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A novel multivalent tuberculosis vaccine confers protection in a mouse model of tuberculosis

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Abstract

Mycobacterium tuberculosis infects one third of the world's population. Due to variable efficacy of the Bacille Calmette Guerin (BCG) vaccine, development of novel TB vaccines remains a priority. Here, we demonstrate the protective efficacy of a novel multivalent DNA vaccine, which contains 15 synthetic antigens targeting the *Mtb* ESX secretion system.

Key words

Tuberculosis, vaccine, DNA vaccine

Tuberculosis (TB) is the leading cause of death from a single infectious agent in the world today[1]. The only currently-licensed vaccine against TB, *M.bovis* Bacille Calmette Guerin (BCG), protects against TB meningitis in infants and children, but has variable efficacy in protecting against adult pulmonary TB.[2] Development of a novel TB vaccine is therefore of paramount importance for the status of global health. Although a clear correlate of vaccine-induced protection has yet to be identified for TB, both interferon (IFN)-γ and interleukin-17A (IL-17) production by $CD4^+$ T cells are targeted in vaccine-induced immunity to *Mtb* infection.[3, 4] In addition, since mice lacking major histocompatibility complex (MHC)-I processing machinery or $CD8^+$ T cells are more susceptible to TB disease,[5, 6] $CD8^+$ T cells have been implicated as having a role in control of *Mtb* infection. The novel multivalent DNA TB vaccine, RSQ-15, is designed using the pVax1 vector. The vector was used to express 15 synthetic consensus antigens of the *Mtb* ESX gene family (esxO, esxR, esxF, esxB, esxC, esxU, esxH, esxA, esxT, esxD, esxQ, esxE, esxV, esxS, and esxW). The antigens were selected based on diversity and cross-reactivity,[7] with the unique goal of inducing an immune response across a broad range of *Mtb* antigens, namely all 23 members of the ESX secretion system. The ESX secretion system is a family of *Mtb* proteins associated with mycobacterial virulence[8] and contains several epitopes able to induce T cell responses in both humans and in animal models.[9, 10] RSQ-15 is delivered intramuscularly (i.m.) followed by electroporation at the site of immunization in order to improve the immunogenicity of the DNA vector.[7, 11] This vector/electroporation combination is approved for use in humans and is in clinical trials for treatment of Human Papilloma Virus-induced cervical disease, with good safety data thus far.[12] Previous results using RSQ-15 in mice have shown robust induction of multifunctional peripheral *Mtb*-specific $CD4^+$ and $CD8^+$ T cells.[7] On the basis of these preliminary immunogenicity results, here we investigate the protective efficacy of RSQ-15 immunization in the mouse model of TB. Our new results show that this novel multivalent TB vaccine confers protection to levels similar to BCG immunization. Thus, our study highlights novel strategies that can be targeted to design new vaccines against TB.

RSQ-15 immunization induces IFN-γ and tumor necrosis factor-α production by $CD4^+$ and $CD8^+$ T cells in the periphery.[7] Thus, we aimed to investigate whether RSQ-15 could also induce mucosal cytokine responses in the lungs. Eight week old C57BL/6 mice were immunized with two doses of 5μg RSQ-15, prepared as described in,[7] delivered intramuscularly (i.m.) followed by electroporation at the site of immunization.[7, 11] One week after the final immunization, lungs were harvested and processed to a single cell suspension.[13] IFN-γ and IL-17 production was measured by antigen-driven ELISpot,[13] using 2.5μg/mL of a pool of all peptides represented in the RSQ-15 vaccine.[7] We observed robust *Mtb*-specific IFN-γ (Figure 1A) but not IL-17 production (data not shown) by lung cells, indicating that RSQ-15 is a potent inducer of mucosal IFN-γ responses in the lungs, but that the antigens or DNA vaccine formulations are not permissive for induction of mucosal Th17 responses.

