Trials in Vaccinology 5 (2016) 88–91

Contents lists available at ScienceDirect

Trials in Vaccinology

journal homepage: www.elsevier.com/locate/trivac

Short communication

Immunogenicity and protection conferred by a recombinant *Mycobacterium marinum* vaccine against Buruli ulcer

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ARTICLE INFO

Article history:
Received 17 March 2016
Accepted 19 April 2016
Available online 4 May 2016

Keywords:
Buruli ulcer
Mycobacterium ulcerans
Vaccine
M. marinum
Ag85A

ABSTRACT

Mycobacterium ulcerans (MU) infection causes the disfiguring necrotic skin disease, Buruli ulcer (BU). While vaccination with *Mycobacterium bovis* BCG provides nominal antigenic cross-reactivity for induction of immunity against experimental MU infection, a mycobacterial species with greater genetic homology to *Mycobacterium ulcerans* may serve as a richer source of cross-protective immunogens and lack the pathological features of MU-based vaccines. *Mycobacterium marinum*, a highly homologous genetic relative of MU, could be used to satisfy these criteria and, as such, we have generated a recombinant *M. marinum* strain expressing the immunodominant, protective MU-Ag85A. The immunogenicity and protection achieved by murine vaccination with this strain are superior to standard BCG vaccination and may serve as a foundation for developing more effective BU vaccines.

Buruli ulcer (BU), one of 17 neglected tropical diseases (NTDs) prioritized by the WHO, has become an emerging health problem largely centered to Sub-Saharan Africa, Australia, and Japan [1–3]. Over 50% of BU cases afflict children less than 15 years of age [4]. Despite the progress made in early detection and treatment of BU, surgical excision of the lesions combined with skin grafting remains the only treatment for advanced ulcers. There are currently no specific prophylactic treatments for BU, and thus an efficacious BU vaccine would be imperative to protect at-risk populations in hyper-endemic areas.

Attempts to generate an efficacious vaccine for BU have met with limited success, although *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccination transiently protected both humans and mice in experimental models [5–9]. Additionally, use of a DNA vaccine encoding the immunodominant *Mycobacterium ulcerans* mycolyl transferase, Ag85A (MU-Ag85A), imparted temporary protection similar to BCG vaccination [10]. We have recently shown that a subcutaneous recombinant BCG vaccine which heterologously expresses the MU-Ag85A protein conferred superior protection against murine Buruli ulcer compared to BCG or Ag85A vaccination by significantly increasing length of survival post-challenge [11]. While vaccination with BCG provides nominal antigenic cross-reactivity for induction of protective memory against *M. ulcerans* infection, a mycobacterial species with greater genetic homology to *M. ulcerans* may serve as a richer source of cross-protective immunogens. Indeed, prior inoculation with the attenuated mycolactone-deficient MU 5114 also confers short-term protection against challenged mice [12]. However, despite a reduction in virulence, this strain may still retain factors which inhibit the development of fully effective immunological memory [13].

We hypothesized that a more efficacious MU vaccine design strategy would involve a mycobacterial strain which lacks both the virulence and potential immunomodulatory properties associated with an MU-based vaccine, while still expressing high levels of orthologous antigens. To satisfy these criteria, we chose the close genetic relative, *Mycobacterium marinum*, which shares up to 99.6% nucleotide identity with MU [14–17]. Despite this extensive genetic homology, infection with *M. marinum* causes comparatively minor and typically indolent, granulomatous skin lesions.

In the present study, we hypothesized that vaccination with *M. marinum* or recombinant strains expressing MU-Ag85A would provide immunogenic antigens but lack the immunosuppressive factors accompanying the more virulent MU background. A study by Fenner et al. published in the late 1950's supported the potential use of *M. marinum* to induce protective immunity against Buruli ulcer [18]. Mice that were inoculated intradermally in the foot pad with *Mycobacterium balnei* (later renamed *M. marinum*) and

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http://dx.doi.org/10.1016/j.trivac.2016.04.001
1879-4378/

challenged with MU nine weeks later exhibited reduced swelling compared to those primed intradermally with BCG [18]. We were interested in expanding upon this work by examining the following parameters not investigated in this previous study: (1) the use of lower-dose, subcutaneously administered (a more physiologically relevant route) *M. marinum* and recombinant *M. marinum* strains; (2) the effects of vaccination on lifespan of MU-challenged mice; (3) the assessment of vaccine immunogenicity.

