of live bacteria immunostained with mouse antisera against recombinant (r)FBA (22,23). FBA has also been shown to function as an adhesin in *S. pneumoniae* and in *Neisseria meningitidis* (19,24). Furthermore, Flamingo cadherin receptor (FCR), a cell adhesion molecule, was found to be a *S. pneumoniae* FBA target receptor. Additionally, a peptide derived from FCR was found to inhibit *S. pneumoniae* adhesion *in vitro* and prevent disease development *in vivo* in a mouse model (24). It has been found that FBA elicits an age-dependent antigenicity in infants and that it is immunogenic in mice (22,23). Finally, immunizing mice with rFBA in the presence of alum has been shown to elicit a protective immune response against a lethal challenge with *S. pneumoniae* (22). Thus, rFBA emerges as a promising vaccine candidate against *S. pneumoniae*.

Aluminum-based compounds are the major adjuvants used, to date, in human vaccination. Although they induce a good Th2 antibody response to the immunogen, they also inhibit humoral and cellular Th1 and Th17 responses. Alum salts are potent inhibitors of Toll-like receptor (TLR) agonist-induced interleukin (IL)-12 secretion, an effect that is mediated by alum-driven phosphoinositide (PI)3 kinase signaling. Moreover, alum salts have been shown to reduce the ability of the immune system to promote Th17 responses due to their effect on the production of IL-23 (25). Thus, we hypothesized that the protective effects elicited by alum salts are not unsurpassed.

In the present study, we explored the ability of rFBA to protect mice against an *S. pneumoniae* challenge and investigated the cytokine profiles elicited in mice immunized with rFBA. In addition, we evaluated the production of functional protective antibodies capable of protecting the host against an *S. pneumoniae* challenge.

Materials and methods

Reagents. Unless otherwise stated, all chemicals and biochemicals were of the highest purity available and were purchased from Sigma-Aldrich (St. Louis, MS, USA).

Bacterial strains and growth conditions. The encapsulated *S. pneumoniae* serotype 3 strain WU2 (25) was grown as previously described (26). Briefly, pneumococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or on blood agar plates.

Mice. Seven-week-old female BALB/cOlaHsd mice (n=121; thereafter referred to as BALB/c mice; Harlan Laboratories, Rehovot, Israel) and 7-week-old CBA/CaHN-Btk^{xid} mice (CBA/N^{xid}; Jackson Laboratories, Bar Harbor, ME, USA) were used in this study. The experiments were performed at one of two locations: i) at the animal facility of Ben-Gurion University of the Negev, Beer-Sheva, Israel, in accordance with the guidelines and approval of its Institutional Animal Care and Use Committee (IACUC) (permit no. 53.08.08); or ii) at the University of Mississippi Medical Center, Jackson, MS, USA, in accordance with the guidelines and approval of the relevant IACUC (permit no. 00163).

In all the experiments, the mice were euthanized with CO_2. In the survival experiments, the mice were monitored for fur appearance, social involvement, and their eating and drinking ability. Any mouse demonstrating an apparent illness, manifested by the appearance of bristled fur, social disengagement, or an inability to eat or drink, was euthanized with CO_2.

Cloning, expression and purification of rFBA. HAT-tagged rFBA was cloned as previously described (22). To prevent any alterations in the immune profile, an untagged protein was cloned, purified and then used for examining the cytokine profile elicited by rFBA and for the analysis of the IgG1/IgG2a experiments. The gene from the locus tag SP_RS02975 that codes for FBA was amplified from the TIGR4 strain with the following primers: forward, 5'-ATGGGATCCATATGGCGA TTGTGTCTGCAGAA-3' (*Nde*1); and reverse, 5'-CATGGAGC TCGCTCAGCTTATTACGCTTTGCCTTCG-3' (*Bpu*11021). The amplified product was inserted into the pET32a+ vector and transformed in DH5α UltraMAX ultracompetent *Escherichia coli* (*E. coli)* cells (Invitrogen, San Diego, CA, USA). Ampicillin-resistant transformants were selected and the vector was purified with a Qiagen HiSpeed Plasmid Maxi kit (Qiagen GmbH, Hilden, Germany). The pET32a+fba vector was transformed into *E. coli* host expression strain BL21(DE3)

pLysS (Novagen, Jaffrey, NH, USA). The identity of the inserts was confirmed by sequencing. The protein was prepared under denaturing conditions and refolded under physiological conditions. rFBA was purified by anion-exchange and size-exclusion chromatography. rFBA identity and purity were confirmed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The lipopolysaccharide (LPS) concentration in the rFBA protein was <8.9 EU/mg.

Preparation of DNA vaccine. The gene SP_0605 coding for FBA was amplified from the TIGR4 strain and the following primers were used: forward, 5'-TCGGATCCATGGCAATCG TTTCAGCAGA-3' (*Bam*H1); and reverse, 5'-TCGAATTCT GCTTTACCTTCTGAACCGA-3' (*Eco*R1). The amplified and *Bam*H1- and *Eco*R1- (Takara Bio Inc., Otsu, Japan) digested DNA fragments were cloned into the pVAC plasmid, and the pVAC^{fba} was transformed in DH5α UltraMAX ultracompetent *E. coli* cells (both from Invitrogen). Zeocin™-resistant transformants were cultured and the vector was purified from the DH5α cells with a Qiagen HiSpeed Plasmid Maxi kit (Qiagen GmbH).

Immunization of mice with rFBA for the survival assay. The BALB/c mice were immunized in one of three ways: i) subcutaneously (SC) with 25 μg of HAT-rFBA plus complete Freund's adjuvant (CFA) for the first immunization (day 0) and with incomplete Freund's adjuvant (IFA) in two subsequent booster immunizations (days 14 and 28; n=25); ii) intramuscularly (IM) with 50 μg pVAC^{fba} (n=21); or iii) intraperitoneally (IP) with 25 μg of HAT-rFBA and 75 μl of the Imject Alum adjuvant (Pierce Biotechnology, Inc., Rockford, IL, USA; n=15) on days 0, 14 and 28. The control mice were immunized with the respective adjuvant (CFA/IFA/IFA n=34, pVAC, empty plasmid n=14, Imject alum, n=6). The results shown are composed of the three different experiments performed at different times; in each experiment, all three adjuvants were tested.

Immunization of mice with rFBA for cytokine profile determination. The BALB/c mice were immunized on day 0 with 10 μg of untagged rFBA alone or with untagged rFBA plus CFA. Booster immunizations of rFBA alone or of rFBA with IFA were administered on days 7 and 21. The control mice were immunized with CFA/IFA alone. All mice were injected SC on both inner thighs, 25 μl/thigh.

Inoculation of immunized mice. The immunized BALB/c mice were inoculated intranasally with a lethal dose of 10^8 CFU of WU2. Survival was monitored daily.

Preparation of antigen-presenting cells (APCs). The mononuclear cells obtained from the spleens of naïve BALB/c mice were treated as previously described (27). Briefly, they were treated with rat anti-mouse monoclonal anti-CD4 (GK1.5), anti-CD8 (YTS169) and anti-Thy1.2 (30-H-12) antibodies, followed by the addition of a low-toxicity rabbit serum, as a source of complement. The monoclonal antibodies and the hybridomas were kindly provided by Professor L. Eizenbach from the Weizmann Institute of Science, Rehovot, Israel. The cells were treated with mitomycin C. The verification of CD4 and CD8 cell depletion and of the purity of the APCs was performed by flow cytometry.

