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A Multi-valent Vaccinia Virus-based Tuberculosis Vaccine Molecularly Adjuvanted with Interleukin-15 Induces Robust Immune Responses in Mice

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Abstract

Tuberculosis caused by *Mycobacterium tuberculosis* is responsible for nearly two million deaths every year globally. A single licensed vaccine derived from *Mycobacterium bovis*, bacille Calmette-Guerin (BCG) administered perinatally as a prophylactic vaccine has been in use for over 80 years and confers substantial protection against childhood tuberculous meningitis and miliary tuberculosis. However, the BCG vaccine is virtually ineffective against the adult pulmonary form of tuberculosis that is pivotal in the transmission of tuberculosis that has infected almost 33% of the global population. Thus, an effective vaccine to both prevent tuberculosis and reduce its transmission is urgently needed. We have generated a multi-valent, vectored vaccine candidate utilizing the modified virus Ankara (MVA) strain of vaccinia virus to tandemly express five antigens, ESAT6, Ag85A, Ag85B, HSP65 and Mtb39A of *Mycobacterium tuberculosis* that have been reported to be protective individually in certain animal models together with an immunostimulatory cytokine interleukin 15 (MVA/IL-15/5Mtb). Although, immunological correlates of protection against tuberculosis in humans remain to be established, we demonstrate that our vaccine induced comparable CD4⁺ T cell and greater CD8⁺ T cell and antibody responses against *Mycobacterium tuberculosis* in vaccinated mice in a direct comparison with the BCG vaccine and conferred protection against an aerogenic challenge of *M. tuberculosis*, thus warranting its further preclinical development.

Keywords

Tuberculosis; vaccine; IL-15

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Introduction

The World Health Organization estimates that *M. tuberculosis* infects one person every second and kills two million people annually. It is believed that about one-third of the global population amounting to two billion people are infected with *M. tuberculosis*, although in many of these infected individuals the infection remains latent without any overt disease [1]. *M. tuberculosis* is also the major opportunistic pathogen associated with HIV disease contributing significantly to HIV-associated mortality especially in certain geographic areas in the world. The recent emergence of extensively drug resistant (XDR) strains in addition to multi-drug resistant (MDR) strains of *M. tuberculosis* that have been in circulation for some time has severely diminished the treatment options available for this deadly disease [1]. *M. tuberculosis* is a highly successful human pathogen that exploits its 4.4 Mb genome with a coding capacity for over 4000 proteins to ensure its survival and persistence in its human host [2]. Nonetheless, the ability of the immune system to mount an effective anti-tubercle bacilli immune response is evident by a number of observations. A large proportion of infected individuals remain disease free life-long attesting to the effective immune control of *M. tuberculosis* in these individuals. In addition, individuals with immune deficiencies such as AIDS or individuals with genetic mutations in the interferon gamma or IL-12 signaling pathways are highly susceptible to recurrent mycobacterial infections highlighting the importance of IL-12 and interferon gamma in controlling tuberculosis (TB) [3–5]. Moreover, individuals undergoing anti-TNF-alpha treatment for autoimmune disorders such as rheumatoid arthritis or Crohn's disease encounter frequent reactivation of latent TB infections underscoring the importance of TNF alpha in the immune control of *M. tuberculosis* [6]. Collectively, these observations support the notion that the induction of immune responses capable of preventing infections or suppressing reactivation is achievable and the development of vaccines capable of inducing such immune responses are realistic and feasible. The only licensed vaccine against TB, a derivative of *M. bovis*, bacille Calmette-Guerin (BCG) offers protection against disseminated childhood tuberculosis whereas it is virtually ineffective against the adult pulmonary disease that is the major cause of TB mortality globally. Therefore, a more efficacious vaccine especially against the pulmonary disease is urgently needed.

