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Anti-tuberculosis immunity induced in mice by vaccination with *Mycobacterium smegmatis* over-expressing Antigen 85B is due to the increased influx of IFN γ -positive CD4 T cells into the lungs

Devin R. Lindsey, Subramanian Dhandayuthapani, and Chinnaswamy Jagannath

SUMMARY

BCG vaccine is unsafe for use in patients with AIDS. *Mycobacterium smegmatis* (Msm), an avirulent species unlike virulent *Mycobacterium tuberculosis* (H37Rv, Mtb) has been used as a carrier vaccine with ambiguous results due to the elicitation of poor immune responses to antigens in mice. In this study, we over-expressed the immunodominant antigen 85B in *M. smegmatis* (Msm-OEAg85B) and compared the immunogenicity of Msm-OEAg85B with that of wild-type Msm. Mice which were vaccinated with either Msm or Msm-OEAg85B and challenged 2 weeks later with Mtb. Vaccine-induced protection and lung T cell responses were evaluated post vaccination and post challenge. Unlike wild-type Msm that elicited minimal T cell responses in mice, MsmOE-Ag85B induced enhanced CD4+IFN γ + T cell responses that leveled off over 2 weeks. After virulent challenge at 2 weeks, Mtb grew progressively in the lungs of naive mice and mice vaccinated with wild-type Msm, but showed reduced growth ($<0.6 \log_{10}$) and therefore protection in Msm-OEAg85B-vaccinated mice. Lungs of Msm-OEAg85B-vaccinated mice showed increased numbers of CD4+IFN γ + T cells suggesting that the reduced bacterial growth was likely due to the enhanced T cell response in lungs. Since wild-type Msm was unable to protect but Msm-OEAg85B was, we suggest that Msm can be genetically manipulated to over-express selected Mtb antigens, thereby paving the way for safer vaccines that can be used in immunodeficient patients.

1. Introduction

Tuberculosis is the leading cause of death due to a single infectious agent. The etiologic agent *Mycobacterium tuberculosis* is also a major cause of accelerated death in AIDS patients. Prevention of tuberculosis using vaccines is therefore a major focus of research. The BCG vaccine has been used for inducing anti-tuberculosis immunity in different parts of the world. While it protects children against tuberculosis with a variable efficacy, it is ineffective against adult tuberculosis. Furthermore, BCG vaccine causes disseminated infection in AIDS patients who have a deficient CD4 T cell response. Since tuberculosis–HIV1 coinfections are a serious threat to the control of both tuberculosis and AIDS, safer vaccines that can induce stronger anti-tuberculosis immunity are required for use in AIDS patients. *Mycobacterium smegmatis* (Msm) is an attenuated species of *Mycobacterium* that is a harmless saprophyte of humans and is amenable for genetic manipulation. It has been previously used as a carrier of vaccine-effective antigens against tuberculosis with ambiguous results.^{1–4} It has also been used to deliver cytokines that can skew Th1 immunity in mice.⁵ Despite these efforts it is yet find a general use as a vaccine platform presumably because the mechanisms of antigen export from Msm and pathways for antigen processing are still poorly understood. We recently described that over-expression of an immunodominant Mtb-derived antigen in Msm subsequently leads to the induction of autophagy when Msm is internalized by in mouse macrophages. We found

that this in turn enhanced Msm-induced immunity. In this study, we demonstrate that regulated expression of a Mtb-derived antigen in a non-vaccinogenic strain of Msm can enhance Th1 responses that contributes to increased anti-tuberculosis immunity.

2. Materials and methods

2.1. Bacteria

Msm (wild-type strain MC2) and Msm-OEAg85B were prepared as described previously.⁶ They were grown in 7H9 broth for 3 days without (Msm) or with 25 µg/mL kanamycin (Msm-OEAg85B) and log phase organisms were washed in saline and adjusted to contain 10⁷ CFU per mL.