Enrichment of CD4$^+$ T-cells by positive selection. CD4$^+$ T-cells from the inguinal lymph nodes, popliteal lymph nodes and from the spleen were obtained from the mice in all immunization groups. The mice were euthanized and the lymph nodes and spleens were mechanically disrupted. CD4$^+$ T-cells were enriched using magnetic beads conjugated to rat anti-mouse CD4$^+$ (L3T4) (Mini MACS system; Miltenyi Biotec, Bisley, UK). CD4$^+$ enrichment (95%) was verified by flow cytometry.

Bone marrow-derived dendritic cells (BMDCs). The BMDCs were generated from bone marrow cells obtained from BALB/c mice as described previously (28). Briefly, BMDCs were generated from bone marrow, which was obtained from BALB/c mice by flushing femoral cells with a 23-gauge needle with RPMI. Low-density mononuclear bone marrow cells were isolated using red blood cell lysing buffer (Sigma-Aldrich) and centrifuged. Cells ($5x10^6$) were cultured in 10-cm tissue dishes with the fresh complete Iscove's Modified Dulbecco's Medium (cIMDM) with recombinant GM-CSF (100 ng/ml) or 20% lymphocyte conditioned medium (LCM), respectively. The medium was changed every 2-3 days and replaced with fresh medium supplemented with GM-CSF (100 ng/ml) or 20% LCM respectively. The following modification to the original protocol is: the cells were grown in a complete RPMI medium and were allowed to differentiate for 9 days prior to co-culture with CD4$^+$ T-cells.

Enrichment of CD4$^+$ T-cells by negative selection. CD4$^+$ T-cells were harvested from the spleens and from the inguinal and popliteal lymph nodes of mice immunized with rFBA plus adjuvant, or with phosphate-buffered saline (PBS) plus adjuvant. The mononuclear cells were first separated with a standard Ficoll separation protocol. CD4$^+$ T-cells were negatively selected using the EasySep CD4$^+$ T-cell enrichment kit (StemCell Technologies, Inc., Vancouver, BC, Canada). The enrichment of CD4$^+$ T-cells (87-90% CD4$^+$ cells out of 23,609 events) was verified by flow cytometry.

Antibodies used for flow cytometry. In the present study, phycoerythrin (PE)-conjugated rat anti-mouse CD11c, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse B220, FITC-conjugated anti-mouse CD11b and allophycocyanin-conjugated rat anti-mouse major histocompatibility complex (MHC II) (IAbdq, I-Edk), all purchased from Pharmingen (San Diego, CA, USA), constituted the antibodies used for the purity verification of the APCs. The depletion of CD4$^+$ and of CD8$^+$ T-cells was verified with a FITC-conjugated rat anti-mouse CD4 and rat anti-mouse CD8 (YTS169) respectively, detected using a FITC-conjugated AffiniPure F(ab')2 fragment of mouse anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The negative selection of CD4$^+$ T-cells was verified using PE-conjugated rat anti-mouse CD4 and allophycocyanin-conjugated rat anti-mouse CD45 (eBioscience, San Diego, CA, USA). Stained cells were analyzed using either FACSCalibur or FACS Canto II (Becton-Dickinson, Mountain View, CA). The data were then analyzed using CELLQuest™ software (version 3.3; BD Biosciences, San Jose, CA, USA) or

with FlowJo software (version 6.3.4; Tree Star Inc., Ashland, OR, USA). Fluorescence data were acquired using logarithmic amplification and the reported fluorescence intensity units represent conversion of channel values according to the logarithmic scale.

In vitro stimulation of CD4$^+$ T-cells

CD4$^+$ T-cell proliferation. The APCs (1x10^5 cells) treated with mitomycin C were incubated with increasing concentrations of rFBA (0-2 μg/ml) for 30 min in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS). An enriched CD4$^+$ T-cell population was added to the rFBA-pulsed APCs (1:1) and incubated for 72 h at 37˚C. CD4$^+$ T-cell proliferation was evaluated as previously described (27), with a slight modification, namely, the extent of proliferation was determined using the BrdU ELISA kit (Hoffman-La Roche, Nutley, NJ, USA) according to the manufacturer's instructions. In these experiments we used 3 mice in each group and the results are a summary of 2 independent experiments, thus, 12 mice were used to perform these experiments.

Cytokine secretion. BMDCs, differentiated as previously described (28), were matured on cRPMI (penicillin 100 U/ml, streptomycin 100 μg/ml, L-glutamine 2 mM, 2-mercaptoethanol 50 μm, FCS 10%) containing 100 ng/ml LPS for 5 h, and then pulsed with 10 ng/ml rFBA for 24 h at 37˚C. The enriched CD4$^+$ T-cells, obtained from rFBA-immunized and PBS-immunized mice (5x10^6 cells), were co-cultured with 10-day-stimulated BMDCs (1x10^6 cells) in triplicate wells. The supernatants were collected at the denoted times and were frozen at -20˚C for later analysis. The cytokine concentrations were determined with a Bio-Plex cytokine (11-plex) assay (Bio-Rad Laboratories, Berkeley, CA, USA) according to the manufacturer's instructions. In addition, the concentration of interferon-γ (IFN-γ) was determined using an ELISA kit (Becton-Dickinson) according to the instructions provided by the manufacturer. In these experiments we used 3 mice in each group and the results are a summary of 3 independent experiments, thus, 18 mice were used to perform these experiments.

Quantitative analysis of IgG1 and IgG2a by ELISA. Two weeks after the final immunization, the BALB/c mice were euthanized and individually bled from the heart. ELISA was carried out in a 96-well plate coated with rFBA. Individual samples of mouse serum were added to the rFBA-coated plates. The concentrations of anti-rFBA mouse IgG1 and anti-mouse IgG2a were determined with biotin-conjugated goat anti-mouse IgG1 and biotin-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA), respectively, together with streptavidin-horseradish peroxide (HRP; Jackson ImmunoResearch Laboratories, Inc.). Standard curves were generated for mouse IgG1 and IgG2a concentrations (Biolegends, San Diego, CA, USA). In these experiments we used 3 mice in each group and the results are a summary of 3 independent experiments, thus, 18 mice were used to perform these experiments.

Immunization of rabbits with rFBA for neutralization assay. Three-month-old New Zealand white (NZW) rabbits (Harlan Laboratories) were immunized as previously described (26). Briefly, 3-month-old white albino rabbits (Harlan Laboratories) were immunized intramuscularly (IM) with 200 μg HAT-rPtsA emulsified with CFA (1:1) in the first immunization or with IFA in booster immunizations in 2-week intervals. Two weeks after their final immunization, the rabbits were exsanguinated, and sera were prepared. The specificity of the antisera was confirmed by immunoblotting and ELISA.

Ex vivo neutralization of S. pneumoniae with anti-rFBA antisera. The WU2 strain was incubated at 37˚C for 1 h either with diluted rabbit pre-immune or rabbit anti-rFBA antiserum. Subsequently, 500 CFU of the bacteria were IP-inoculated into the BALB/c mice. Survival was monitored daily. In this experiment 10 mice were inoculated with *S. pneumoniae* treated *ex vivo* with sera obtained from rFBA/CFA/IFA immunized rabbits and 10 mice were inoculated with *S. pneumoniae* treated *ex vivo* with sera obtained from pre-immune sera from rabbits.