We have generated a multi-valent, vectored vaccine candidate utilizing the modified virus Ankara (MVA) strain of vaccinia virus to tandemly express five antigens, ESAT6, Ag85A, Ag85B, HSP65 and Mtb39A of *Mycobacterium tuberculosis* that have been reported to be protective individually in certain animal models, together with an immunestimulatory cytokine interleukin 15 (MVA/IL-15/5Mtb) and demonstrate that our vaccine induces a robust immune response in vaccinated mice that is qualitatively superior to the licensed BCG vaccine and confers protection against an aerogenic challenge of *M. tuberculosis*.

Materials and Methods

Construction of vaccinia-based vaccine candidate

M. tuberculosis genomic DNA from H37Rv strain was isolated by standard procedures [7] and the coding segments of *HSP65*, *Ag85B*, *ESAT6*, and *Mtb 39A* genes were amplified individually by polymerase chain reaction (PCR). The 5' primers contained a synthetic early-late vaccinia promoter added prior to the initiator ATG codon and the 3' primer contained a vaccinia transcription terminator sequence TTTTCT added after the gene specific translation terminator codon for each of the genes amplified. When constructing the expression cassette of *Mtb39A* gene, two additional codons (TCG CGA) that are not in the native sequence were added prior to the terminator TGA codon. In the case of the *Ag85A* gene, first we amplified the gene segment that encodes the mature polypeptide and then a synthetic DNA cassette that contained an early-late vaccinia promoter followed by a segment that encodes a 40-amino acid polypeptide corresponding to the murine immunoglobulin *kappa* light chain signal sequence

along with an epitope derived from the hemagglutinin polypeptide of influenza virus for which specific monoclonal antibodies are available commercially (METDTLLLWVLLLWVPGSTGDYPYDVPDYAGA QADLPGDG) was positioned in-frame, 5' to the mature coding segment of *Ag85A* gene. Furthermore, in addition to the *ESAT6* gene amplified from the strain H37Rv, we also synthesized a codon-optimized version of *ESAT6* gene *de novo* for expression in mammalian cells with a 5' vaccinia early-late promoter and a 3' TTTTCT element immediately after the TAA terminator codon. The coding segment of human *IL-15* gene with a 5' vaccinia early-late promoter and a 3' TTTTCT transcriptional terminator sequence has been described previously [8]. A seed stock of modified vaccinia virus Ankara (MVA) generated in the year 1974 before the bovine spongiform encephalopathy era was kindly provided by Dr. Bernard Moss from the National Institute of Allergy and Infectious Diseases. To create recombinant vaccinia viruses pTFHA transfer vector with a 1.8 Kb DNA fragment encompassing the hemagglutinin gene of vaccinia virus and *E. coli gpt* gene were used [8]. MVA recombinant viruses with five *M. tuberculosis* genes *HSP65*, *Ag85A*, *Ag85B*, *Mtb 39A*, native *ESAT6* and a codon-optimized *ESAT6* gene along with *IL-15* were created by first cloning all seven genes as a head to tail concatamer into the pTFHA transfer vector by standard cloning techniques. Recombinant viruses were then generated by standard procedures as described previously [7] by transfecting the relevant transfer plasmid into MVA infected cells and selecting plaques that were resistant to mycophenolic acid. The MVA strain of vaccinia and its recombinant derivatives were grown in a BHK-21 cell line from ATCC.

Western Blot analysis

MVA recombinants were grown in BHK-21 cells and when infected cells displayed 75% CPE, infected monolayers were harvested and cell pellets were resuspended in RIPA buffer with protease inhibitors to yield a final protein concentration of 10 mg/ml. Infected cell lysates were subjected to SDS-PAGE (10% acrylamide gels for *Ag85A*, *Ag85B*, *HSP65* and *Mtb39A* whereas for *ESAT6*, a 15% gel was used) and the separated proteins were transferred to PVDF membranes for immunoblotting. The following primary antibodies were used for the detection of mycobacterial antigens: monoclonal antibody for *ESAT6* (clone HYB 076-08); monoclonal antibody for *HSP65* (clone 4HG11); chicken polyclonal antibody for *Ag85A*; rabbit polyclonal antibody for *Ag85B*, all purchased from Abcam Inc., Cambridge, MA. For detecting *Mtb39A*, an in-house generated polyclonal rabbit antibody was used.

