Effects of DNA- and *Mycobacterium bovis* BCG-Based Delivery of the Flt3 Ligand on Protective Immunity to *Mycobacterium tuberculosis*

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The control of intracellular pathogens such as *Mycobacterium tuberculosis* is dependent on the activation and maintenance of pathogen-reactive T cells. Dendritic cells (DCs) are the major antigen-presenting cells initiating antimycobacterial T-cell responses in vivo. To investigate if immunization strategies that aim to optimize DC function can improve protective immunity against virulent mycobacterial infection, we exploited the ability of the hematopoietic growth factor Fms-like tyrosine kinase 3 ligand (Flt3L) to expand the number of DCs in vivo. A DNA fusion of the genes encoding murine Flt3L and *M. tuberculosis* antigen 85B stimulated enhanced gamma interferon (IFN-γ) release by T cells and provided better protection against virulent *M. tuberculosis* than DNA encoding the single components. Vaccination of mice with a recombinant *Mycobacterium bovis* BCG strain secreting Flt3L (BCG:Flt3L) led to early expansion of DCs compared to immunization with BCG alone, and this effect was associated with increased stimulation of BCG-reactive IFN-γ-secreting T cells. BCG and BCG:Flt3L provided similar protective efficacies against low-dose aerosol *M. tuberculosis*; however, immunization of immunodeficient mice revealed that BCG:Flt3L was markedly less virulent than conventional BCG. These results demonstrate the potential of in vivo targeting of DCs to improve antimycobacterial vaccine efficacy.
munity against aerosol *M. tuberculosis* infection in mice. In addition, secretion of Flt3L by a recombinant form of the BCG vaccine augments immunogenicity and improves the safety of BCG in immunodeficient animals without altering the protective efficacy.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* BCG strain Pasteur were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase or on solid Middlebrook 7H11 medium (Difco Laboratories) supplemented with oleic acid-albumin-dextrose-catalase. When required, the antibiotic kanamycin was added at a concentration of 25 μg/ml.

**Construction of DNA vaccines and BCG strains.** The pCDNA3 vector expressing the *M. tuberculosis* Ag85B gene has been previously described (18). The gene encoding *M. tuberculosis* Ag85B was amplified from *M. tuberculosis* genomic DNA and cloned into the BamHI/HindIII sites of pCDNA/mflt3L (16), yielding pFlt3L, which allows detection of recombinant proteins by a C-terminal epitope tag (37). BCG strains containing pJEX65 alone (BCG-Ct) was used as the control strain for vaccination experiments. Vectors were transformed into BCG as previously described (31). BCG strains were grown as described previously (29), and culture supernatants were concentrated approximately 10-fold using a Nanosep 3K Omega centrifugal column (Full Corporation, Ann Arbor, MI). The total protein concentration was determined using a bichinchoninic acid protein assay kit (Pierce, Rockford, IL). Lysates and supernatants were analyzed by enzyme-linked immunosorbent assay using the anti-myc monoclonal antibody (MAB) 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) or by a murine Flt3L-specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

**Transfection of HEK 293 cells.** Six-well plates were seeded with human embryonic kidney HEK 293 cells. DNA constructs were transfected into HEK 293 cells using FuGene (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. Supernatant was collected after 48 h, and the Flt3L concentration was determined by an ELISA (R&D Systems).

**DC culture and flow cytometry.** DC precursors were derived from murine bone marrow as described previously (9). DC precursors (2 × 10⁵ cells/well) were incubated for 6 days with supernatants from H37Rv-transfected cells or granulocyte-macrophage colony-stimulating factor (2.5 ng/ml; Peprotech, Rocky Hill, NJ) plus IL-4 (5 ng/ml; Peprotech). The expression of cell surface marker proteins was analyzed with a FACScalibur (BD Biosciences, San Jose, CA) using the CellQuest software (BD Biosciences). Phycoerythrin-conjugated major histocompatibility complex class II (MHC-II), allopelycystein-conjugated CD11b, biotin-conjugated CD11c, fluorescein isothiocyanate-conjugated CD80, and streptavidin-PerCP were all purchased from BD Pharmingen (San Diego, CA).

**Animal infections.** For immunogenicity studies, mice were inoculated intramuscularly with plasmid DNA (three inoculations of 100 μg in saline at 2-week intervals) or subcutaneously with BCG strains (5 × 10⁵ CFU in saline), and immune responses were analyzed at the specified time points. IFN-γ release by splenocytes after DNA vaccination was determined by incubating 5 × 10⁵ splenocytes with 3 μg/ml of *M. tuberculosis* Ag85B protein and 72 h after determining the level of IFN-γ released by ELISA. IFN-γ-secreting T cells were detected by incubating 5 × 10⁵ splenocytes or DNL cells with BCG lysate (1 or 10 μg/ml) and performing an enzyme-linked immunosorbent assay as previously described (29).