The WU2 strain was incubated at 37˚C for 1 h either with 1:10 diluted CBA/N^{xid} mice pre-immune serum or with 1:25 diluted serum obtained from rFBA-immunized CBA/N^{xid} mice. Subsequently, 10^4 CFU of bacteria were IV-inoculated into the BALB/c mice. Survival was monitored continuously. In these experiments 10 CBA/N^{xid} were immunized with rFBA and 10 were immunized with the adjuvant only as described above. Sera were drawn and 10 mice were inoculated with bacteria treated *ex vivo* with sera obtained from rFBA immunized mice and 10 mice were inoculated with bacteria treated *ex vivo* with sera obtained from adjuvant immunized mice.

Statistical analysis. Log-rank and Wilcoxon signed-rank test, as determined by the GraphPad 6 software, were used to evaluate survival. Differences in cytokine levels or cell proliferation rates between two groups were analyzed with a two-tailed Student's t-test. Values of $p<0.05$ were considered to indicate a statistically significant difference.

Results

rFBA as a potential vaccine. The BALB/c mice immunized with rFBA + CFA/IFA/IFA [namely, with CFA and then with two IFA boosters] or with the adjuvants only (control) were challenged intranasally with a lethal dose of encapsulated *S. pneumoniae* serotype 3 strain WU2. The survival rates of the mice immunized with rFBA + CFA/IFA/IFA were significantly higher than those of the control mice (Fig. 1A; log-rank test $p<0.01$). The survival rates of the mice immunized with a DNA vaccine (namely, with *fba* inserted into the pVAC plasmid; pVAC^{fba}) were also significantly higher than those of the mice immunized with the respective adjuvant alone (Fig. 1B; log-rank test $p<0.05$); however, the DNA vaccine was less protective (19% survivors) than immunization with FBA + CFA/IFA/IFA (36% survivors). The mice immunized with rFBA + alum demonstrated 26% survival, in comparison with 0% survival in the mice immunized with alum alone (Fig. 1C; Wilcoxon, $p<0.05$).

CD4$^+$ T-cell proliferation. To examine the extent of CD4$^+$ T-cell proliferation in response to rFBA stimulation, CD4$^+$ T-cells obtained from the rFBA + CFA/IFA/IFA-immunized mice were co-cultured with naïve APCs treated *in vitro* with rFBA. The control groups included CD4$^-$ T-cells derived from the mice immunized with rFBA + CFA/IFA/IFA, CD4$^+$ T-cells obtained from the mice immunized with the adjuvant alone,

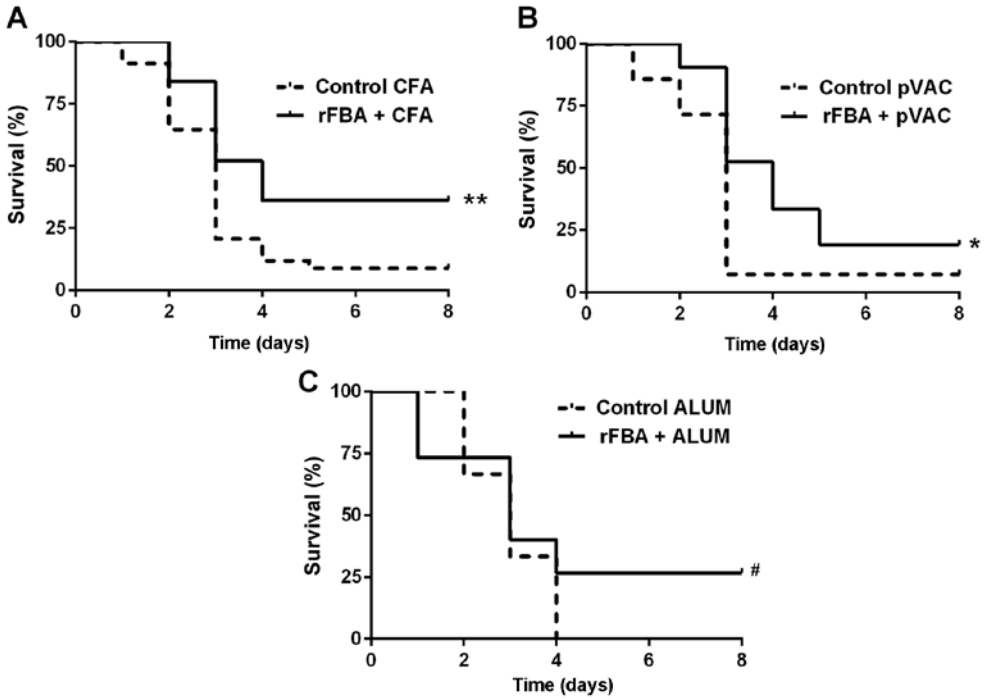

Figure 1. Vaccine potential of recombinant fructose-1,6-bisphosphate aldolase (rFBA). BALB/c mice were immunized subcutaneously (SC) with rFBA + complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA)/IFA [(A) n=25], intramuscularly with pVAC^{fba} [(B) n=21], or intraperitoneally (IP) with Imject Alum [(C) n=15]. Control mice were immunized with the respective adjuvants: SC with CFA/IFA [(A) n=34] or with an empty plasmid [(B) n=14], or IP with Imject Alum [(C) n=6]. Immunized mice were inoculated intranasally with 10^8 CFU of the *S. pneumoniae* WU2 serotype. Survival was monitored daily. A Mann-Whitney U test revealed significantly higher survival for the rFBA + CFA/IFA/IFA- and pVAC^{fba}-immunized groups as compared with the respective control groups [(A) CFA/IFA/IFA, long-rank test $^{**}p<0.01$; (B) pVAC^{fba}, long-rank test $^{*}p<0.05$). rFBA + Imject Alum group demonstrated increased protection that did not reach significance by log-rank but reached significance by Wilcoxon test [(C) $^{\#}p<0.05$] in comparison with their respective controls. The results presented are a summary of two independent experiments.

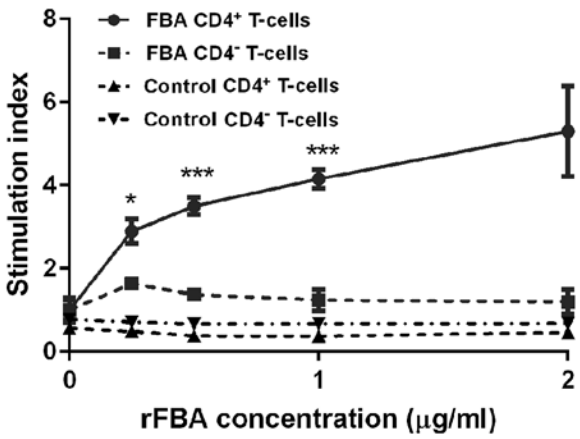

Figure 2. Memory CD4$^+$ T-cell proliferation in response to recombinant fructose-1,6-bisphosphate aldolase (rFBA) re-stimulation. The extent of proliferation, determined with a BrdU ELISA kit, was examined in the following groups of cells: i) CD4$^+$ T-cells from mice immunized with rFBA + complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA)/IFA (FBA CD4$^+$ T-cells). All the following groups served as control groups: ii) CD4$^+$ T-cells derived from mice immunized with an adjuvant only (control CD4$^+$ T-cells). In addition, mononuclear cells depleted of CD4$^+$ T-cells, derived from iii) mice immunized with rFBA (control FBA CD4$^-$ T-cells) or from iv) mice immunized with an adjuvant only (control CD4$^-$ T-cells). All cells were co-cultured with mitomycin-treated spleen-derived naïve antigen-presenting cells (APCs) and incubated for 72 h with increasing concentrations of rFBA (0-2 μg/ml). The values represent the means of quadruplicate wells ± standard deviation, and are expressed as the stimulation index, namely, the ratio of the extent of proliferation in the presence of rFBA to that in the absence of rFBA. Significant differences were found with the Student's t-test between the FBA CD4$^+$ T-cells group and the control groups ($^{*}p\leq0.05$ and $^{***}p<0.001$). Results are the summary of two independent experiments.