Detection of IL-15 activity

BHK-21 cells were infected with vaccinia virus at a multiplicity of infection of 10 and infected cells were cultured for 3 days prior to harvesting the supernatants for IL-15 activity. Harvested supernatants were irradiated (3000 rad) to eliminate infectivity and then tested for IL-15 activity using a commercial ELISA kit for human IL-15 (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Bioactivity was determined by the ability of supernatants to support the growth of IL-2/IL-15-dependent NK-92 cell line as reported previously [9]. After addition of test supernatants cells were incubated for 48 hours then pulsed with 1 microCi/ml of ³H-thymidine for 6 additional hours. Triplicate samples were counted by scintillation. Cells incubated with media alone served as a negative control and cells stimulated with recombinant IL-15 served as a positive control. The proliferative index was calculated as the fold-increase in ³H-thymidine uptake above the media control.

Mice and Immunizations

Specific pathogen free female BALB/c and C57BL/6 mice (6–10 weeks old) were used for immunization studies. All animal procedures were carried out under institutionally approved protocols. Mice were immunized subcutaneously at the base of the tail with 1×10⁷ plaque-forming units (pfu) of vaccinia virus in a volume of 100 microliters. A second booster dose

was given 4 weeks later. Each experimental group consisted of 6 animals. For comparative analysis mice were given two doses of Pasteur strain of BCG [1×10^6 colony forming units (CFU)] subcutaneously 4 weeks apart in a volume of 100 microliters. For protection efficacy studies, mice were vaccinated with MVA/IL-15/5Mtb (2 doses of vaccine 1×10^7 pfu given 4 weeks apart). The controls included in the experiments were wild-type MVA group (2 doses of 1×10^7 pfu given 4 weeks apart), BCG vaccinated group (single dose of 1×10^6 CFU given subcutaneously) and an unvaccinated naïve group. Vaccinated animals were aerogenically challenged 4 weeks after vaccination with the *M. tuberculosis* Erdman strain (WHO standard) suspended in PBS at a concentration known to deliver 200 CFU to the lungs over a 30-min exposure time in a Middlebrook chamber (GlasCol, Terre Haute, IN) and the bacterial burdens in the lungs and spleens of the challenged mice were determined, a month later by standard CFU (colony forming units) plating procedures as reported previously [10].

Assays for immune responses

An enzyme linked immunosorbent assay (ELISA) was used to measure the levels of mycobacterial antigen-binding antibodies in sera collected from vaccinated mice essentially as described previously except for the antigen preparations used for coating ELISA plates [8]. For coating ELISA plates, a sonicated lysate of *M. tuberculosis* strain H37Rv was used at a concentration of 1 mcg/ml in bicarbonate coating buffer. For detecting cellular response against mycobacterial antigens, an *in vitro* co-culture assay was used. Splenocytes from three animals within a group were pooled and CD4⁺ and CD8⁺ T lymphocytes were purified negatively using CD4⁺ or CD8⁺ T cell isolation kit from Miltenyi Biotech, Auburn, CA according to the manufacturer's instructions. Purified cells were plated at 2×10^6 cells per well in 24 well clusters in triplicate. To syngeneic splenocytes from age matched naïve mice, a sonicated lysate of *M. tuberculosis* was added at a final concentration of 1 mcg/ml and incubated for six hours followed by irradiation (3000 rad), prior to adding them at 1×10^6 cells per well to coculture with purified CD4⁺ or CD8⁺ lymphocytes from vaccinated mice. After 72 hours of co-culture, supernatants were harvested and the interferon gamma (IFN γ) levels were measured using a commercial ELISA kit for mouse IFN γ (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. All samples were tested in triplicate in the assays.

