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Original Paper

The Effect of Delivery Systems on the Induction of T Helper 1 Cell Response to an ESAT6-Like Protein Rv3619c and Identification of Its Immunodominant Peptides

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Highlights of the Study

- This study evaluated the effects of antigens and delivery systems on the induction of Th1 cytokines in response to *Mycobacterium tuberculsosis*-specific protein Rv3619c in mice.
- Four delivery systems were evaluated; chemical adjuvants (incomplete Freund's adjuvant and aluminum hydroxide), recombinant *Mycobacterium smegmatis* and DNA plasmid.
- Th1 immune responses were induced in mice in response to the pool of peptides as well as to individual peptides covering the sequence of Rv3619c antigen.

Abstract

Objective: This study determined the effects of chemical adjuvants, incomplete Freund's adjuvant (IFA) and aluminum hydroxide (Alum), mycobacteria, and a DNA plasmid as delivery systems on the induction of protective Th1 (interferon-gamma (IFN-γ)) and nonprotective Th2 (IL-5) and Treg (IL-10) cytokine responses to Rv3619c and its peptides. Rv3619c is an immunodominant *Mycobacterium tuberculosis*-specific antigen and belongs to the early-secreted antigenic target of 6 kDa-family of proteins. Delivery systems are needed to deliver such antigens in animal models and induce protective immune responses. ***Methods:*** The *rv3619c* gene was amplified from the genomic DNA of *M. tuberculosis* and cloned into appropriate vectors for expression in *Escherichia coli*, *Mycobacterium smegmatis*, and eukaryotic cells. Spleen cells from mice immunized with *rv3619c* using different delivery systems were stimulated in vitro with synthetic peptides (P1 to P6) of Rv3619c, and secreted cytokines were estimated by ELISA. ***Results:*** The recombinant *M. smegmatis* and DNA plasmid induced the secretion of the protective cytokine IFN-γ in response to peptide-pool of Rv3619c and all the individual peptides, whereas rv3619c/IFA induced the secretion of IFN-γ in response to the peptide pool, and the peptides P5 and P6. However, the secretions of the nonprotective cytokines IL-5 and IL-10 were induced to none of the peptides with the delivery systems used. ***Conclusion:*** Rv3619c is a major antigen of *M. tuberculosis* with multiple immunogenic epitopes; however, immune responses to individual epitopes can vary based on delivery systems used.

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Introduction

The early-secreted antigenic target of 6 kDa (ESAT6) is an immunodominant protein of *Mycobacterium tuberculosis* [1]. It was isolated from the short-term culture filtrate of *M. tuberculosis* and was shown to be a major protein antigen stimulating T-cell responses in a mouse model of memory immunity after infection with *M. tuberculosis* [1]. In human studies, ESAT6 was immunodominant and recognized by T cells from tuberculosis patients but not from BCG-vaccinated healthy subjects [2]. Other studies have shown that immunization with ESAT6 protected animals against challenge with *M. tuberculosis* [3].

The gene for ESAT6 (*esxA*) is located in the *M. tuberculosis* genomic region known as the region of difference 1 (RD1), which is present in the pathogenic *M. bovis* but absent in all strains of BCG currently used as vaccines in different parts of the world, hence the antigens encoded by RD1 are considered *M. tuberculosis*-specific [4]. Another *M. tuberculosis*-specific protein encoded by RD1 is the culture filtrate protein of 10 kDa (CFP10, *esxB*). As *esxA* and *esxB* are specific for *M. tuberculosis*/*M. bovis* and immunodominant [4], they have been recommended for the specific diagnosis of infections caused by *M. tuberculosis* in both interferon-gamma (IFN-γ) release assays [5], and tuberculin-type skin tests [6]. Furthermore, immunization with a vaccine preparation containing ESXA and ESXB has been shown to protect against challenge with *M. tuberculosis* in both humans and animal models of TB [7]. However, ESXA and ESXB cannot be used for both vaccination and diagnosis because their application in diagnosis among people vaccinated with preparations containing ESXA and ESXB does not differentiate between the effects of vaccination versus infection with *M. tuberculosis*. As the diagnostic use of ESXA and ESXB is well established at the global level [5], other appropriate antigens should be exploited for vaccine applications.