To this end, *M. marinum* (Aronson) was transformed with an empty vector (pHA) or an overexpression construct encoding MU-Ag85A, and large quality-controlled vaccine accession lots were generated as previously described [19]. Briefly, quality control was performed on at least three random vials of each vaccine accession lot by multiple methods. These included Western analysis of heterologous antigen expression, quantification of colony forming units, lack of contamination, and assessment of plasmid stability by gel electrophoresis and sequencing of the recombinant insert.

To determine if vaccination with *M. marinum* MU-Ag85A could generate antigen-specific adaptive immune responses, C57BL/6 mice were primed with 10^7 bacilli by intravenous (IV) injection to observe maximal immune responses. All animal studies were approved by the Duke University IACUC. At intervals ranging from 1 to 8 weeks post-prime, MHCII tetramer staining of peripheral lymphocytes was used to quantify the percentage of $CD4^+$ T cells which recognized Ag85A. In order to assess if a heterologous mycobacterium could be used to boost initial T cell proliferation, an intravenous dose of 10^7 *Mycobacterium smegmatis* expressing MU-Ag85A was given at 8 weeks post-prime. As seen in Fig. 1A, *M. marinum* MU-Ag85A induced significantly larger populations of Ag85A-specific helper T cells compared to *M. marinum* pHA vaccinated or unprimed mice at several time points post-prime and boost. Background tetramer-positive T cell populations during *M. marinum* pHA priming were most likely due to endogenous expression of Ag85A. To determine if the antigen-specific T cells produced during vaccination could generate T_h1 responses, C57BL/6 mice were vaccinated as in Fig. 1A. $IFN\gamma^+$ splenocytes were then quantified by ELISPOT following *in vitro* stimulation with either MU-Ag85A peptide (FQAAYNAAGGHNAVWNFDDN) or heat-killed *M. ulcerans* 1615 (HKMU). Vaccination with *M. marinum* and *M. marinum* MU-Ag85A both significantly increased the number of $IFN\gamma^+$ splenocytes compared to unprimed mice, suggesting that genetic identity may heighten immune recognition of shared antigens between MU and *M. marinum* (Fig. 1B). Interestingly, *M. marinum* MU-Ag85A yielded even greater levels of $IFN\gamma^+$ splenocytes compared to *M. marinum* pHA ($p < 0.001$). This suggests that antigen expression in the *M. marinum* background amplified the responsiveness of functional T_h1 cells expanded by vaccination to endogenous levels of Ag85A expressed by *M. ulcerans* bacteria.

Importantly, subcutaneous priming with *M. marinum* MU-Ag85A also yielded significantly greater antigen-specific T cell populations compared to *M. marinum* alone, though this route induced lower absolute percentages which peaked at a later time point compared to the intravenous priming (Fig. 1C). However, upon examination of the fold increase of antigen-specific $CD4^+$ T cells induced by *M. marinum* MU-Ag85A compared to *M. marinum* vaccination at the two strongest respective time points for each route, subcutaneous priming yielded higher fold-increases for weeks 3 and 4 post-prime (2.1 and 2.5-fold, respectively) versus weeks 2 and 3 following IV priming (1.7 and 1.8-fold, respectively).

Protective efficacy of the *M. marinum* vaccine was examined in the murine footpad challenge model. To recapitulate a more physiologically relevant vaccination route as used in humans, mice were subcutaneously primed with 10^7 *M. marinum* or *M. marinum* MU-Ag85A and boosted with *M. smegmatis* MU-Ag85A. Mice were then challenged with 10^5 virulent MU 1615 via an intradermal footpad route two weeks post-boost. Footpad tissue sections were stained with hematoxylin and eosin at 12 weeks post-challenge (Fig. 2A). Strikingly, while extensive tissue swelling, loss of epidermal integrity, and necrosis were observed in unprimed mice, both *M. marinum* and *M. marinum* MU-Ag85A vaccinated animals exhibited less pathology. Additionally, diminished tissue edema was observed within histological footpad sections from *M. marinum* MU-Ag85A vaccinated mice compared to *M. marinum* vaccination.