Statistical analysis

Analysis of variance was used to determine the effect of different immunization protocols on the magnitude of cellular and antibody responses. Student's t test was used to compare immunization protocols and significance levels were set at a *P* value of 0.05.

Results and Discussion

Accumulating evidence supports the notion that the development of a highly effective vaccine against *M. tuberculosis* infection is achievable. However, because of the complexity of the large *M. tuberculosis* genome with a coding capacity for more than 4000 genes, such a vaccine should be able to induce not only a robust immune response quantitatively but that response should also be sufficiently broad to target multiple antigens of the pathogen to deliver a "multi-hit" attack. We have designed and generated a multivalent candidate vaccine. Our vaccine (MVA/IL-15/5Mtb) consists of three key elements that collectively contribute to induce a superior immune response: i) the use of a viral vector, Modified virus Ankara vaccinia virus to deliver *M. tuberculosis* antigens, ii) the incorporation of five *M. tuberculosis* antigens that have been proven individually to induce protective immune responses in animal models, and iii) the tandem expression of immune stimulatory cytokine IL-15 as a molecular adjuvant.

An MVA live virus-based delivery system was selected due to the greater potency of live viruses at stimulating adaptive immune responses than peptides, proteins, or DNA vaccines, as a result of their ability to mimic a natural infection and to induce innate immune responses via Toll like receptors (TLRs) and provide an inflammatory milieu for antigen capture by dendritic cells [11]. Antigen-capture in this inflammatory milieu results in the maturation and expression of co-stimulatory molecules by these dendritic cells and ultimately to prime T cells robustly and to induce superior immune responses. Many MVA vectored vaccine candidates are currently being evaluated in clinical trials and its use as a pre-vaccine in over one hundred thousand individuals during the smallpox eradication campaign attests to its safety.

Our vaccine expresses five *M. tuberculosis* genes simultaneously that have been individually validated previously to induce protective immune responses and includes Ag85A, Ag85B, ESAT-6, HSP65 and Mtb39A antigens. Each of these antigens is being evaluated in clinical trials currently or is ready to enter trials shortly as single agent entities or in combination [12]. Our design strategy was to robustly express a spectrum of protective antigens simultaneously to induce a broad immune response that that can deliver a “multi-hit” attack.

IL-15 is a powerful immune stimulatory cytokine with a wide range of biological activities [13]. It is involved in the activation, proliferation and differentiation of CD8⁺T-cells and NK-cells, and the maintenance of CD8⁺ memory T cells in addition to supporting the survival of mature DC. Our previous work has shown that the incorporation of IL-15 into vaccinia-based vaccines induces high avidity, long lived antigen specific memory cytotoxic T lymphocytes (CTL) as well as persistent antigen specific antibody responses thereby conferring a durable effective immunity against vaccine antigens [8,14–16]. Collectively, these properties of IL-15 make a compelling rationale to incorporate it as a molecular adjuvant in developing a superior TB vaccine. Since the protection from TB requires a sustained cell-mediated immune response to the infection, the incorporation of IL-15 as a molecular adjuvant in our vaccine therefore is likely to improve the vaccine induced responses both qualitatively and quantitatively. A robust mucosal immune response in addition to a systemic cell-mediated response is likely to be important for an effective TB vaccine that can be both preventive as well as therapeutic. In this regard, it is important to note that IL-15 is a pivotal cytokine in orchestrating the immune networks operational in mucosal surfaces and is required for the generation of intraepithelial lymphocytes, gamma/delta T cells, and the differentiation of B-1 cells and the development of IgA responses [13,16].