Molecular adjuvants are small molecules, such as cytokines, that can be co-administered with vaccines and can act as adjuvants. DNA constructs expressing the molecular adjuvant IL-33 have previously been shown to enhance cytokine production by T cells,[11, 14] including responses to the *Mtb* antigen 85B (Ag85B).[11] Given the absence of IL-17 induction following RSQ-15 immunization alone, we also wanted to address whether inclusion of a construct expressing IL-23, a critical mediator of vaccine-induced IL-17 responses in *Mtb* infection models,[4, 15] in the RSQ-15 vaccine could induce IL-17 responses. Finally, to further expand the antigen repertoire of RSQ-15, we also included a plasmid expressing Ag85B (pAg85B), a mycolyl transferase expressed in a system unrelated to the ESX secretion system.[16] Mice were immunized as described previously, with the inclusion of constructs expressing mtrIL-33, IL-23 or Ag85B. Adjuvanting RSQ-15 with mtrIL-33 or IL-23 alone did not have a significant effect on mucosal antigen-specific IFN-γ production (Figure 1B). Furthermore, including IL-23 in the RSQ-15 immunization regimen did not induce any detectable antigen-specific IL-17 responses (data not shown). Including pAg85B along with

mtrIL-33 in the RSQ-15 vaccine, however, significantly improved mucosal antigen-specific IFN-γ production (Figure 1B).

Given the induction of high levels of *Mtb*-specific mucosal IFN-γ production in RSQ-15-immunized mice (Figure 1A,B), we next assessed the protective efficacy of the vaccine following *Mtb* H37Rv aerosol challenge in B6 mice. Mice were immunized with RSQ-15 as described previously. Control B6 mice received $1x10^6$ cfu BCG delivered subcutaneously.[13] Four weeks after the final immunization, mice were infected with 100 cfu aerosolised *Mtb* H37Rv using a Glas-Col aerosol exposure system.[13] Infectious dose was determined at 24 hours post-infection by plating lung homogenates on 7H11 agar plates. Lungs of infected mice were harvested 30 days post-infection and homogenized and plated in serial dilutions on 7H11 agar plates to quantify bacterial burden. Subcutaneous immunization with BCG conferred ~1 log protection over unimmunized control mice (Figure 1C). The RSQ-15 vaccine alone also conferred significant protection, with levels of protection not significantly different to that conferred by BCG. Despite improved induction of lung-resident antigen-specific IFN-γ responses (Figure 1B), however, immunization with RSQ-15.pAg85B.mtrIL-33 had no improved effect on vaccine efficacy compared that conferred by RSQ-15 alone (Figure 1C). The RSQ-15 vaccine has previously been demonstrated to boost BCG-induced immunogenicity.[7] Due to this, as well as the ability of pAg85B to enhance cytokine production in the lungs (Figure 1B), we aimed to determine whether boosting BCG with RSQ-15.pAg85B.mtrIL-33 would enhance the protective efficacy of BCG. We found, however, that mice immunized with BCG + RSQ-15.pAg85B.mtrIL-33 did not improve protection when compared to BCG alone (Figure 1C).

In this study, we sought to investigate the protective efficacy of RSQ-15, a novel synthetic vaccine expressing 15 consensus antigens of the ESX secretion system, with the goal of inducing T cell responses to all 23 *Mtb* ESX members. The antigens are expressed in a plasmid vector, the immunogenicity of which is enhanced by administering electroporation to the site of immunization. With the exception of BCG-based vaccines, such a broad

antigen repertoire in a TB vaccine is unprecedented. A striking feature of RSQ-15-mediated immunogenicity is the induction of potent antigen-specific IFN-γ-producing cells in the lungs of immunized mice. Unless a vaccine is delivered mucosally, pre-clinical vaccine studies rarely assess the immunogenicity and induction of cytokine-producing cells in the lungs. In a study using adenovirus 5 expressing Ag85A (Ad5Ag85A) as a boost to intradermally (i.d.)-delivered BCG, i.d.-delivered AdAg85A induced cytokine-producing cells in the lungs, although not to levels induced by intranasal (i.n.) administration.[17] In a similar study comparing BCG administered s.c. followed by either i.m. or i.n.-administered AdAg85A, IFN-γ production in the bronchoalveolar lavage fluid was significantly lower in mice receiving a parenteral boost compared to those receiving a mucosal boost.[18] Thus, the ability of parenterally-delivered RSQ-15 to induce potent IFN-γ production in the lungs is, to our knowledge, unique.

