Improved Protection against Disseminated Tuberculosis by *Mycobacterium bovis* Bacillus Calmette-Guérin Secreting Murine GM-CSF Is Associated with Expansion and Activation of APCs

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**References**

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Improved Protection against Disseminated Tuberculosis by 

*Mycobacterium bovis* Bacillus Calmette-Guérin Secreting Murine GM-CSF Is Associated with Expansion and Activation of APCs

Anthony A. Ryan,* Teresa M. Wozniak,* Elena Shklovskaya,* Michael A. O’Donnell,§ Barbara Fazekas de St. Groth,* Warwick J. Britton,* and James A. Triccas2*,‡

Modulating the host-immune response by the use of recombinant vaccines is a potential strategy to improve protection against microbial pathogens. In this study, we sought to determine whether secretion of murine GM-CSF by the bacillus Calmette-Guérin (BCG) vaccine influenced protective immunity against *Mycobacterium tuberculosis*. BCG-derived GM-CSF stimulated the in vitro generation of functional APCs from murine bone marrow precursors, as demonstrated by the infection-induced secretion of IL-12 by differentiated APCs, and the ability of these cells to present Ag to mycobacterium-specific T cells. Mice vaccinated with BCG-secreting murine GM-CSF (BCG:GM-CSF) showed increased numbers of CD11c+MHCII+ and CD11c−CD11b+F4/80+ cells compared with those vaccinated with control BCG, and this effect was most apparent in the draining lymph nodes at 7 and 14 days postvaccination. Vaccination with BCG:GM-CSF also resulted in enhanced expression of costimulatory molecules on migratory dendritic cells in the draining lymph nodes. The increased APC number was associated with an increase in the frequency of anti-mycobacterial IFN-γ-secreting T cells generated after BCG:GM-CSF vaccination compared with vaccination with control BCG, and this effect was sustained up to 17 wk in the spleens of immunized mice. Vaccination with BCG:GM-CSF resulted in an ~10-fold increase in protection against disseminated *M. tuberculosis* infection compared with control BCG. This study demonstrates the potential of BCG-secreting immunostimulatory molecules as vaccines to protect against tuberculosis and suggests BCG:GM-CSF merits further appraisal as a candidate to control *M. tuberculosis* infection in humans. The Journal of Immunology, 2007, 179: 8418–8424.

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nti-mycobacterial immunity is established in the lymph nodes that drain the site of infection, triggered by the presentation of mycobacterial Ags via the MHC class I and II pathways (1). Presentation of mycobacterial peptides by professional APCs to T cells is responsible for bridging the gap between innate and acquired immunity (2, 3). The recruitment of Ag-specific IFN-γ-secreting CD4+ T cells to the site of infection is a critical component of immunity to tuberculosis, as it leads to the release of soluble factors such as IFN-γ, activating infected macrophages and inhibiting the replication of *M. tuberculosis* (4). Dendritic cells (DCs) are recognized as the most potent stimulators of naive T cells, as they are specialized for Ag uptake in the periphery, upon which they migrate via afferent lymphatics to the draining lymph nodes (DLNs) (5). During this process, DCs up-regulate chemokine receptors and costimulatory molecules that are required for migration and effective stimulation of T cells (1, 6). Mature DCs are defined by a high surface expression of MHC class II in conjunction with the expression of the integrin-αx chain, CD11c, and high surface expression of costimulatory molecules (7). Jiao et al. (8) showed that following high dose i.v. infection of mice with bacillus Calmette-Guérin (BCG), DCs are the most potent subset of cells at presenting Ag to splenic T cells. DCs rapidly distribute into the T cell zone after BCG infection, produce IL-12p40, and up-regulate costimulatory molecules including CD40, CD80, and CD86 (8). Depletion of CD11c+ cells in vivo delays the onset of an Ag-specific T cell response to *M. tuberculosis* and impairs the control of the infection (9). Similarly, depletion of activated macrophages resulted in impaired resistance of mice to pulmonary *M. tuberculosis* infection (10). Therefore, modulating APC function may prove an effective vaccine strategy to control infection with *M. tuberculosis*.