To confirm the expression of Ag85A, Ag85B, ESAT-6, HSP65 and Mtb39A, a cell lysate from MVA/IL-15/5Mtb virus-infected BHK-21 cells was subjected to SDS-PAGE analysis followed by immunoblotting with specific antibodies for each individual *M. tuberculosis* antigen. As shown in Fig. 1, all 5 antigens were expressed at detectable levels in infected cell lysates. No extensive degradation was noted for expressed ESAT6 and HSP65 polypeptides although the size of ESAT6 polypeptide expressed by MVA/IL-15/5Mtb appeared to be slightly larger than the expected 11.5 kDa. Two prominent bands were seen for Mtb39A of which the smaller band is likely to be a degradative product of the correctly sized Mtb39A of approximately 39 kDa. The major products for Ag85A and Ag85B expression correlated with the expected sizes of 35 kDa for these two polypeptides when polyclonal antibodies were used for their detection (Fig 1). Because of extensive cross reactivity that exists between Ag85A and Ag85B with commercially available antibodies specific for the Ag85 complex of *M. tuberculosis*, and because of their similar molecular weights, we used a monoclonal antibody specific for the influenza virus hemagglutinin epitope (YPYDVPDYA) that had been added to the N-terminus of Ag85A expressed in our MVA/IL-15/5Mtb (see *Materials and Methods* section) to confirm its expression (data not shown), in addition to what is shown in Fig. 1 with an Ag85A specific polyclonal antiserum. We also reconfirmed the expression of Ag85B, using antiAg85B antiserum generated by injecting mice with a DNA expression construct of Ag85B (data not

shown). As shown in Table 1, the bioactivity of MVA/IL-15/5Mtb-expressed human IL-15 was confirmed by the ability of MVA/IL-15/5Mtb-infected, irradiated BHK-21 culture supernatants to induce robust proliferation of an IL-2/IL-15 cytokine dependent human NK cell line (NK-92) that was inhibitable by the addition of human IL-15 neutralizing antibodies. We further confirmed the antigenicity and quantitated the amount of IL-15 present in the MVA/IL-15/5Mtb infected culture supernatant of BHK-21 cells by an ELISA assay as shown in Table 1.

We next assessed the immunogenicity of our multi-valent, molecularly adjuvanted vaccine with the immune stimulatory IL-15 cytokine in mice and compared the immune responses induced by our MVA vaccinia-based vaccine candidate with that of the currently licensed BCG vaccine. Groups of mice (C57BL/6) were vaccinated with BCG or MVA/IL-15/5Mtb (two doses of vaccine were given 4 weeks apart). Serum was collected and pooled from the vaccinated mice and assessed for the presence of antibodies against *M. tuberculosis* using an ELISA where the coated antigen was a sonicated, irradiated, lysate of *M. tuberculosis*. As shown in Fig 2A, the MVA/IL-15/5Mtb vaccine induced significantly higher levels of IgG antibodies against *M. tuberculosis* antigens in vaccinated animals than the licensed BCG vaccine in immunized mice. The protective role of antibodies against *M. tuberculosis* which is a facultative intracellular pathogen has been controversial. The potential role of locally synthesized TB-specific IgG or secretory IgA in eliminating *M. tuberculosis* in droplet infection in the lower respiratory tract remains to be rigorously addressed. Nonetheless, it is conceivable that antibodies could still impart beneficial effects especially if these vaccine-induced antibodies are of Th1-associated IgG2a and/or IgG2b subclasses via opsonization, complement activation and mediate antibody-dependent cellular cytotoxicity or enhance antigen presentation even in the case of intracellular pathogens such as *M. tuberculosis* where their cellular location precludes direct binding to antibodies [17]. Therefore, to determine the extent of Th1-associated IgG2a and IgG2b subclass distribution against *M. tuberculosis* in the sera from vaccinated mice, we repeated the ELISA assay instead of using an anti-mouse total IgG specific secondary antibody as was done in Fig. 2A, with subclass-specific anti IgG2a and IgG2b secondary antibodies separately. As shown in Fig. 2B, the predominant anti *M. tuberculosis* antibody response induced by our MVA/IL-15/5Mtb vaccine was of Th1-associated IgG2a subclass specificity. In vaccinated BALB/c mice similar antibody profiles were also detected (data not shown).