The pVax1 expression system along with electroporation has primarily been used for immunization against viruses, and as such has been shown to be a potent inducer of $CD8^+$ T cells.[12, 14, 19] RSQ-15 is also a potent inducer of $CD8^+$ T cells, although $CD4^+$ T cells are also induced, albeit to a lesser extent.[7] Although we did not assess which T cell subset was responsible for cytokine production in the lungs, it is likely, based on T cell subsets induced in the periphery, that the major cytokine-producing subset is $CD8^+$ T cells. RSQ-15 is therefore unique in that it is the first multi-antigen TB vaccine to induce predominantly $CD8^+$ T cells. Indeed, even protection conferred by the recombinant BCG Δ*ureChly*$^+$ vaccine, initially designed to induce $CD8^+$ T cells,[20] was later thought to be mediated by $CD4^+$ T cells.[21, 22] The molecular adjuvant IL-33 has previously been shown to boost cytokine responses by both $CD8^+$ and $CD4^+$ T cells in a pVax1-based immunization regimen using *Mtb*, LCMV and HIV antigens.[11, 14] Although administering RSQ-15 with mtrIL-33 alone had no significant effect on vaccine immunogenicity, co-administration of RSQ-15 + mtrIL-33 along with a plasmid expressing Ag85B greatly enhanced cytokine production in the lungs. Similar results were observed following immunization with plasmids expressing Ag85B alone

adjuvanted with mtrIL-33,[11] suggesting that some feature of the Ag85B protein makes it a good candidate for being adjuvanted by mtrIL-33, perhaps due to the signalling mechanism of the protein through toll-like receptors or other pattern recognition receptors.[23, 24] ESAT-6, a member of the ESX secretion system, and one of the proteins expressed in RSQ-15, has been shown to inhibit cytokine production by T cells;[25] it is therefore possible that the presence of ESAT-6 or other ESX proteins interferes with mtrIL-33 signalling through its receptor, ST2, and that the presence of Ag85B is required to overcome this.

Although RSQ-15 induces potent IFN-γ production, we detected no IL-17 induction. Given the importance of IL-17 in vaccine-induced protection against TB disease,[4, 15] we aimed to induce the cytokine using a plasmid expressing IL-23, a cytokine essential to the proliferation of Th17 cells. Previous studies using DNA immunization and electroporation for the treatment of tumors used a plasmid expressing IL-23 to enhance Th17 responses.[26] Here, however, our attempt to induce IL-17 following RSQ-15 immunization using a plasmid expressing IL-23 failed. The reason for this is unclear, and could be due to a number of factors. It is possible that the antigens used are not conducive for the induction of Th17 cells, however this is unlikely, as we have previously shown that both ESAT-6 and Ag85B are able to induce IL-17 production.[27] Alternatively, it could be that the dose and length of electroporation either does not target Th17-inducing antigen-presenting cells, or induces non-responsiveness of antigen-presenting cell subsets responsible for Th17 induction. Finally, it is possible that RSQ-15 does not induce cytokines such as IL-1β or IL-6, which are important for the induction of Th17 cells,[28] and that IL-23 therefore needs to be administered along with other Th17-polarizing cytokines required for induction of Th17 cells for successful induction of IL-17-producing cells.

Electroporation as a method for adjuvanting DNA vaccine responses is growing in use, with promising results. The principle of the approach is to a) increase the uptake of the administered plasmid by cells at the site of injection, and b) possibly to influence local inflammation and the infiltration of antigen-presenting cells to the site of immunization.[29]

Previous results using DNA vectors expressing TB antigens conferred limited protection against *Mtb* [30, 31] or BCG challenge,[32] with the level of DNA vaccine-induced protection not equalling that induced by BCG. In contrast to these studies, following aerosol challenge with *Mtb* H37Rv, RSQ-15 conferred ~1 log protection in the lungs. In spite of the addition of Ag85B and IL-33 to enhance the immunogenicity of the vaccine, however, protective efficacy was not improved. In an attempt to improve protection over BCG alone, RSQ-15.pAg85B.mtrIL-33 was administered as a boost to BCG, but again did not improve protection over levels induced by BCG alone. Given previous data showing the importance of IL-17 in control of *Mtb* infection,[4, 15] the lack of IL-17 induction by RSQ-15 could account for the fact that even with the molecular adjuvant mtrIL-33 enhancing IFN-γ responses in this study, based on the immunization scheme tested, RSQ-15 did not improve on the protection conferred by BCG. It would therefore be worth exploring the use of a mucosal boost in an attempt to induce IL-17-producing cells in the lungs of RSQ-15-immunized mice, and hence improve vaccine-induced protection.