and CD4$^-$ T-cells obtained from the mice immunized with the adjuvant alone. The CD4$^+$ T-cells derived from the mice immunized with rFBA + CFA/IFA/IFA showed a dose-dependent increase in the proliferation response upon re-stimulation with rFBA-pulsed APCs (Fig. 2; $p<0.05$). The CD4$^+$ T-cells that were derived from the mice immunized with the adjuvant alone and from the CD4$^+$ T-cell-depleted groups did not respond to rFBA-pulsed APCs. These results demonstrate that rFBA induces an antigen-specific CD4$^+$ T-cell proliferative response.

Cytokine secretion from memory CD4$^+$ T-cells in response to re-stimulation with rFBA. To evaluate the nature of the immune response following immunization with rFBA, we measured *in vitro* the cytokine secretion by CD4$^+$ T-cells in response to stimulation with rFBA-pulsed BMDCs. Two types of co-cultures were examined: CD4$^+$ T-cells obtained from the mice immunized with rFBA + CFA/IFA/IFA ('FBA' culture), and CD4$^+$ T-cells obtained from the mice immunized with the adjuvant alone ('CFA' culture). Additional controls included cultures with BMDCs only ('DC-only' culture), CD4$^+$ T-cells only, obtained from rFBA + CFA/IFA/IFA-immunized mice ('CD4-only' culture) and CD4$^+$ T-cells stimulated with concanavalin A (ConA) ('CD4$^+$ConA' culture).

The supernatant of the FBA culture contained levels of the Th1-related cytokines IFN-γ, IL-2 and tumor necrosis factor-α (TNF-α) that were significantly higher than those in the supernatant of any of the other cultures (Fig. 3). More specifically, in the FBA culture, the levels of IFN-γ were significantly higher than baseline (Fig. 3A; $p<0.01$) between 12 and 72 h

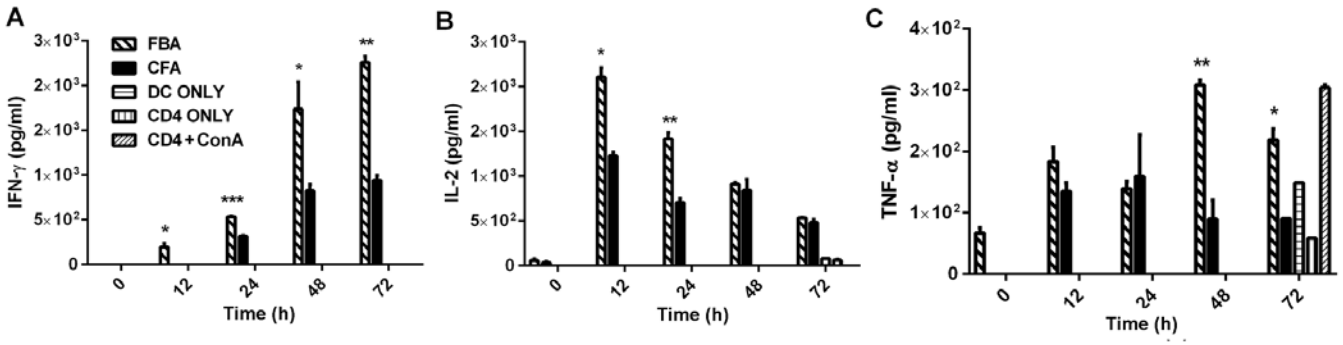

Figure 3. T helper (Th)1-type cytokine secretion from memory CD4+ T-cells in response to recombinant fructose-1,6-bisphosphate aldolase (rFBA) re-stimulation. CD4+ T-cells were obtained from BALB/c mice immunized with rFBA + complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA)/IFA ['FBA' cultures; the legend for the bar fill format that appears in (A) also applies to (B and C)], or from mice immunized with adjuvant alone ('CFA' cultures), followed by negative selection. CD4+ T-cells that were obtained from BALB/c mice immunized with rFBA and CFA/IFA were used for the 'CD4+ only' control culture; BMDC cultures were used for the 'DC only' control cultures; and CD4+ T-cells that were obtained from mice immunized with the adjuvant alone and stimulated with concanavalin A (ConA) were used for the 'CD4+ConA' control culture. CD4+ T-cells in the control groups were stimulated with ConA. All CD4+ T-cells were co-incubated with rFBA-pulsed BMDCs for 72 h. The levels of cytokine secretion [(A) IFN-γ; (B) interleukin (IL)-2; (C) tumor necrosis factor (TNF)-α] was measured by multiplex ELISA. The results represent the means ± standard deviations of triplicate wells. A two-tailed Student's t-test found significant differences ($p<0.05$) between the 'FBA' cultures and 'CFA': cultures ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$). Results show one representative experiment out of two independent experiments.