To assess the cellular immune responses induced by our vaccine, CD4⁺ and CD8⁺ T cells were purified from the spleens of vaccinated C57BL/6 mice 8 days after the final vaccination and then co-cultured with syngeneic splenocytes pulsed with a lysate of *M. tuberculosis*. Interferon gamma (IFN- γ) levels in the supernatants of co-cultured cells were then measured by an ELISA after 72 hours. As shown in Fig 3, both BCG vaccine and MVA/IL-15/5Mtb vaccine induced a dominant CD4⁺ T cell-mediated immune response in vaccinated C57BL/6 mice against mycobacterial antigens as reflected by the production of IFN- γ upon antigenic stimulation. In contrast, the magnitude of CD8⁺ T cell-mediated responses against *M. tuberculosis* antigens was notably less intense than the CD4⁺ T cell responses as shown in Fig. 3 (right panel). Nonetheless, the CD8⁺ responses induced by our MVA/IL-15/5Mtb vaccine were significantly higher than those induced by the licensed BCG vaccine. The use of *M. tuberculosis* lysate to stimulate CD4⁺ and CD8⁺ T lymphocytes from the vaccinated animals precluded defining the ability of each of the five antigens expressed by MVA/IL-15/5Mtb to induce a robust cellular immune response. However, perhaps except for the Ag85A antigen [18–20], only limited information exists as to the presence and distribution of dominant murine MHC class I and class II epitopes in the antigens expressed by our vaccine. In order to verify that our vaccine MVA/IL-15/5Mtb is indeed capable of eliciting a strong immune response against one of the more extensively-studied, dominant protective antigens of *M. tuberculosis* namely Ag85A, splenocytes from vaccinated BALB/c mice were cocultured with either an Ag85A-specific

dominant class II peptide (LTSELPGWLQANRHVKPTGS) or a dominant class I peptide (MPVGGQSSF restricted by H-2L^d) separately. As a negative control, we included a dominant H-2K^d restricted peptide TYQRTRALV from the nucleoprotein of influenza virus [21]. Under these conditions copious amounts of IFN- γ was produced as shown in Table 2, thus confirming that our vaccine induces both CD4⁺ and CD8⁺ T cell responses against the immunodominant Ag85A antigen. As expected splenocytes from vaccinated C57BL/6 mice that carry I-A^b class II and K^b and D^b class I MHC haplotypes did not respond to the above peptides.

Having confirmed the induction of a strong cellular and humoral response against *M. tuberculosis* in vaccinated mice we next evaluated the ability of our vaccine to confer protection against an aerogenic challenge of *M. tuberculosis*. As shown in Table 3, the MVA/IL-15/5Mtb vaccinated C57BL/6 mice that were challenged 4 weeks after immunization displayed reductions in the *M. tuberculosis* bacterial burden both in the lungs and spleens that were comparable to the reductions seen in BCG vaccinated mice thus illustrating the protective efficacy of MVA/IL-15/5Mtb.

One of the leading TB vaccine candidates that has undergone successful phase I evaluation and is now in phase II trials in South Africa is a monovalent MVA recombinant that expresses the immunodominant Ag85A antigen. This vaccine elicits potent polyfunctional CD4 memory responses in individuals who have been previously vaccinated with BCG [22,23]. Our MVA/IL-15/5Mtb pentavalent vaccine adjuvanted with IL-15 which expresses four additional antigens in concert with Ag85A is likely to represent a further improvement of the version of MVA vaccine currently undergoing phase II evaluation on the basis of having a greater antigenic repertoire presented in a priming milieu with a potent immunostimulatory cytokine.