2.2. Mouse vaccination and challenge

Msm (wild-type strain MC2) and Msm-OEAg85B were used at 10⁷ CFU per mouse given as one subcutaneous dose. Vaccinated and naive mice were divided into two groups. One group was challenged with an aerosol dose of 100 CFU of Mtb as described before.⁶ Two weeks later mice were sacrificed and analyzed for bacterial counts of lungs by plating organ homogenates on 7H11 agar plates. A second group of mice were sacrificed at 1 and 2 weeks post challenge and lungs homogenized to prepare T cells as described before. They were stained for CD4 T cells expressing IFNγ and analyzed using flow cytometry.⁷

3. Results and discussion

Antigen 85 complex proteins A and B are powerful antigens of *M. tuberculosis* and BCG vaccine that induce both T cell and antibody responses in animal models and humans.^{3, 8} We recently described that Ag85B-induced immunogenicity is a strong correlate of vaccine-induced immunity in mice.⁶ We therefore used antigen 85B to study development of Msm vaccine platforms. Even though wild-type Msm contains genes that encode the proteins of the antigen 85 complex, secretion of these proteins is minimal and the culture filtrates do not contain these proteins comparable to those of BCG. Perhaps because of this, wild-type Msm is unable to induce protection against challenge with tuberculosis in mice even when it expresses Mtb antigens.¹ Interestingly, wild-type Msm has also been used to deliver other types of antigens to immunize mice, again with ambiguous results. For example, a tumor antigen delivered by the slow growing BCG elicited CD8 responses in mice while the same antigen expressed in Msm failed to do so, indicating that different pathways of processing may determine vaccine efficacy.⁹ On the other hand, Msm was able to elicit CD8 responses while expressing full-length HIV 1-derived *Env* antigen.^{10, 11} Although many investigators have used Msm to express Mtb-derived antigens, the MHC-II dependent mechanisms of peptide presentation and induction of CD4 responses remain unclear.

In our previous studies, we analyzed the mechanisms of mycobacterial antigen processing in mouse macrophages and dendritic cells. We first found that the pH of the BCG phagosome was near neutral that precluded an effective presentation of Ag85B.¹² Mtb-derived mutant phagosomes like those of H37Ra acquired vATPase, acidified the phagosomes and were more effective presenters of Ag85B. Secondly, direct expression of pH-independent Cathepsin-S in BCG enhanced the macrophage-mediated presentation of Ag85B.¹³ More recently we found that hyperexpression of Ag85B in Msm led to autophagy that increased its MHC-II-dependent presentation to T cells. These observations suggested that genetic modulation can be used to enhance the ability of Msm to deliver antigens into macrophages and induce more effective immunity. Consistent with this hypothesis, Msm-OEAg85B but not wild-type Msm was able to immunize mice and elicit stronger CD4+IFNγ+ T cell responses (Fig. 1). Unlike wild-type Msm, Msm-OEAg85B also induced protection against challenge with tuberculosis (Fig. 2)

which correlated with a strong recall of CD4+IFN γ + T cells in the lungs (Fig. 3). These data suggest that Msm can be genetically engineered to induce novel types of immune responses in mice and perhaps humans.

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Fig. 1.

Mycobacterium smegmatis over-expressing antigen 85B (Msm-OEAg85B) induces a stronger T cell response in mice after vaccination. Mice were vaccinated with *Mycobacterium smegmatis* and Msm-OEAg85B given as one subcutaneous dose at 10^7 CFU per mouse. Spleen-derived T cells were stained for CD4 T cells secreting IFN γ and analyzed using flow cytometry with a BD FACSscan. CD4+IFN γ + cells were enumerated and plotted against time after vaccination. *p* values shown as numbers above bars are for comparisons between Msm- and Msm-OEAg85B-vaccinated mice. Data are from analysis of 4 mice per group analyzed at each week (*t* test). Significant levels of CD8 T cells secreting IFN γ were not found after vaccination and not shown.

Fig. 2.

Msm-OEAg85B protects mice against tuberculosis. Mice were vaccinated with Msm and Msm-OEAg85B as in Fig. 1. Two weeks after immunization, mice were aerosol challenged with 100 CFU of virulent *M. tuberculosis* H37Rv. Growth of H37Rv after challenge was determined using colony counts of lungs at intervals shown.

Fig. 3.

Msm-OEAg85B induces a stronger CD4 T cell recall response in vivo. Mice were vaccinated and challenged as in Fig. 2. At 1 and after challenge, the lung-derived T cells were purified, stained for CD4 and CD8 phenotype and intracellular IFN γ before analysis using flow cytometry. Data show that only Msm-OEAg85B-vaccinated mice show a strong recall expansion of CD4+IFN γ + T cells.