The analysis of the *M. tuberculosis* genome has shown the existence of 21 additional ESAT6-like proteins [8]. Four of them are located in two *M. tuberculosis*-specific genomic regions, i.e., RD7 encoding Rv2346c (ESXO) and Rv2347c (ESXP), and RD9 encoding Rv3619c (ESXV) and Rv3620c (ESXW) [8]. These antigens have demonstrated the induction of protective immune responses in vaccinated mice [9]. However, the strongest Th1 responses were induced by Rv3619c [9], and immunization of mice with Rv3619c protected them against infection with *M. tuberculosis* [10] and the ovalbumin-induced airway pathology in an allergic murine model of asthma [11].

This study was aimed at identifying the effects of four delivery systems, i.e., two chemical adjuvants (incomplete Freund's adjuvant [IFA] and aluminum hydroxide [Alum]), a mycobacterial vector (*M. smegmatis*), and a DNA plasmid (pUMVC6) on the induction of Th1-type immune responses to Rv3619c. Furthermore, peptides containing immunodominant epitopes of Rv3619c were identified by using an overlapping synthetic peptide approach.

Methods

Bacterial Strains, Plasmids, and Cloning of Rv3619c Gene in Vectors

Genomic DNA isolated from *M. tuberculosis* H37Rv (obtained from the American Type Culture Collection, Rockville, MD, USA) served as the source for the amplification and subsequent cloning of the *rv3619c* gene, as previously described [9]. In brief, DNA corresponding to the *rv3619c* gene was amplified by PCR using genomic DNA isolated from *M. tuberculosis* and gene-specific primers (ThermoFisher Scientific, Ulm, Germany) (online suppl. Table S1; see www.karger.com/doi/10.1159/000525136 for all online suppl. material) and then ligated to appropriated cloning and expression vectors i.e., expression vector pGES-TH1 [9], shuttle vector pDE22 [12], and DNA plasmid vector pUMVC6 [12] (Aldevron, Fargo, ND, USA), as described previously [9, 11, 12] for expression in *E. coli* BL-21 (Novagen, Madison, WI, USA), *M. smegmatis* (ATCC 700084/mc(2)155, ATCC, Manassas, VA, USA), and DNA plasmid vector pUMVC6, respectively.

Recombinant Proteins and Mycobacteria

The expression vector pGES-TH1 was used for high-level expression of the Rv3619c fusion protein in *E. coli* [9, 11]; the expression of the recombinant protein was determined by Western blotting using anti-GST antibodies (diluted 1:1,000 in TBST) (Abcam, Cambridge, UK) [9]. The recombinant protein was purified to homogeneity using affinity chromatography and analyzed by SDS-PAGE [9, 11]. The recombinant plasmid pDE22/*rv3619c* was electroporated into *M. smegmatis* and the expression of the *rv3619c* gene in recombinant (r)*M. smegmatis* was determined by reverse-transcriptase-PCR, as described previously [9, 11]. The amplified products were analyzed by Agarose gel electrophoresis.

Synthetic Peptides

Peptides (25-mers overlapping with neighboring peptides by 10 residues) covering the sequences of Rv3619c protein were synthesized by solid-phase peptide synthesis using fluorenylmethoxy-carbonyl chemistry (online suppl. Table S2) [9, 12]. The peptides were dissolved in sterile phosphate-buffered saline (PBS) (pH 7.0) and frozen at −20°C in aliquots, as described previously [9, 12].

Immunization of Mice

Six- to 8-week-old female pathogen-free BALB/c mice were used in this study. Mice were divided into 6 groups (online suppl. Fig. S1). Mice in groups 1 and 2 (5 mice in each group, *n* = 5) were immunized intraperitoneally with 2 μg of Rv3619c purified recombinant protein emulsified (1:1 ratio) with either IFA (Sigma-Al-
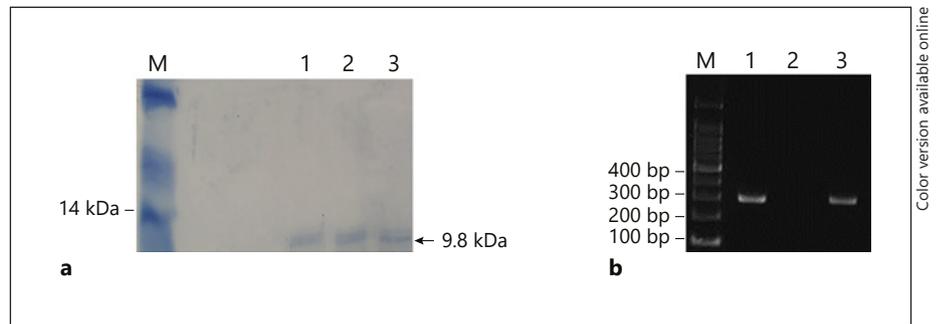
Fig. 1. a SDS-analysis of purified Rv3619c protein (9.8 kDa). Lane M: Prestained low molecular weight marker. Lanes 1–3: GST-free Rv3619c proteins. **b** Agarose gel electrophoresis of PCR-amplified products using genomic DNA isolated from r*M. smegmatis* transformed with pDE22/*rv3619c*, DNase-treated genomic DNA, and cDNA (reverse-transcriptase-PCR). Lane M: 100 bp marker. Lane 1: Genomic DNA amplified with Rv3619c primers (285 bp product). Lane 2: Genomic DNA treated with DNase and amplified with *rv3619c* primers (no product) Lane 3: cDNA amplified with *rv3619c* primers (285 bp product).