Though two billion people are infected with *M. tuberculosis* globally, the correlates of immune protection for this deadly disease remain poorly defined and constitute a major impediment for developing and efficacy testing of potential vaccines that can either replace BCG or one that can be used in conjunction with a primary BCG vaccination to prevent adult pulmonary TB. However, ravaging TB occurs most often as a consequence of reactivated latent infection in HIV infected individuals with low CD4⁺ T cell counts, and attests to a critical role for these CD4⁺ T cells in the immune control of *M. tuberculosis* in humans. Furthermore, evidence from CD8 gene deleted mouse models has implicated a pivotal role for CD8⁺ T cells in controlling *M. tuberculosis* in mice. The IL-15 knockout mice also have been shown to display impaired long-term protective immunity to BCG due to loss of CD8⁺ memory responses in the lungs of these mice as well as failure of CD8⁺ T cells to accumulate in draining lymph nodes and at the sites of infection [24,25]. Consistent with these observations in animal models, robust CD8⁺ responses against *M. tuberculosis* in latently infected individuals have been demonstrated. In addition, recent evidence suggests that IL-15 plays a critical role in orchestrating antimicrobial pathways involving cathelicidin in eliminating *M. tuberculosis* in infected human macrophages [26].

Despite the long-held view that a CD4-mediated Th1 immune response is likely to be pivotal in controlling *M. tuberculosis*, there is a growing appreciation that CD8⁺ T cells play a critical non-redundant role in protective immunity against tuberculosis which has fueled new strategies to design vaccines that can induce such robust CD8⁺ responses [reviewed in ref 27]. Ultimately, a vaccine that combines the functional synergy of both CD4 and CD8 components of the cellular immunity is likely to have the best chance of preventing tuberculosis. It is remarkable that our vaccine with five antigens induces a CD4⁺ T cell response that approximates the CD4⁺ T cell response induced by BCG that expresses myriad of mycobacterial genes. Even more impressive is the observation that the CD8⁺ response is superior to that of BCG. There is an abundance of evidence that the molecular adjuvant IL-15 incorporated in our vaccine plays a pivotal role in CD8-mediated immune responses including the maintenance of memory

although its role in the CD4-mediated immune responses appears to be marginal in mice that could account for the more modest CD4 response elicited by our vaccine. Nonetheless, emerging evidence from *in vivo* primate studies and *in vitro* studies with human cells, suggest that IL-15 may impact both CD4⁺ and CD8⁺ T cell subsets in humans [28,29].

Collectively, the above observations indicate that our IL-15 adjuvanted, multi-valent tuberculosis vaccine MVA/IL-15/5Mtb induces a robust immune response that is qualitatively and quantitatively on par, if not superior to the currently licensed BCG vaccine thus warranting its further preclinical development as a stand-alone vaccine or as a booster vaccine with BCG priming in appropriate animal models of TB for its protective efficacy with the final goal of testing this vaccine in humans.

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Figure 1.

The expression of integrated *M. tuberculosis* genes by recombinant MVA/IL-15/5Mtb virus. MVA/IL-15/5Mtb infected BHK-21 cell lysates were subjected to SDS-PAGE on either 15% (for ESAT6) or 10% (for Ag85A, Ag85B, HSP65 and Mtb39A) acylamide gels and Western blot analyses were performed. For detecting ESAT6, a mouse MAb (clone HYB 076-08) was used; for Ag85A, a polyclonal chicken antibody was used; for Ag85B and Mtb39A specific polyclonal rabbit antibodies were used; HSP65 was detected with a mouse Mab (clone 4HG11). For each antigen the correct band is indicated by an asterisk and on the left side of each panel, arrowheads indicate the position of molecular weight markers relevant to assessing the size of the expressed protein. Lane 1, lysates infected with MVA/IL-15/5Mtb; lane 2, lysates infected with parental MVA virus.

Figure 2.