**RESULTS**

Flt3L-Ag85B fusion protein is secreted by plasmid DNA in a functional form. DNA vaccines were constructed which expressed either mouse soluble Flt3L (pFlt3L), the *M. tuberculosis* Ag85B protein (p85B), or a fusion of the two proteins (pFlt-85) (Fig. 1A). Flt3L could be detected in supernatants from H37Rv 293 cells transfected with pFlt3L or pFlt-85, although the fusion protein was secreted at lower levels than Flt3L alone (Fig. 1B). In order to determine if Flt3L was secreted in a functional form, supernatants from transfected H37Rv 293 cells were cultured with mouse bone marrow cells for 6 days. Cells displaying a DC phenotype (CD11c⁺ MHC-II⁺) were readily generated from bone marrow cultures supplemented with pFlt3L and pFlt3L-85B supernatants (Fig. 1C and D). The same population was not present in cultures supplemented with supernatants from pCDNA3- and p85B-transfected H37Rv 293 cells. Therefore, Flt3L can be delivered as a fusion with Ag85B in a biologically active form.

Delivery of Flt3L-Ag85B by DNA vaccination improves protective efficacy against aerosol *M. tuberculosis* infection. We next determined if fusion of the Flt3L molecule can augment the protective effect of the Ag85B protein. The immunopotentiating effect of Flt3L on antigen-specific immunity was investigated by vaccinating mice with DNA vectors and measuring the release of IFN-γ by Ag85B-reactive splenocytes. Vaccination with the plasmid expressing the antigen alone (p85B) resulted in the release of IFN-γ by splenocytes in response to the Ag85B protein (Fig. 2A). Vaccination with DNA encoding the fusion protein (pFlt-85) significantly enhanced antigen-specific IFN-γ release compared to vaccination with DNA encoding Ag85B alone (Fig. 2A). In order to determine if the improved immunogenicity imparted by the Flt3L-Ag85B fusion translated into enhanced protective efficacy, mice were vaccinated with DNA vectors and challenged 6 weeks after the last injection with aerosol *M. tuberculosis* H37Rv. Delivery of Flt3L alone conferred significant protection against *M. tuberculosis* challenge, and the level was equivalent to the level of protection afforded by p85B. The protective effect of Flt3L and Ag85B was significantly improved by fusion of the two proteins in the form of pFlt-85 (Fig. 2B). Therefore, Flt3L is able to augment the immunogenicity and protective efficacy of DNA vaccines encoding the *M. tuberculosis* Ag85B protein.

Seerion of Flt3L by BCG improves antimycobacterial immu- nity after vaccination. While delivery of Flt3L fused to the Ag85B protein resulted in significant protective efficacy against aerosol *M. tuberculosis* infection, the level did not reach that achieved by delivery of the BCG vaccine (Fig. 2B). We hypothesized that delivery of Flt3L during BCG vaccination may influence the numbers of antigen-presenting cells and increase the protective capacity of the BCG vaccine. To investigate this, an rBCG strain that secreted soluble murine Flt3L (BCG: Flt3L) was developed. Flt3L was readily detected in cell lysates

Survival was calculated using a Kaplan-Meier nonparametric survival plot, and significance was assessed by the Mantel-Cox log rank test.

**Statistical analysis.** Statistical analyses of the results from immunological assays and log-transformed bacterial counts were conducted using analysis of variance (ANOVA). Fisher’s protected least-significant-difference ANOVA post hoc test was used for pairwise comparison of multigroup data sets. A P value of <0.05 was considered significant.

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of BCG:Flt3L by Western blotting with the anti-c-myc MAb 9E10 (Fig. 3A) or in culture supernatants by Flt3L-specific ELISA (Fig. 3B).

To determine if secretion of Flt3L influences the immunogenicity of BCG, mice were vaccinated with BCG:Flt3L or BCG containing the empty vector (BCG:Ct), and at various time points the numbers of IFN-γ-secreting splenocytes or DLN cells produced in response to BCG antigens were determined by flow cytometry.