drich, St. Louis, MO, USA) or Alum (ThermoFisher Scientific Inc., Waltham, MA, USA), respectively, and boosted twice with the protein at 2-week intervals in 200 μL suspension, as described previously [9, 11]. Mice in group 3 ($n = 5$) were immunized and boosted three times (at 2-week intervals) intraperitoneally with 5×10^7 colony forming units (CFU) of r*M. smegmatis* expressing the *rv3619c* gene in 100 μL suspension, as described previously [9]. Mice in group 4 ($n = 5$) were immunized and boosted twice (at 3-week intervals) intramuscularly with 100 μg rDNA plasmid in 100 μL suspension, as described previously [10]. Mice in group 5, the negative control group ($n = 5$), were injected three times (at 2-week intervals) intraperitoneally with PBS (placebo). Mice in group 6 were immunized with and boosted three times (at 2-week intervals) intraperitoneally with 5×10^7 CFU of wild-type *M. smegmatis*. Two weeks (in case of the chemical adjuvants [IFA and Alum], recombinant mycobacteria, and negative control groups) and 3 weeks (in case of the rDNA plasmid group) after the last booster, mice were euthanized, and spleen cells were collected aseptically according to standard procedures [9].

Spleen Cell Cultures for Cytokine Assays

Mitogen- and peptide-induced secretions of Th1 cytokine (IFN-γ), Th2 cytokine (IL-5), and Treg cytokine (IL-10), from mouse spleen cells, were determined as described previously [9, 11]. In brief, 5×10^6 cells/mL spleen cells were stimulated in triplicates with the mitogen Concanavalin A (Con A [0.25 μg/well], experimental positive control) (Sigma Aldrich), the peptide pool of Rv3619c, and its individual peptides (P1 to P6) at the optimal concentration, 25 μg/mL in a round-bottom 96-well plate (ThermoFisher Scientific Inc.) [9, 11]. The negative control wells did not receive any of these stimulants to test for antigen-nonspecific secretion of cytokines. Spleen cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture supernatants were collected for cytokine assays after appropriate time intervals, as described previously [9, 11]. The culture supernatants of spleen cells from each group of mice ($n = 5$) stimulated with the peptide pool or the individual peptides (P1 to P6) were pooled together and tested in duplicates for concentrations of Th1 cytokine IFN-γ, Th2 cytokine IL-5, and Treg cytokine IL-10 using ELISA kits (ThermoFisher Scientific Inc.), as specified by the manufac-

turer. The average cytokine concentrations from the duplicates were calculated. The minimum detectable concentrations of IFN-γ, IL-5, and IL-10 using the kits were 95.25 pg/mL, 69.92 pg/mL, and 66.20 pg/mL, respectively.

*Statistical Analysis*

The numerical values were expressed as means. The concentrations of IFN-γ, IL-5, and IL-10 in response to a given stimulant were considered significant with quantities >100 pg/mL and the ratio of experimental (stimulated)/control (nonstimulated) >2 [9, 11, 12]. The ratios of IFN-γ:IL-5 and IFN-γ:IL-10 exceeding 2 were considered as Th1-biased, 1 to 2 no bias, and less than 1 as Th2 and Treg-biased, respectively [9, 11, 12].