M. tuberculosis specific antibody profiles of mice vaccinated with either MVA/IL-15/5Mtb or BCG. Groups of mice (n=6) were vaccinated twice four weeks apart subcutaneously with a dose of 1×10^7 pfu MVA/IL-15/5Mtb or 1×10^6 cfu of BCG. Animals were bled 4 weeks after the second dose of vaccine and sera separated and pooled within each group. *M. tuberculosis*-specific antibodies were detected by an ELISA. A sonicated, irradiated *M. tuberculosis* lysate was used as the plate bound antigen. In panel A, bound antibodies were detected with an anti mouse IgG (total IgG) secondary antibody. In panel B, pooled sera from MVA/IL-15/5Mtb vaccinated mice were used and to determine the subclass specificities of *M. tuberculosis*

reactive antibodies, either secondary antibody specific for mouse IgG2a or mouse IgG2b was used.

Figure 3.

Cellular immune response against *M. tuberculosis* antigens in vaccinated mice. Groups of mice were vaccinated twice four weeks apart subcutaneously with MVA, MVA/IL-15/5Mtb or BCG. Three animals from each group were euthanized 8 days after booster vaccinations and splenocytes were harvested and pooled. From pooled splenocytes, CD4⁺ and CD8⁺ T lymphocytes were purified by negative selection with magnetic microbeads. Purified cells were then plated at 2×10^6 cells per well in triplicate. Age matched, naïve syngeneic splenocytes mixed with a sonicated, irradiated lysate of *M. tuberculosis* were added to each well at 1×10^6 cells per well (denoted by +). Splenocytes (2×10^6 cells per well) from vaccinated animals cultured alone in parallel without any added *M. tuberculosis* lysate served as controls (denoted by -). After a 72-hour period of co-culture, INF- γ levels in the culture supernatants were determined using a commercial ELISA kit for mouse INF γ .

TABLE 1

Characterization of MVA/IL-15/5Mtb vaccinia-expressed IL-15.

Agent	Quantitation of IL-15 by ELISA	Bioactivity as detected by proliferative index [*]
MVA	None detected	1.2
MVA/IL-15/5Mtb	2000 picog/ml	93.9
MVA/IL-15/5Mtb+IL-15Ab ^{**}	Not done	6.8
Culture medium	None detected	1.0

* Proliferative index was calculated as the fold-increase in ³H-thymidine uptake above the media control.

** Virus infected culture supernatant was mixed with IL-15 neutralizing monoclonal antibody (final concentration of 1 mcg/ml) before adding to NK-92 cells.

TABLE 2Splenocytes from MVA/IL-15/5Mtb vaccinated mice respond to Ag85A peptides^a.

Strain	Peptide	IFN gamma pg/ml
BALB/c	LTSELPGWLQANRHVKPTGS	328.4±13.0
	MPVGGQSST	181.4±6.7
	TYQRTRALV	not detected
C57BL/6	LTSELPGWLQANRHVKPTGS	not detected
	MPVGGQSST	not detected
	TYQRTRALV	not detected

^a Splenocytes isolated 8 days post vaccination from immunized mice were cocultured with the indicated peptides in triplicate at a final concentration of 2 mcg/ml. IFN gamma in the culture supernatants were measured 72 hours later by an ELISA.

TABLE 3

M. tuberculosis growth in the lungs and spleens of vaccinated and control mice^a

Immunization Group	Lungs		Spleens	
	Mean log ₁₀ CFU ± SD	Difference in CFU	Mean log ₁₀ CFU ± SD	Difference in CFU
Naïve	5.99 ± 0.08		5.60 ± 0.11	
BCG	5.46 ± 0.09	(-0.45) *	5.07 ± 0.15	(-0.53) *
MVA	5.82 ± 0.11		5.28 ± 0.09	
MVA/IL-15/5Mtb	5.50 ± 0.15	(-0.49) *	4.88 ± 0.19	(-0.72) *

^aProtective efficacy in C57BL/6 mice immunized with MVA vaccines (two doses given 4 weeks apart) or BCG (single dose). Vaccinated mice were challenged 4 weeks later with *M. tuberculosis* and bacterial burdens were determined after 1 month.

* Asterisks show statistical significance (p<0.05) compared to naïve controls.