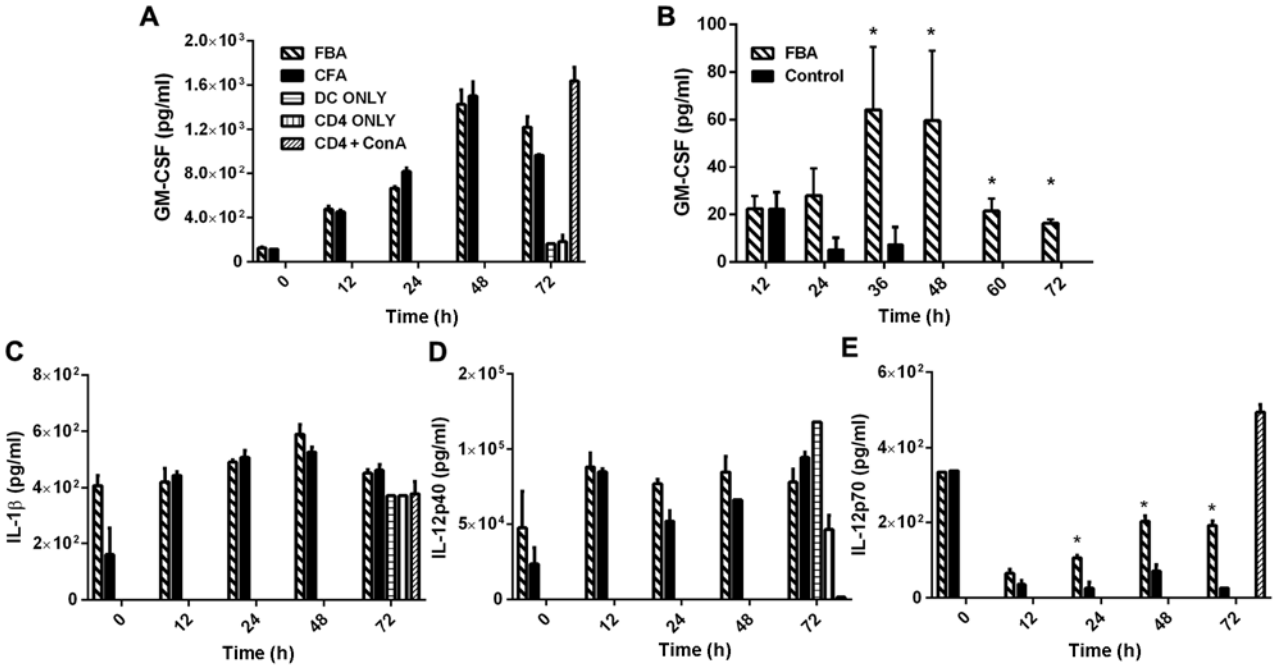

Figure 4. Pro-inflammatory-type cytokine secretion from memory CD4+ T-cells in response to recombinant fructose-1,6-bisphosphate aldolase (rFBA) re-stimulation. CD4+ T-cells were obtained from BALB/c mice immunized with rFBA + complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA)/IFA ['FBA' cultures; the legend for the bar fill format that appears in (A) applies also to (C-E)] or from mice immunized with the adjuvant alone ('CFA' cultures), followed by negative selection. CD4+ T-cells that were obtained from BALB/c mice immunized with rFBA and CFA/IFA were used for the 'CD4+ only' control cultures, and CD4+ T-cells that were obtained from mice immunized with the adjuvant alone were stimulated with 5 μg/ml of concanavalin A (ConA) and used for the 'CD4+ConA' control cultures. CD4+ T-cells were co-incubated with rFBA-pulsed BMDCs for 72 h for determination of the level of the cytokine secretion measured by multiplex ELISA. The results represent the means ± standard deviation of triplicate wells. A two-tailed Student's t-test revealed no significant differences between the FBA and CFA cultures for (A) granulocyte-macrophage colony-stimulating factor (GM-CSF) (C) IL-1β, (D) IL-12p40. (E) By contrast IL-12p70 demonstrated high levels at time 0 h that decreased at 12 h and significantly increased at 24, 48 and 78 h ($p<0.05$). Results are from one representative experiment out of two independent experiments. (B) GM-CSF/APC. CD4+ T-cells were obtained from BALB/c mice immunized with rFBA + CFA/IFA/IFA ('FBA' cultures), or from mice immunized with the adjuvant alone ('CFA' cultures), followed by negative selection. CD4+ T-cells were co-incubated with rFBA-pulsed spleen-derived naïve antigen-presenting cells (APCs) for 72 h. The results represent the mean ± standard deviation of triplicate wells. Significant differences ($p<0.05$) were determined by a two-tailed Student's t-test between the 'FBA' cultures and the 'CFA' cultures. Results are combined from two independent experiments.

following re-stimulation, and they peaked at 48-72 h (Fig. 3A); the levels of IL-2 peaked at 12 h (Fig. 3B; $p<0.05$) and declined thereafter; and the levels of TNF-α peaked at 48 h (Fig. 3C; $p<0.01$) and were then reduced at 72 h (Fig. 3C; $p<0.05$). Of note, the TNF-α level increased at 72 h in the CD4+ ConA group (as shown in Fig. 3C). No increase was observed in the secretion of IFN-γ, IL-2 or TNF-α in the supernatants of the CFA, DC-only, CD4-only, or CD4+ConA cultures.

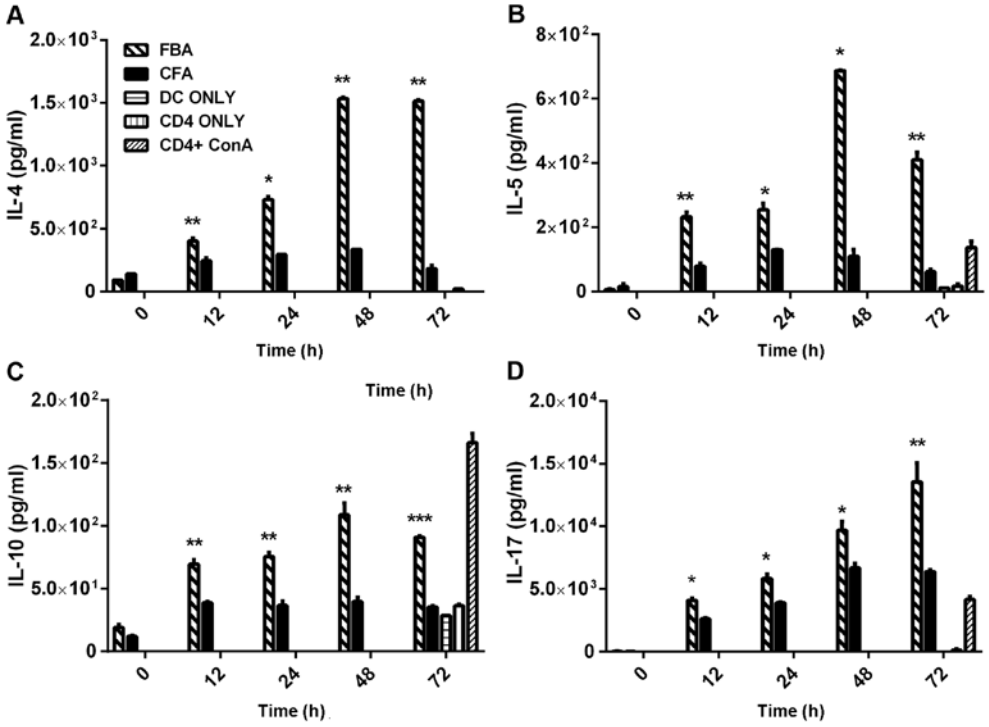

Figure 5. T helper (Th)2- and Th17-type cytokine secretion from memory CD4+ T-cells in response to recombinant fructose-1,6-bisphosphate aldolase (rFBA) re-stimulation. CD4+ T-cells were obtained from BALB/c mice immunized with rFBA + complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA) booster/IFA booster ['FBA' cultures; the legend for the bar fill format in (A) applies also to (B-D)], or from mice immunized with adjuvant alone ('CFA' cultures), followed by negative selection. CD4+ T-cells that were obtained from BALB/c mice immunized with rFBA and CFA/IFA were used for the 'CD4+ only' control cultures, and CD4+ T-cells that were obtained from mice immunized with the adjuvant alone were used for the 'CD4+ConA' control cultures. CD4+ T-cells stimulated with 5 μg/ml of concanavalin A (ConA) served as CD4+ ConA group. CD4+ T-cells were co-incubated with rFBA-pulsed BMDCs for 72 h. The levels of cytokine [(A) IL-4; (B) IL-5; (C) IL-10; and (D) IL-17] secretion were measured by multiplex ELISA. The results represent the means ± standard deviation of triplicate wells. Significant differences between groups were calculated with a two-tailed Student's t-test ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$). Results are from one representative experiment out of two independent experiments.