Results

Cloning and Sub-Cloning of Genes and the Expression and Purification of Rv3619c Protein

The DNA fragment corresponding to the *rv3619c* gene was PCR-amplified from the genomic DNA of *M. tuberculosis* using gene-specific primers and cloned into appropriate expression vectors. Western-blot analysis, using anti-GST antibodies, cell lysates from *E. coli* cells transformed with pGES-TH1/*rv3619c* showed the expression of fusion proteins corresponding to the expected size (data not shown). The induced recombinant *E. coli* cells containing pGES-TH1/*rv3619c* were sonicated and the proteins were solubilized by 1 mM dithiotheritol and then loaded onto and released from the Glutathione-Sepharose affinity matrix, and the eluted proteins were loaded onto and released from Ni-NTA agarose columns and analyzed by SDS-PAGE. The results showed that the purified protein corresponded to the expected size of GST-free Rv3619c, i.e., 9.8 kDa (Fig. 1a).
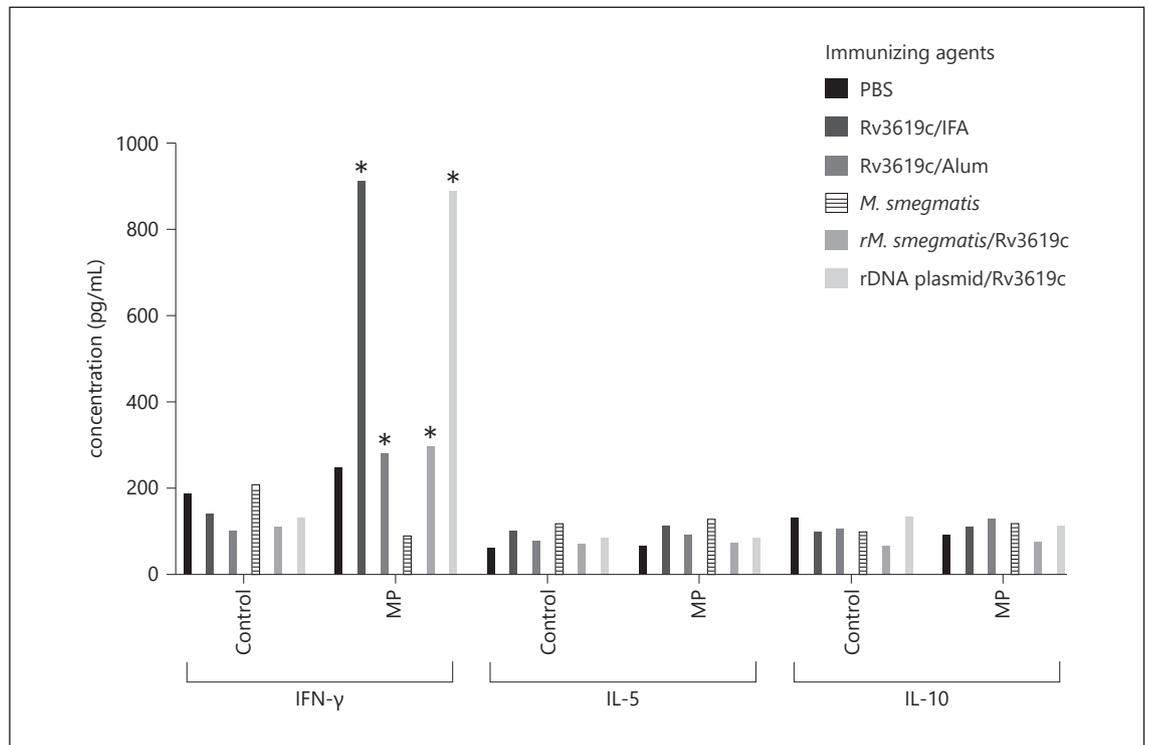
Fig. 2. Cytokine concentrations (pg/mL) in supernatants of cultured spleen cells from mice administered with PBS, Rv3619c/IFA, Rv3619c/Alum, wild-type *M. smegmatis*, r*M. smegmatis*/*rv3619c*, and rDNA plasmid/*rv3619c* and stimulated with the peptide pool of Rv3619c. Spleen cells obtained from immunized mice were cultured in triplicates in the absence of any stimulant (control) and in the presence of stimulants (experimental), i.e., pool of Rv3619c peptides (P1 to P6 [PP]) covering the sequences of Rv3619c protein. The supernatants were collected on day 6. The culture supernatants were tested for secreted IFN-γ, IL-5, and IL-10 in duplicate wells of 96-well plates by ELISA. The concentration of cytokines in response to pool of Rv3619c peptides were considered significant (*) with quantities >100 pg/mL and the ratio of experimental/control >2 [9, 11]. E/C, experimental/control.