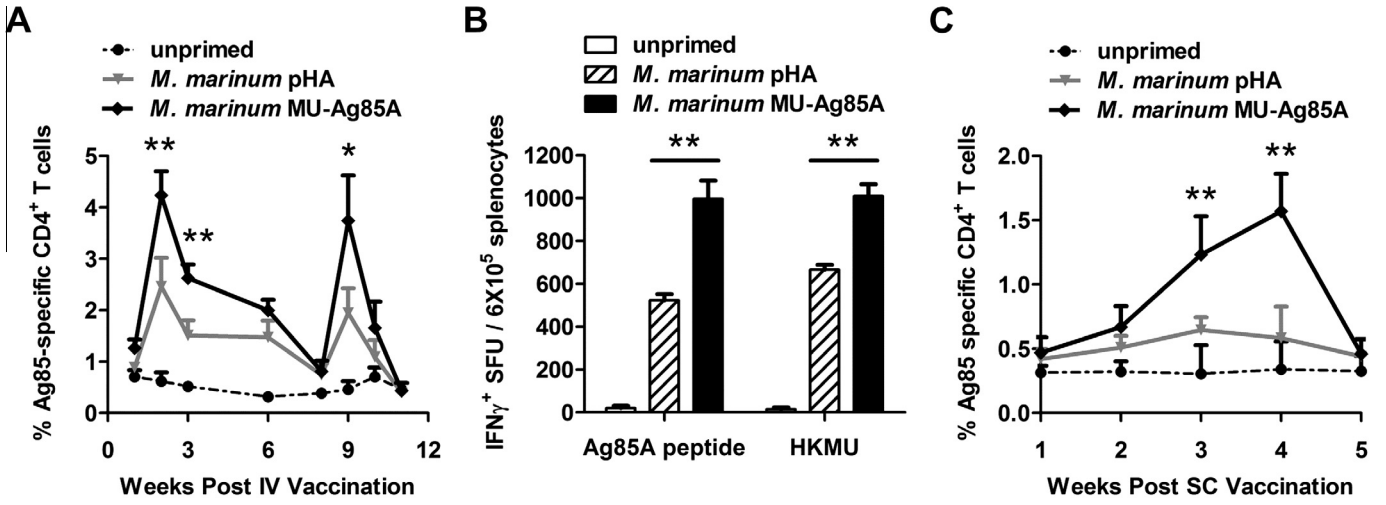

Fig. 1. Expression of MU-Ag85A by *M. marinum* exhibits enhanced immunogenicity. A. C57BL/6 mice were left unprimed (hashed black) or were intravenously (IV) primed using 10^7 *M. marinum* pHA (gray) or *M. marinum* MU-Ag85A (black) ($n = 5$ mice for each group). At various time points, PBMCs were isolated and flow cytometric analysis was performed to quantify levels of $CD4^+$ T cells bound to MHCII-Ag85 tetramer. At 8 weeks post-prime (black arrow), mice were intravenously boosted with 10^7 *Msmeg* expressing MU-Ag85A. Error bars represent standard deviation of the mean. Asterisks indicate statistical analysis by the Student's *t*-test comparing the *M. marinum* pHA to *M. marinum* MU-Ag85A groups. $^*p < 0.01$, $^{**}p < 0.003$ B. C57BL/6 mice were left unprimed (white) or were intravenously vaccinated using 10^7 *M. marinum*-pHA (hashed) or *M. marinum* MU-Ag85A (black) and boosted with 10^7 *M. smegmatis* expressing MU-Ag85A 8 weeks later. Two weeks following the boost, splenocytes were isolated for stimulation with MU-Ag85A peptide or heat killed *M. ulcerans* 1615 (HKMU). ELISPOT spot forming units (SFU) were used to quantify $IFN\gamma^+$ splenocytes following 24 h of stimulation. Error bars represent standard deviation of the mean. Asterisks indicate statistical analysis using the Student's *t*-test ($n = 5$ mice for each group). $^{**}p < 0.001$ C. C57BL/6 mice were left unprimed (hashed black) or were subcutaneously (SC) primed using 10^7 *M. marinum* pHA (gray) or *M. marinum* MU-Ag85A (black). PBMCs were isolated and flow cytometric analysis was performed to quantify levels of $CD4^+$ T cells bound to MHCII-Ag85 tetramer. Error bars represent standard deviation. Asterisks indicate statistical analysis by the Student's *t*-test ($n = 5$ mice for each group) comparing the *M. marinum* pHA to *M. marinum* MU-Ag85A groups. $^{**}p < 0.001$.