High levels of the Th1-related cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β IL-12p40, co-cultured with the rFBA-stimulated BMDCs, were found in the supernatants of the FBA and CFA cultures (Fig. 4A, C and D). No such stimulation was observed in the BMDC-only or in the CD4-only cultures. Notably, in the supernatant of CD4+ T-cells that were obtained from the rFBA-immunized mice and co-cultured with naïve spleen-derived APCs treated with rFBA, the levels of GM-CSF significantly increased at 36 and 48 h following co-culture initiation (Fig. 4B; $p<0.05$) and declined thereafter (Fig. 4). However, high levels of GM-CSF were observed in the CD4+ConA cultures (Fig. 4A). IL-1β and IL-12p40 were detected in the supernatant of DC-only cultures (Fig. 4C and D, respectively) and in the supernatant of CD4-only cultures (at <3%), probably due to residual APCs in the supernatant following the negative selection procedure. Seventy-two hours following the initiation of the cultures, the levels of IL-1β increased to a similar extent in all the control cultures, while the levels of IL-12p40 decreased in the CD4+ConA culture. The IL-12p70 levels were elevated in the FBA and CFA cultures at the time of initiation of the co-cultures and declined at 12 h after culture initiation. The levels of IL-12p70 in the FBA culture co-cultured with BMDCs increased from the reduced levels at 12 h following the initiation of the co-cultures and reached a plateau at 48 h (Fig. 4E; $p<0.05$) ; this pattern was not observed in any of the control cultures.

The supernatants of the FBA cultures demonstrated significantly higher levels of the Th2-related cytokines, IL-4, IL-5 and IL-10, at 12-48 h after co-incubation with the rFBA-pulsed BMDCs (Fig. 5A-C, respectively). As compared with their baseline levels, the levels of each of those three cytokines peaked at 48 h ($p<0.05$); then, the levels of IL-4 remained elevated (Fig. 5A; $p<0.05$), whereas the levels of IL-5 (Fig. 5B) and IL-10 (Fig. 5C) declined, remaining higher than their baseline levels ($p<0.05$ for each cytokine). The secretion of IL-4, IL-5 and IL-10 was significantly decreased in all control cultures, except for an increase in IL-10 in the CD4+ConA culture (Fig. 5C).

The levels of the Th17-related cytokine, IL-17A, increased significantly (from 34 pg/ml to 13,500 pg/ml; Fig. 5D; $p<0.05$) in the FBA culture at 72 h following the co-incubation of the CD4+ T-cells with the rFBA-pulsed BMDCs (Fig. 5D). Such an increase was not observed in any of the control cultures.

Concentrations of anti-rFBA IgG1 and IgG2a subtypes and protective ability of anti-rFBA antiserum. In addition to the cytokine profile expressed *in vitro*, we determined the concentrations of IgG1 and IgG2a antibodies in sera obtained from the mice immunized with rFBA + CFA/IFA/IFA or with CFA/IFA/IFA alone (control). The mice immunized with rFBA + CFA/IFA/IFA showed anti-rFBA IgG1 (Fig. 6A; $p<0.001$) and anti-rFBA IgG2a (Fig. 6A; $p<0.05$) titers that were significantly higher than those in the control mice (Fig. 6A).

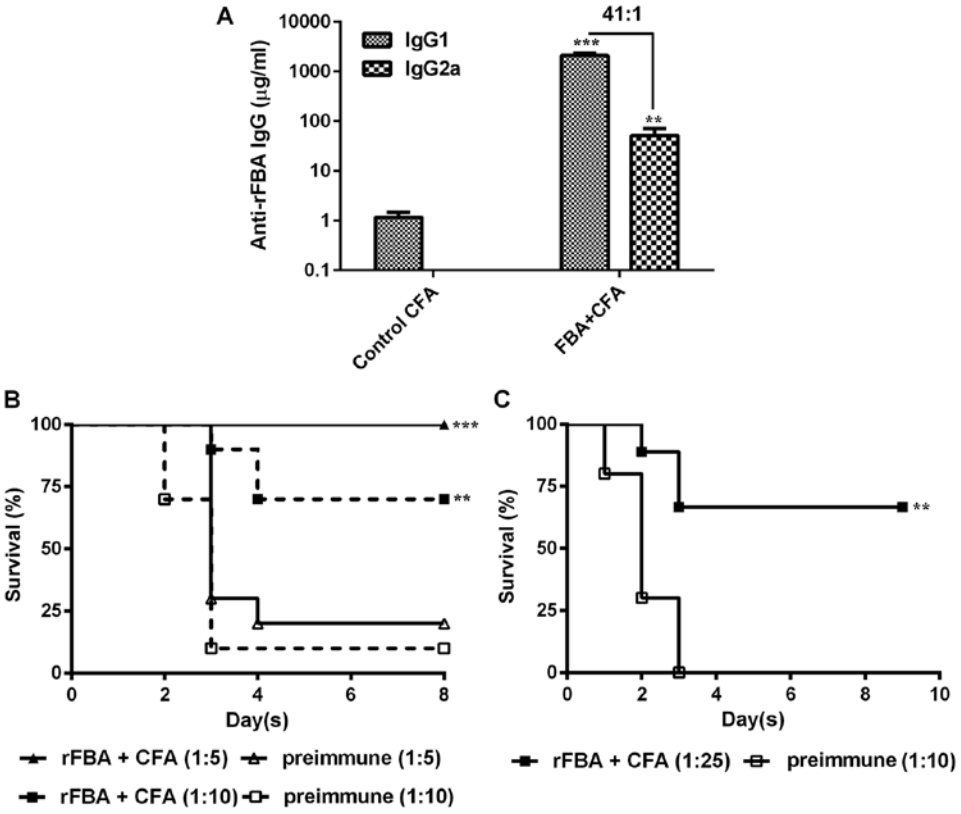

Figure 6. Anti-recombinant fructose-1,6-bisphosphate aldolase (rFBA) antibodies: IgG1 and IgG2a subtypes and their *S. pneumoniae* neutralizing ability. (A) BALB/c mice were immunized with rFBA + complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA)/IFA. Control mice were immunized with CFA/IFA/IFA alone. Two weeks following the third immunization, serum concentrations of IgG1 and IgG2a antibodies to rFBA were determined. A two-tailed Student's t-test revealed significant differences in the concentrations of IgG1 ($p<0.001$) and IgG2a ($p<0.05$) in sera obtained from rFBA-immunized mice, as compared with the negative control. The presented results were summarized from two independent ELISA experiments. Two or three mice were immunized in each group. (B) *S. pneumoniae* serotype 3 strains WU2 were incubated at 1:5 or 1:10 dilutions with either preimmune rabbit serum or with rabbit anti-rFBA antiserum. Then, 500 CFU of WU2 were inoculated intraperitoneally in BALB/c mice and survival was monitored daily for 7 days. Mice inoculated with anti-rFBA antiserum survived significantly longer than mice inoculated with pre-immune-treated bacteria (1:5 dilution, log-rank test $^{***}p<0.001$; 1:10 dilution (log-rank test $^{**}p<0.01$). Each group comprised 10 mice. (C) WU2 strains were incubated at a 1:10 dilution with preimmune serum obtained from CBA/N^{xid} mice or at a 1:25 dilution with serum obtained from CBA/N^{xid} mice immunized with rFBA. Then, 10^4 CFU of WU2 strain were inoculated intravenously in BALB/c mice and survival was monitored continuously. BALB/c mice inoculated with bacteria incubated *ex vivo* with sera obtained from rFBA-immunized CBA/N^{xid} survived significantly longer than mice inoculated with preimmune-treated bacteria (log-rank test $^{*}<0.01$). Each group comprised 10 mice.

Analysis of the concentration ratio of IgG1 to IgG2a antibodies showed that immunization with rFBA + CFA/IFA/IFA resulted in an IgG1/IgG2a ratio of 41.