Expression of Rv3619c Gene in rM. Smegmatis

PCR with genomic DNA of *M. smegmatis* electroporated with the rplasmid pDE22/*rv3619c* showed the presence of the cloned gene (Fig. 1b, lane 1), and its treatment with DNase destroyed the cloned gene (Fig. 1b, lane 2). The reverse-transcriptase-PCR experiments with RNA isolated from *M. smegmatis* electroporated with the rplasmid pDE22/*rv3619c* showed the expression of the *rv3619c* gene in r*M. smegmatis* at the mRNA level (Fig. 1b, lane 3).

Secretion of Th1 (IFN-γ), Th2 (IL-5), and Treg (IL-10) Cytokines by Spleen Cells of Mice Immunized with Rv3619c using Different Delivery Systems in Response to Various Stimuli

Spleen cells from all groups of mice secreted Th1 (IFN-γ), Th2 (IL-5), and Treg (IL-10) cytokines in response to the nonspecific stimulant Con A (online suppl. Table S3), showing that the experimental conditions

were appropriate. Mice immunized with Rv3619c secreted Th1 cytokine IFN-γ in response to peptide pool of Rv3619c using all four delivery systems but did not secrete significant concentrations of Th2 (IL-5) and Treg (IL-10) cytokines (Fig. 2). Furthermore, mice immunized with the placebo (PBS) and *M. smegmatis* did not secrete significant concentrations of any of the cytokines in response to the peptide pool of Rv3619c (Fig. 2). Further experiments to identify the individual peptides stimulating IFN-γ production using pools of supernatants showed that spleen cells of mice immunized with Rv3619c recombinant protein emulsified with IFA induced the secretion of IFN-γ in response to the peptides P5 and P6 (Fig. 3a). However, spleen cells of mice immunized with Rv3619c recombinant protein emulsified with alum did not induce the secretion of IFN-γ in response to any individual peptide (Fig. 3b). Interestingly, spleen cells of mice immunized with r*M. smegmatis* and the rDNA plasmid construct induced a significant increase in the
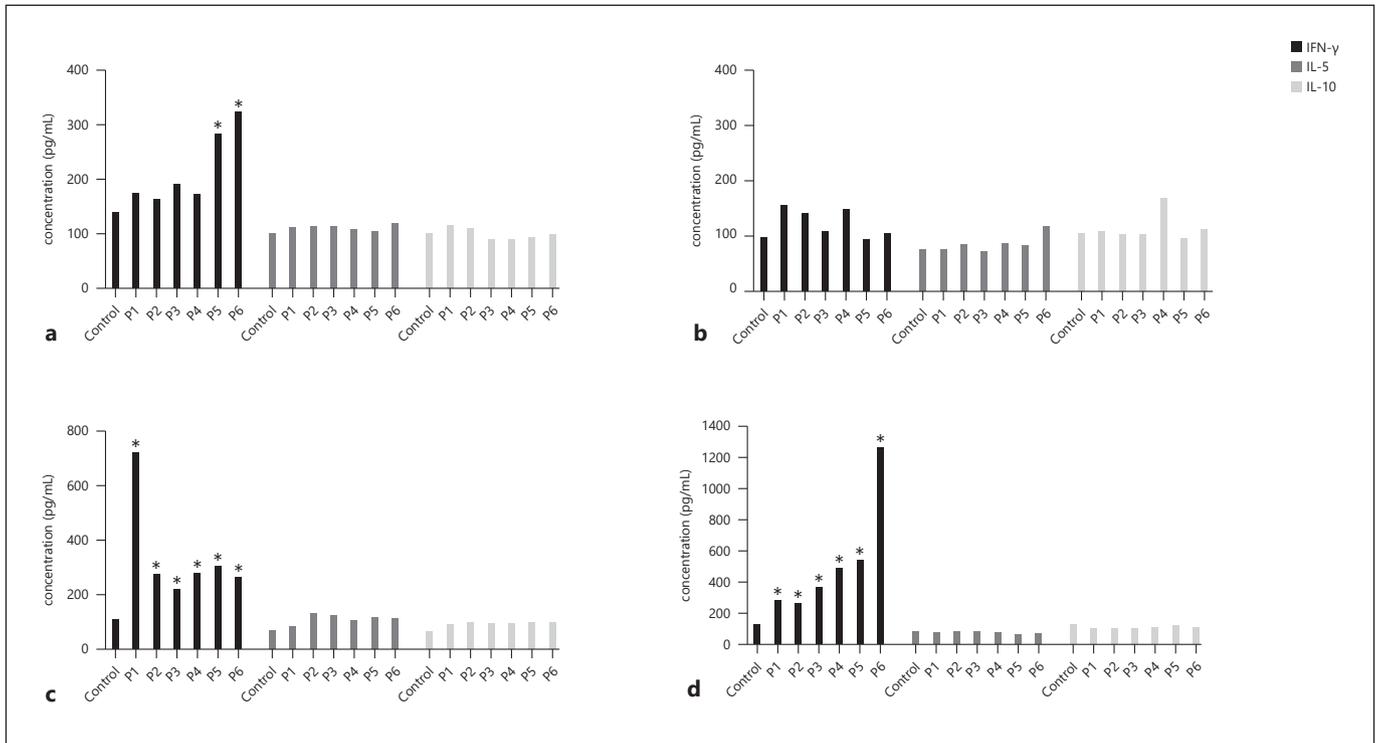
Fig. 3. Cytokine concentrations (pg/mL) in supernatant of cultured spleen cells from mice immunized with Rv3619c/IFA (**a**), Rv3619c/Alum (**b**), r*M. smegmatis*/*rv3619c* (**c**), and rDNA plasmid/*rv3619c* (**d**) and stimulated with the individual peptides (P1 to P6) of Rv3619c. Spleen cells obtained from immunized mice were cultured in triplicates in the absence of any stimulant (control) and in the presence of stimulants (experimental), i.e., individual peptides of Rv3619c (P1 to P6) covering the sequences of Rv3619c protein. The supernatants were collected on day 6. The culture supernatants were tested for secreted IFN-γ, IL-5, and IL-10 in duplicate wells of 96-well plates by ELISA. The concentration of cytokines in response to pool of Rv3619c peptides were considered significant (*) with quantities >100 pg/mL and the ratio of experimental/control >2 [9, 11]. E/C, experimental/control.