FIG. 1. Flt3L-Ag85B is expressed by DNA vaccines in a functional form. (A) Schematic representation of pCDNA3 vectors expressing Flt3L, M. tuberculosis Ag85B (p85B), and the Flt3L-Ag85B fusion protein (pFlt-85). (B) Secretion of Flt3L by DNA-transfected HEK 293 cells. Flt3L in the culture medium of cells 3 days after transfection was detected by ELISA. (C) Generation of DCs from bone marrow progenitors using culture medium from DNA-transfected HEK 293 cells. The generation of CD11c+ MHC-II+ cells on day 6 after addition of HEK 293 supernatants to bone marrow cells was determined by flow cytometry. (D) Total number of CD11c+ MHC-II+ cells in culture.

FIG. 2. Immunogenicity and protective efficacy of DNA encoding murine Flt3L and M. tuberculosis Ag85B. (A) Splenocytes from immunized mice were cultured with 3 μg/ml Ag85B, and the level of IFN-γ released was determined by ELISA. (B) For determination of protective efficacy, 4 weeks following the final vaccination mice were infected by the aerosol route with 100 CFU of M. tuberculosis H37Rv. Four weeks after challenge, the bacterial load, expressed as the log10 CFU (mean ± standard error of the mean), was analyzed in the lung. The significance of differences between groups was determined by ANOVA. The error bars indicate standard errors of the means, and the data are representative of two separate experiments.
mined. At day 7 postinfection, mice vaccinated with BCG:Flt3L displayed heightened IFN-γ responses compared with those of BCG:Ct-vaccinated animals, and the difference was significant in the DLNs (Fig. 3C and 3D). The difference was less apparent at day 14, and no difference was observed at day 56 postinfection in either the spleen or the DLNs. Therefore, secretion of Flt3L had an early effect on immunogenicity. To determine if this effect was associated with expansion of DCs at these sites, cells from vaccinated mice were stained for cell surface markers and analyzed by flow cytometry. In the spleen, expression of Flt3L in BCG had no significant effect on the number of CD11c+ MHC-II+ cells at any of the time points examined (Fig. 4A). In the DLNs an increased number of CD11c+ MHC-II+ cells was observed at 7 days after infection with BCG:Flt3L compared to the number of cells after infection with BCG:Ct (Fig. 4B). The increase was apparent in both the CD11c+ MHC-II+ B220− subset (conventional DCs) and CD11c+ MHC-II+ B220− cells (plasmacytoid DCs) (Fig. 4F). No significant difference was detected at later time points (Fig. 4B, D, and F). Therefore, secretion of Flt3L augmented antimycobacterial immunity generated by rBCG early after vaccination, particularly in lymph nodes draining the site of immunization.

Vaccination with BCG secreting Flt3L improves vaccine safety without altering the protective efficacy. In order to determine if secretion of Flt3L by BCG influenced the protective efficacy of the vaccine, mice were vaccinated with BCG:Flt3L and 12 weeks later were challenged with aerosol *M. tuberculosis*. Both rBCG strains provided significant protection against *M. tuberculosis* compared to the results obtained for nonimmunized animals, and this effect was apparent in both the lung (Fig. 5A) and the spleen (Fig. 5B). Secretion of Flt3L did not significantly improve the protective efficacy of BCG, as BCG:Ct and BCG:Flt3L protected mice to similar extents. However, i.v. inoculation of the two strains and monitoring of the bacterial loads in organs indicated that BCG:Flt3L appeared to be more attenuated for in vivo growth than the control BCG (Fig. 5C and 5D). The difference was most apparent in the lung at 14 and 28 days postinfection. This effect was not due to toxicity of Flt3L expressed in BCG, as BCG:Ct and BCG:Flt3L grew equally well in anaxic culture (data not shown). To determine if the increased clearance of BCG:Flt3L in vivo reduced the virulence of the vaccine, immunodeficient RAG-1−/− mice were infected with either BCG:Flt3L or BCG:Ct and survival was monitored. RAG-1−/− mice survived approximately two times longer after infection with BCG:Flt3L than after infection with BCG:Ct (Fig. 6A). Infection of RAG-1−/− mice with the rBCG strains and analysis of early changes in the numbers of DC revealed a trend toward increased numbers of CD11c+ MHC-II+ cells in the DLNs after vaccination with BCG:Flt3L compared to the results obtained with BCG: Ct, which was not observed in the spleen (Fig. 6A and 6B).
Together, these results indicate that secretion of Flt3L improves the safety profile of BCG without altering the protective efficacy against virulent *M. tuberculosis* infection.