We then investigated the ability of the serum obtained from the rFBA-immunized rabbits to neutralize *S. pneumoniae* virulence. The BALB/c mice inoculated IP with WU2 bacteria that were pre-incubated *ex vivo* with rabbit anti-rFBA antiserum survived significantly longer (Fig. 6B; $p<0.01$) than the mice inoculated with bacteria treated with pre-immune rabbit serum (Fig. 6B). In addition, the BALB/c mice inoculated intravenously with a lethal dose of WU2 pre-incubated *ex vivo* with CBA/N^{xid} mouse anti-rFBA antiserum survived significantly longer than the mice inoculated with bacteria pre-incubated *in vitro* with mouse pre-immune serum (Fig. 6C; $p<0.01$) .

Discussion

The surface localization of FBA has been previously demonstrated by immunoblotting and MALDI-TOF analysis of thge bacterial cell wall (22) and by flow cytometric analysis of live bacteria stained with rabbit anti-rFBA antibodies (24). rFBA in the presence of alum has been shown to elicit a protective immune response in a mouse lethal challenge model (22). Thus, similar to other glycolytic enzymes (26,27), FBA was suspected to have important functions other than its principal role as a cytoplasmic enzyme in glycolysis. Indeed, FBA was later found to mediate the adhesion of *S. pneumoniae* to host cells, as FBA and anti-FBA antibodies inhibited *S. pneumoniae* adhesion to A549 cells (24). In addition, using a peptide combinatorial library expressed in the filamentous phage, FCR was identified as the putative receptor of FBA. A peptide derived from FCR inhibited *S. pneumoniae* adhesion to A549 cells *in vitro* and prevented disease development *in vivo* in the mouse model following *S. pneumoniae* challenge (24). In the present study, we aimed to elucidate the nature of the protective immune response elicited by rFBA.

A major challenge in the development of subunit vaccines is the development of an efficient adjuvant or of a lymph node delivery system that would replace the highly potent CFA adjuvant (28-31). Aluminum salts elicit purely Th2-type responses (28), DNA vaccines elicit mainly Th1-type immune responses (by interacting with TLR9) (29), and CFA/IFA adjuvants elicit both a Th1-type (30) and a Th2-type (31) immune

responses. A Th17-type response was previously observed in experimental autoimmune encephalomyelitis induced by immunization with myelin-derived peptides in CFA (32), and, therefore, we suspected that a Th17-type response will be induced in the presence of CFA as the adjuvant. Th17 has recently been shown to be associated with protective immunity against *S. pneumoniae* (34). Despite extensive efforts to develop new adjuvants, none has thus far surpassed the adjuvancy capacity of CFA (5,33). CFA/IFA adjuvants are widely used in preclinical studies investigating candidates for a *S. pneumoniae* protein-based vaccine (34-36).

In the present experiments, the protection observed against a lethal *S. pneumoniae* challenge following immunization with rFBA in the presence of CFA/IFA surpassed the protection observed following immunization with pVAC^{fba} or of rFBA in the presence of alum salt. Intranasal inoculation of mice with a whole cell vaccine (WCV) in the presence of cholera toxin or labile toxin, or of their non-toxic mutated derivatives, has been shown to elicit protection against intranasal inoculation with *S. pneumoniae* (37), which was Th17-dependent and antibody-independent (38). However, parenteral immunization with a WCV in the presence of alum as the adjuvant, either subcutaneously or intramuscularly, elicited a mucosal and systemic protection that was antibody dependent and T-cell-independent (39). WCVs contain pathogen-associated molecular patterns (PAMPs), such as lipoprotein and lipoteichoic acid that interact with TLR2, pneumolysin that interacts with TLR4, or peptidoglycan that interacts with nucleotide-binding oligomerization domain containing 2 (NOD2), which may contribute to the protective immune response elicited by the host (40). Lipidated membrane proteins have also been shown to elicit an increase in Th17 response and improve protection in comparison with non-lipidated proteins as a result of TLR2 activation (41). We suspected that the better protection following immunization with a single protein and with CFA/IFA as the adjuvant will elicit protection of the three arms of the immune response due to the mycobacterial components of CFA (40).

In this study, initial experiments performed on CD4$^+$ T-cells co-incubated with rFBA-stimulated APCs revealed a significantly increased proliferation of memory CD4$^+$ T-cells in comparison with the following three control groups: CD4$^-$ T-cells obtained from the rFBA-immunized mice, CD4$^+$ T-cells obtained from the mice immunized with the adjuvant only, and CD4$^-$ T-cells obtained from the mice immunized with the adjuvant only. The extent of CD4$^+$ T-cell proliferation was similar to that described for other bacterial protein immunogens (42,43).

The nature of the adaptive immune response to rFBA was examined by analyzing the cytokine secretion profile in the co-cultures of rFBA-pulsed naïve BMDCs and CD4$^+$ T-cells obtained from rFBA-immunized mice. In the supernatant of those cultures, the levels of IL-2, IFN-γ, TNF-α, IL-12p70, IL-4, IL-5, IL-10 and IL-17 were significantly higher than those in the supernatants of the co-cultures of rFBA-treated BMDCs with CD4$^+$ T-cells obtained from the mice immunized with the adjuvant only. The supernatants of the CD4$^+$ T-cells obtained from the rFBA-immunized mice in the absence of BMDCs did not show an increased cytokine secretion. Similarly, the CD4$^-$ T-cells obtained from either the rFBA + CFA/IFA/IFA or the adjuvant only-immunized mice did not show increased cytokine secretion. This finding confirms the importance of the cross-talk between CD4$^+$ T-cells and rFBA-treated dendritic cells for the secretion of those cytokines, which are known to be secreted from Th1-, Th2- and Th17-type memory CD4$^+$ T-cells (44,45).

The secretion of IL-2 peaked as early as 12 h following the initiation of the co-culture, and subsequently decreased. This temporal pattern is in accordance with the known secretion pattern of IL-2, which is rapid and transient, and the decrease in IL-2 production was due to the negative feedback loop required to limit IL-2 production during helper T-cell differentiation (46). The significantly increased secretion of IFN-γ that was detected in this study is consistent with that of previous studies on children, which demonstrated the importance of IFN-γ for the naturally acquired immune response to *S. pneumoniae* (47) and for the acquired immune response to *S. pneumoniae* proteins in mice (48). In this study, the secretion of IL-2 and IFN-γ cytokines suggests the existence of a Th1-type response to immunization with rFBA. The significantly increased secretion of the proinflammatory cytokine TNF-α is in accordance with previous research that demonstrated the importance of TNF-α in the development of the adaptive humoral immune response to rPspA following immunization with unencapsulated R36A (49).

The levels of GM-CSF, IL-12p40 and IL-1β observed in the cultures of CD4$^+$ T-cells obtained from the rFBA-immunized mice were insignificant compared to those in CD4$^+$ T-cells obtained from the adjuvant-immunized mice. Dendritic cell maturation was performed by exposing the cells to LPS prior to stimulation with rFBA. Thus, the increased levels of the GM-CSF, IL-12p40 and IL-1β cytokines were probably due to the effect of LPS on the dendritic cells, as was described previously (50). Indeed, a significant increase was observed in the levels of GM-CSF in the supernatant of CD4$^+$ T-cells that were obtained from the rFBA-immunized mice and co-cultured with APCs pulsed with rFBA but not treated with LPS, as compared with those observed in the supernatant of CD4$^+$ T-cells that were obtained from the adjuvant-immunized mice and co-cultured with rFBA-pulsed APCs (Fig. 4B).