Table 1. Cytokine ratios in supernatants of spleen cells obtained from mice immunized with Rv3619c/IFA, Rv3619c/Alum, r*M. smegmatis*/*rv3619c*, and rDNA/*rv3619c*, and stimulated with the individual peptides of Rv3619c

Stimulant	INF-γ:IL-10				INF-γ:IL-5			
	Rv3619c/ IFA	Rv3619c/ Alum	r*M. smegmatis*/ *rv3619c*	rDNA/ *rv3619c*	Rv3619cIFA	Rv3619c/ Alum	r*M. smegmatis*/ *rv3619c*	rDNA/ *rv3619c*
P1	1.51	1.44	7.82	2.78	1.56	2.05	8.47	3.65
P2	1.48	1.38	2.74	2.63	1.45	1.66	2.11	3.24
P3	2.14	1.07	2.32	3.64	1.70	1.50	1.76	4.28
P4	1.93	0.88	2.89	4.51	1.58	1.70	2.60	6.01
P5	3.02	0.99	3.08	4.31	2.73	1.14	2.57	8.10
P6	3.25	0.94	2.67	11.73	2.71	0.89	2.31	17.56

IFN-γ:IL-5 and IFN-γ:IL-10 ratios in the supernatants of spleen cells cultures in response to individual peptides of Rv3619c (P1 to P6) from immunized mice with Rv3619c/IFA, Rv3619c/Alum, r*M. smegmatis*/*rv3619c*, and rDNA/*rv3619c*. The ratios of IFN-γ:IL-5 and IFN-γ:IL-10 exceeding 2 were considered as Th1-bias, 1 to 2 no bias, and less than 1 as Th2 and Treg-biases, respectively.

levels of IFN-γ in response to all individual peptides when compared to either nonstimulated cells or the negative control (Fig. 3c, d). However, mice from all of the groups did not secrete significant concentrations of IL-5 and IL-10 in response to the individual peptides of Rv3619c (Fig. 3a–d.