**DISCUSSION**

Delivery of molecules during vaccination that target important components of the protective host immune response is a powerful vaccine strategy that has been applied to the control of many infectious diseases (19, 30). DCs play a central role in priming immune responses and are particularly important in the generation of Th1-like responses required to combat intracellular infections (13). We considered the possibility that targeting DCs may be a particularly effective way to improve protective immunity to control mycobacterial infection. In this report we demonstrate that vaccine delivery of Flt3L, a well-characterized DC growth factor, could influence the protective efficacy against virulent *M. tuberculosis* infection in mice. The fusion consisting of Flt3L and the *M. tuberculosis* Ag85B protein expanded DCs in vitro (Fig. 1), induced strong Th1-like T-cell responses in vivo, and displayed significant protective efficacy against aerosol infection with *M. tuberculosis* (Fig. 2). Fusion of Flt3L to protective antigens has also been shown to enhance immunogenicity in models of viral immunity (34) and tumor metastasis (16). It appears that fusion of Flt3L to the antigen is necessary for effective induction of immunity, as codelivery of DNA encoding *M. tuberculosis* HSP65 and Flt3L was not effective against *M. tuberculosis* infection (3), and codelivery of Flt3L and Ag85B did not significantly improve the immunogenicity of Ag85B in our model (data not shown). Interestingly, DNA encoding Flt3L alone afforded protection...
against *M. tuberculosis* infection (Fig. 2). This suggests that Flt3L can induce partial protection against infection, which is further enhanced by addition of a specific *M. tuberculosis* antigen. These results are in accordance with previous studies that demonstrated that pretreatment with Flt3L can induce protection against viral (36, 38), parasitic (7), and bacterial infections (12).

DNA-encoded Flt3L-Ag85B was a less potent inducer of DC differentiation in vitro than pFlt3L, and this may have been due to the lower levels of Flt3L-Ag85B detected in supernatants of DNA-transfected HEK 293 cells (Fig. 1). Reduced expression of the fusion protein compared to that of the single components was also apparent upon detection of Ag85B by Western blotting with anti-Ag85B polyclonal antibody, which demonstrated there were reduced levels of Ag85B in HEK 293 cells transfected with pFlt3L-Ag85B compared with the levels in pAg85B-transfected cells (data not shown). This suggests that sustained delivery of low levels of DNA-encoded Flt3L-Ag85B was sufficient to induce protective immune response, which was superior to that induced by delivery of higher levels of Flt3L or Ag85B alone. It is possible that the delivery of low levels of DNA-encoded Flt3L provides an optimal amount of DC expansion/activation, as it has been reported that high-dose Flt3L pretreatment can impair protective immunity to intracellular infections (1). While this report is in conflict with numerous studies that have demonstrated control of infection by Flt3L-induced DC expansion, it does suggest that the number of DCs must be properly regulated to adequately control infections. With this in mind, it would be particularly interesting to modify expression of the Flt3L-Ag85B fusion protein to determine if the protective effect of the subunit vaccine against TB can be further improved.

BCG has been widely used as a vehicle to deliver protective antigens and immunostimulatory agents to improve protection against TB (28). While the vaccine has been engineered to secrete immunostimulatory agents, such as cytokines (20, 25, 27) and, more recently, chemokines (33), we demonstrate here, for the first time, that the hemopoietic growth factor Flt3L can be expressed and secreted by a bacterial system in a functional form (Fig. 3). We hypothesized that BCG secreting Flt3L would act locally on DC precursors to increase DC numbers and improve antigen presentation and antimycobacterial T-cell responses. We observed an early increase in the number of DCs after BCG:Flt3L vaccination, which correlated with improved induction of IFN-γ-secreting cells, especially in lymph nodes draining the site of infection (Fig. 3). This further high-
intracellular pathogens such as *M. tuberculosis*, and this could be addressed by developing BCG strains that express higher levels of Flt3L and/or by employing vectors that permit a more sustained mode of delivery.

New candidate TB vaccines have recently entered phase I clinical trials (35), and it is hoped that a new effective vaccine can be available for use within the next 10 years (40). Any new live vaccine to replace BCG would need to maintain the significant effect that the BCG vaccine has on preventing miliary tuberculosis in humans; however, the vaccine was less virulent in immunodeficient mice (Fig. 6). The reduced virulence correlated with the attenuated phenotype of BCG:Flt3L in the lungs of immunocompetent mice and a trend toward increased DC numbers in the DLNs of RAG-1−/− mice immunized with BCG:Flt3L (Fig. 6). This is in accordance with the capacity of Flt3L to expand DCs and enhance immunity in RAG-1−/− mice (7, 38). These results suggest that manipulating components of host immunity may be a suitable strategy to improve the effectiveness of the BCG vaccine and to deliver new candidates to control TB in humans.

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