The synthetic DNA vaccine studied here delivered with electroporation represents a promising avenue to pursue for development of a novel multivalent TB vaccine, not least due to the fact that the approach has already been shown to be safe and very immune potent in humans.[12, 19] Furthermore, given that pVax1 is a non-replicating vector, RSQ-15 could be safely administered to immunocompromised individuals. This is in contrast to BCG, which is contraindicated in infants exposed to HIV due to the risk of developing 'BCG-osis'. This, along with the novel approach for inducing immunogenicity against a broad range of immunodominant and non-immunodominant antigens, suggests RSQ-15 is a promising vaccine candidate for future TB vaccines.

Abbreviations:

AdAg85A adenovirus expressing Ag85A

Ag85B antigen 85B

BCG	*Mycobacterium bovis* Bacille Calmette Guerin
cfu	colony forming units
ESAT-6	early secretory antigenic target 6
i.d.	intradermal
i.m.	intramuscular
i.n.	intranasal
IFN-γ	interferon gamma
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-17	interleukin 17A
IL-23	interleukin 23
MHC	major histocompatibility complex
mtrIL-33	interleukin 33
Mtb	*Mycobacterium tuberculosis*
RSQ-15	pVax expressing 15 *Mtb* ESX antigens
TB	tuberculosis
Th17	T helper 17

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D.B.W. has grant funding, participates in industry collaborations, has received speaking honoraria, and fees for consulting. This service includes serving on scientific review committees and advisory boards. Remuneration includes direct payments or stock or stock options and in the interest of disclosure therefore he notes potential conflicts associated with this work with Inovio, where he is a member of the SAB, Bristol Myers Squibb, Roche, Ferring, Touchlight, oncosec, Merck, VGXI, and possibly others. Licensing of technology from his laboratory has created over 100 jobs in the private sector in the biotech/pharma industry. The other authors declare no competing financial interests.

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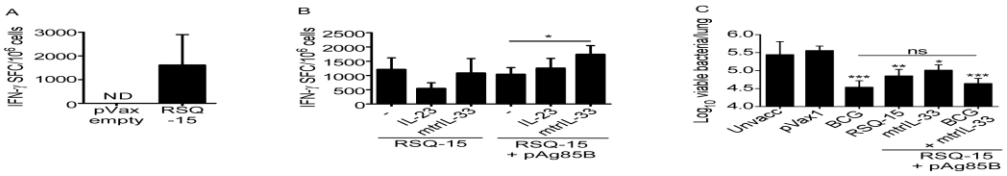

Griffiths & Villarreal Figure 1

Figure 1. RSQ-15 induces potent IFN-γ responses in the lungs and confers protection against *Mtb* infection. (A) C57BL/6 mice were immunized with two doses at a two week interval of RSQ-15 followed by immediate electroporation. One week after the final immunization, lungs were harvested and antigen-specific IFN-γ production was determined by ELISpot measured as spot forming cells (SFC) per 10^6 cells. The pVax1 empty vector was used as a control. **(B)** C57BL/6 mice were immunized with two doses of RSQ-15, RSQ-15.IL-23, RSQ-15.pAg85B, RSQ-15.mtrIL-33, RSQ-15.pAg85B.IL-23, or RSQ-15.pAg85B.mtrIL-33 followed immediately by electroporation, with a two week interval between the doses. One week after the final immunization, lungs were harvested and antigen-specific cytokine production was determined by ELISpot. **(C)** C57BL/6 mice were immunized with either BCG, RSQ-15, RSQ-15.pAg85B.mtrIL-33, or BCG followed four weeks later by two boosts two weeks apart with RSQ-15.pAg85B.mtrIL-33 as described previously. Four weeks following the final immunization, mice were challenged with low dose aerosolized *Mtb* H37Rv, and bacterial burden in the lungs was assessed at 30 days post-infection. n = 5 ± SD, data show a combination of two experiments, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, assessed by one way ANOVA followed by Tukey's post-hoc test.