The relative concentrations of Th1, Th2, and Treg cytokines secreted from the spleen cells of immunized mice in response to the individual peptides of Rv3619c, as calculated by the ratios of Th1:Th2 (IFN-γ:IL-5) and Th1:Treg (IFN-γ:IL-10), are shown in Table 1. The analysis of these results for cytokine biases showed that Th1-biased responses, compared to Th2, Treg, or no biases, were observed with all individual peptides (P1 to P6) of Rv3619c in mice immunized with r*M. smegmatis* and rDNA plasmid (Table 1). In the case of mice immunized with Rv3619c/IFA, the peptides P3, P5, and P6 showed the induction of Th1-biased responses, whereas the peptides P1, P2, and P4 either showed no biases or Th2 and Treg-biases (Table 1). Whereas in mice immunized with Rv3619c/Alum, Th1-bias was observed only with the peptide P1 in relation to IFN-γ:IL-5 ratio, and all other peptides showed either no biases or Th2 and Treg biases (Table 1).

Discussion

In this study, the effects of four delivery systems (two chemical adjuvants [IFA and Alum], *M. smegmatis*, and a DNA plasmid) were studied in mice for the induction of protective Th1 (IFN-γ) and nonprotective Th2 (IL-5) and Treg (IL-10) cytokine responses to *M. tuberculosis*-specific protein antigen Rv3619c and its individual peptides. The results show that with each adjuvant and delivery system, the secretion of protective Th1 cytokine IFN-γ was significant in response to the peptide pool of Rv3619c, and no significant secretion of Th2 (IL-5) and Treg (IL-10) cytokines. However, IFN-γ production in response to individual peptides of Rv3619c varied with the delivery systems used, and only r*M. smegmatis* and rDNA plasmid consistently induced IFN-γ responses to all the individual peptides (P1 to P6) with Th1-biased responses. To our knowledge, this is the first report to study protective and nonprotective cytokine responses to individual peptides of Rv3619c using different delivery systems for immunization of mice with Rv3619c.

Chemical adjuvants are among the first generation of delivery systems that have been identified and used for about 5 decades [13]. IFA is a water-in-oil emulsion that allows small droplets of the recombinant protein to be stabilized and distributed throughout the oil phase, thus prolonging the duration of antigen persistence at the site of injection, which results in the recruitment of antigen-presenting cells at the site of injection and the induction of local inflammation [14]. Alum salts are known to have better safety profiles and increase the stability and immunogenicity of recombinant proteins. They have also been extensively used in studies aimed at developing subunit TB vaccines and as a standard for comparison of efficacies of newly developed adjuvants [15–17]. Alum is currently registered for use in humans; however, there have been some controversial outcomes in preclinical studies involving immunization with immunodominant mycobacterial proteins emulsified with Alum. While Orr and colleagues showed significantly reduced bacterial load in the lungs and spleens of *M. tuberculosis*-infected mice vaccinated with Alum in combination with glucopyranosyl lipid adjuvant expressing ID93 [16], Agger et al. [17] showed that combining Ag85B-ESXA fusion protein with Alum and dimethyldioctadecylammonium as adjuvants did not reduce the bacterial load in the lungs of infected mice. Furthermore, immunization of mice with the chimeric tuberculosis vaccine antigen H56 along with alum induced the secretion of IL-5 and primary B cell responses rather than primary T cell responses and IFN-γ [18]. Our study shows that immunization with Rv3619c along with Alum did not induce protective Th1-biased responses to any of the individual peptides of Rv3619c; instead, several peptides induced nonprotective Th2 and Treg-biased responses.

Even though IFA and alum are used to enhance humoral responses [19], it has been shown that IFA induces the secretion of Th1 rather than Th2 cytokines when emulsified with an immunodominant antigen [9]. In this study, spleen cells from mice immunized intraperitoneally with recombinant proteins in chemical adjuvants showed increased secretion of protective IFN-γ with a Th1 bias, when compared to Th2 and Treg responses. Similarly, Lu et al. have shown that immunization of mice subcutaneously with ESAT-6 emulsified with IFA, induced a strong cellular immunity characterized by the induction of Th1 cells and the secretion of IFN-γ and IL-2 [20]. In another study, immunization with IFA-emulsified ESX-fusion proteins induced the secretion of Th1 cytokines and protected the animals challenged with *Mycobacterium bovis* BCG-Pasteur [21]. In our study, although the concentration of IFN-γ secreted in response to the peptide pool of Rv3619c was the highest with IFA, the response to individual peptides

was limited to only two terminal peptides P5 and P6, and these were the only peptides that consistently induced Th1-biased responses.