The levels of IL-12p70 detected in the culture supernatant of CD4$^+$ T-cells obtained from the rFBA-immunized mice and co-cultured with the rFBA-treated BMDCs were significantly higher than those detected in the culture supernatant of CD4$^+$ T-cells obtained from the adjuvant-immunized mice in the presence of rFBA-treated BMDCs. This phenomenon was not observed in the rFBA-treated BMDCs co-cultured with CD4$^+$ T-cells obtained from the adjuvant-immunized mice, suggesting that a cross-talk exists between CD4$^+$ T-cells and the rFBA-treated BMDCs. Indeed, such a cross-talk is crucial in the development of Th1-type responses, as described previously (51).

A significant increase in the secretion of IL-4, IL-5 and IL-10 was observed in the supernatants of CD4$^+$ T-cells obtained from the rFBA-immunized mice co-cultured with the rFBA-treated BMDCs. The higher levels of IL-4 resemble those found after immunizing mice with rGroEL (52), PspA (53) or PsaA (54). The increased secretion of IL-4, IL-5 and IL-10 suggests that rFBA immunization elicits a Th2-type immune response, which may be important for protection. Recently, IL-10 has been shown to be important to avoid excessive tissue

inflammation and to improve host survival, despite the fact that bacterial dissemination is less efficient in the presence of this cytokine (55).

The secretion of the IL-17 cytokine by $CD4^+$ T-cells co-cultured with the rFBA-treated BMDCs increased continuously throughout the 72 h of the experiment. This finding is in agreement with the findings of recent studies demonstrating the importance of IL-17 in protection against *S. pneumoniae*. The seminal studies of Malley and Anderson [reviewed in (8)] have shown that, in mice, the acquired protective immune response to pneumococcal colonization following intranasal immunization with heat-killed unencapsulated bacteria depends on $CD4^+$ T-cells, and particularly on the Th17 arm of the immune response; however, it is antibody-independent (39).

In the present study, cytokines from the Th1, Th2 and Th17 arms of the immune response were induced following immunization with a single protein, rFBA. These results confirm previous findings obtained *in vivo* in the bronchoalveolar lavage and the serum of mice immunized with *Lactobacillus lactis* expressing the PppA protein from *S. pneumoniae* (56). Other studies performed with human $CD4^+$ T-cells have demonstrated the dominance of both the Th17-type and Th1-type immune responses to *S. pneumoniae* (9,10) or to *S. pneumoniae* proteins (4,50). Parenteral immunization with attenuated WCV in the presence of alum elicited Th1-, Th2- and Th17-type cytokine expression that was an order of magnitude lower than that found in the present study (57). An alum-adjuvanted *Staphylococcus aureus* antigen [(clumping factor A (ClfA)] induced IL-17-dependent protection against Staphylococcal challenge (58). Therefore, the ability of alum to promote Th17-type responses appears to depend on the nature of the vaccine antigen; in particular, antigens with inherent immunomodulatory activity, such as killed bacteria, may synergize with the adjuvant to promote Th17-type responses.

ConA was used in the present study as a polyclonal non-specific stimulator for cytokine secretion. ConA is a member of the legume lectin family and binds specifically to internal and terminal α-D-mannosyl and α-D-glucosyl groups found in various sugars, glycoproteins and glycolipids (59). ConA binds the CD3 component of human T-cell receptors (60) and most of its stimulatory properties are similar to those of anti-CD3 and anti-TCR antibodies (61). The effect of ConA on T-cells is generally attributed to its binding to and cross-linking of the CD3 component of the TCR, thereby mimicking the physiological T-cell receptor ligand MHC-peptide complex (62). However, lectins are more potent stimulators of cell proliferation than anti-CD3 or anti-CD4 antibodies, possibly as a result of simultaneous activation of one or more co-stimulatory receptors. In addition, differences in substrate phosphorylation downstream from TCR following the binding of an MHC-peptide complex, in comparison to ConA activation of $CD4^+$ T-cells, were reported (63). The activation of T-cells with ConA resulted in reduced secretion of IL-2 and IFN-γ in comparison with stimulation with an antigen (64), suggesting that antigen-MHC or ConA activation of CD3 stimulates different levels of cytokine expression.

The type of antibody produced against rFBA and the importance of antibodies to protection were analyzed in the sera obtained from the rFBA-immunized mice. Immunizing mice with rFBA resulted in a median IgG1/IgG2a ratio of 41, suggesting a primary Th2 response resulting in IgG1 antibody production, with a substantial Th1 response resulting in IgG2a antibody production. In a previous study, the immunization of BALB/c mice with rPspA + alum induced an IgG1/IgG2a ratio of 1024, suggesting a strong Th2-biased response. However, immunization with a *pspA* DNA vaccine induced an IgG1/IgG2a ratio of 2, suggesting a strong Th1 bias (65). Th1- and Th2-type immune responses are mutually exclusive in the presence of all the components of the immune system *in vivo*. Thus, the Th1 Th2 exclusion is never absolute but, rather, relative. The induction of both Th1- and Th2-type responses *in vivo* is in accordance with our *in vitro* results, in which Th1- and Th2-related cytokines were found upon exposure of the $CD4^+$ T-cells to rFBA-pulsed BMDCs. The expression of Th1- and Th2-type cytokines appeared simultaneously; suggesting that memory T-cells from these arms of the immune response are present in the rFBA-immunized mice. Activation *in vitro* with the rFBA-pulsed BMDCs elicited cytokine production from the Th1, Th2 and Th17 arms of the immune response. *In vivo*, re-exposure to an immunogen/pathogen may culminate in higher expression of one of the arms of the immune response relative to the others due to activation of control mechanisms that are lacking in the *in vitro* system. Furthermore, rabbit and mouse anti-rFBA antisera pre-incubated *ex vivo* with *S. pneumoniae* significantly neutralized bacterial virulence and thereby protected the mice against a *S. pneumoniae* lethal challenge, as compared with bacteria pre-incubated with pre-immune serum. This neutralization may result from interference in bacterial adhesion to host cells (66) and suggests that the antibodies also enhance phagocytosis of bacteria that shed their capsule during adhesion.

We conducted our experiments with both the rabbit and the mouse sera since different species have different immunoglobulin protein profiles. Humans and mice have five antibody isotypes (IgA, IgD, IgE, IgG and IgM), whereas, in rabbits, four isotypes (IgA, IgE, IgG and IgM) have been identified to date, to the best of our knowledge. In addition, rabbits have only one IgG subclass, whereas mice have five subclasses (IgG1, IgG2a, IgG2b, IgG2c and IgG3) and humans have four (IgG1, IgG2, IgG3 and IgG4). We have previously demonstrated that anti-rFBA antisera inhibit the adhesion of *S. pneumoniae* to cultured lung-derived epithelial cells (24). Similarly, antibodies to other *S. pneumoniae* adhesins, among which are PcpA and PsrP, inhibited the adhesion of *S. pneumoniae* to human respiratory epithelial cells (15,67), and antibodies against PcpA were found to protect mice against a pneumococcal challenge (15). The exposure of bacterial cell-wall proteins occurs during bacterial adhesion to the host as a result of capsular shedding (66), which enables access and binding of the antibodies to their respective proteins and, possibly, facilitates their neutralization. Therefore, the observed protection of mice against a *S. pneumoniae* challenge by using anti-FBA antibodies may result from inhibition of bacterial adhesion to the host.

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