The rapidly growing recombinant nonpathogenic mycobacteria expressing immunodominant *M. tuberculosis* antigens have been broadly used as TB vaccines in preclinical studies due to their closeness to *M. tuberculosis* and the ability to activate and induce the maturation of dendritic cells and memory T cells, which can lead to long-lasting immunity [22]. In addition, the safety of *M. smegmatis* has been well documented, and its efficacy has been studied as vaccines and immunotherapeutic agents for the prevention of TB and anti-cancer therapies in animal models [23, 24]. Furthermore, *M. smegmatis* is nontoxic in animal models lacking NK or T cells [25]. In our study, we evaluated *M. smegmatis* carrying a recombinant shuttle vector (rpDE22) containing *rv3619c* as a live vaccine candidate. We observed that spleen cells obtained from mice immunized with r*M. smegmatis*/rpDE22 induced the secretion of IFN-γ in response to the pool of synthetic peptides and all individual peptides of Rv3619c. Furthermore, Th1-biased responses were observed with all peptides of Rv3619c. These results are in line with other studies which demonstrated the ability of recombinant *M. smegmatis* to express *M. tuberculosis* antigens and peptides, leading to the secretion of Th1 cytokines and enhanced protection against tuberculosis compared to immunization with *M. smegmatis* alone [22, 26]. Moreover, Th1-inducing recombinant *M. smegmatis* has a superior immunotherapeutic effect against persistent tuberculosis infection in an animal model when combined with the anti-tuberculosis drugs rifampicin and isoniazid [27].

Recombinant DNA plasmids harboring the genes of *M. tuberculosis* antigens are strong stimulators of Th1 cell responses [28, 29]. Their ability to secrete proteins expressed from cloned genes as cytoplasmic proteins initiates antigen trafficking from the site of injection to the lymphoid organs where dendritic cells play a major role in inducing cell-mediated responses [28, 29]. Immunization of mice with recombinant DNA plasmids has been shown to induce Th1-biased responses with significantly higher Th1/Th2 ratios when compared to BCG [30]. Similarly, we have found that immunization of mice with the recombinant DNA plasmid, pUMVC6/*rv3619c*, induced the secretion of Th1 cytokines, but not Th2 and Treg cytokines, in response to the peptide pool and all the six individual peptides of Rv3619c. Furthermore, Th1-biased responses were observed with all individual peptides of Rv3619c.

Our results suggest that immunization with Rv3619c using appropriate delivery systems could present the possibility of developing new tuberculosis vaccines. This is supported by a study which showed that immunization with Rv3619c using an archaeosome-based antigen delivery system induced the secretion of protective Th1 cytokines and provided improved and long-term protection against *M. tuberculosis*-challenge in BALB/c mice as compared to BCG [10]. However, to confirm the protective efficacy of Rv3619c with the current adjuvants and delivery systems used, protection experiments should be performed in animal models of tuberculosis.

Conclusion

Stimulation in vitro with a pool of peptides from an immunodominant *M. tuberculosis*-specific antigen Rv3619c induced the secretion of the protective Th1 cytokine IFN-γ from the spleen cells of mice immunized with different delivery systems, in the absence of the nonprotective Th2 and Treg cytokines. However, the breadth of immune responses, as shown by the response to individual peptides, was best with the delivery systems *M. smegmatis* and DNA plasmid, as all peptides of Rv3619c showed a Th1 response in the absence of significant concentrations of Th2 and Treg cytokines. Furthermore, Th1-biased responses were observed with all peptides using both the delivery systems, suggesting that their potential may further be exploited in experiments related to protection against the *M. tuberculosis* challenge.

Statement of Ethics

All experiments in mice were performed in accordance with the principles of NC3Rs' ARRIVE guidelines for reporting humane animal research, the BJP Guidelines, and in accordance with the EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The experimental protocols were approved by the "Health Science Center Animal Welfare Committee" and the project funding body (approval number YM06/15) and complied with regulations for the animal care and ethical use of laboratory animals in the Health Sciences Center, Kuwait University.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

Author Contributions

All the authors conceptualized the study, designed the experiments, and contributed to funding acquisition. Hussain A. Safar conducted the experiments. All the authors contributed to data analysis, manuscript writing, and approved the final manuscript.

Data Availability Statement

Data supporting the findings of this study are available on upon reasonable request.

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