The cytokine GM-CSF plays a pleiotropic role in the generation, proliferation, and differentiation of hemopoietic progenitor cells toward cells of myeloid lineage such as monocytes/macrophages, DCs, neutrophils, and eosinophils (11). GM-CSF also acts locally as a proinflammatory cytokine, both in recruitment of leukocytes and the enhancement of APC function (12). GM-CSF can induce up-regulation of MHC class II and costimulatory molecules such as CD80 and CD86 on APC as well as increasing their phagocytic activity and stimulatory capacity (13–17). Coinmunization with or over-expression of GM-CSF enhances Ag-specific IFN-γ-secreting T cells and increases the protection against a variety of infectious agents (18–21).
GM-CSF-deficient APCs are poor inducers of T cell responses, suggesting that the cytokine is required for optimal generation of T cell immunity (22, 23).

Recombinant BCG strains can be engineered to express immunomodulatory molecules such as cytokines and chemokines in a functional form (24–27). This study aimed to modulate APC function by delivery of GM-CSF via the BCG vaccine. BCG-derived GM-CSF induced in vitro differentiation of APCs with a potent capacity to stimulate naive T cells. In vivo administration of the cytokine-producing strain led to an increase in APC numbers in lymph nodes that drain the site of immunization with elevated immunostimulatory function. BCG-expressing GM-CSF induced an early increase in IFN-γ production, which was maintained up to 17 wk postimmunization. The heightened immune response after BCG-secreting murine GM-CSF (BCG:GM-CSF) immunization translated to increased protection against disseminated infection when mice were challenged with aerosol M. tuberculosis.

Materials and Methods

Bacterial strains and growth conditions

M. tuberculosis H37Rv (ATCC 27294) was grown in Proskauer and Beck liquid medium (BD) for 14 days at 37°C. Recombinant BCG strains were grown in Middlebrook 7H9 broth with 10% albumin-dextrose-catalase (ADC) enrichment (Difco Laboratories). When required, the antibiotics kanamycin (25 µg/ml) and hygromycin B (50 µg/ml) were added to liquid and/or solid medium for rBCG cultures. Mycobacteria were enumerated on Middlebrook 7H11 agar supplemented with 10% oleic acid-ADC enrichment (Difco Laboratories).

BCG:GM-CSF was a gift of Professor Rick Young, Whitehead Institute (Cambridge, MA) (25). Control of the construction BCG strain (BCG-Ct), harboring the pMV261 plasmid, has been previously described (27). To construct BCG expressing the OVA protein, residues 230–259 of OVA (28), resulting in pJEX84. A 1.1 kb fragment containing the Mycobacterium bovis HSP60 promoter, the M. tuberculosis Ag85B signal sequence, OVA230–258, and e-myc epitope tag was excised from pJEX84 and inserted into the mycobacterial integrative vector pNIP-40B (provided by Dr. Nathalie Winter, Pasteur Institute, Paris, France) to yield pJEX87. Plasmid JEX87 was transformed into BCG:Ct and BCG:GM-CSF as previously described (29).

Animals

Female C57BL/6 and male B6.SJL/PtpcA mice aged 6–8 wk were purchased from the Animal Research Centre. OT-II transgenic mice were bred (8, 9), coupled with the well-established ability of GM-CSF to influence the differentiation and activation of APC subsets, suggested GM-CSF may serve to improve the efficacy of anti-Mycobacterium vaccines. To explore this hypothesis, we made use of BCG:GM-CSF (25). We first determined whether GM-CSF secreted by BCG was able to influence the differentiation of APCs in established in vitro systems. Secretion of GM-CSF was only detected in supernatant samples and standards added, and anti-IL-12p40/biotin (R&D Systems), anti-IFN-γ/biotin (XMG1.2), or anti-GM-CSF (R&D Systems) were added followed by streptavidin-HRP. Substrate solution was added and absorbance measured at a dual wavelength of 405/492 nm.

Cytokine assays

Ag-specific IFN-γ-secreting cells were measured by ELISPOT as previously described (29). For cytokine ELISA, plates were coated with anti-IL-12p40, anti-IFN-γ (AN18), or anti-GM-CSF Abs (R&D Systems), supernatant samples and standards added, and anti-IL-12p40/biotin (R&D Systems), anti-IFN-γ/biotin (XMG1.2), or anti-GM-CSF (R&D Systems) were added followed by streptavidin-HRP. Substrate solution was added and absorbance measured at a dual wavelength of 405/492 nm.

CFSE labeling and adoptive transfer

Lymph node cells from donor OT-II mice (Ly5.2+2) were labeled with 5 µM CFSE as previously described (31). Approximately 5 × 10^6 cells were injected i.v. into B6.SJL/PtpcA (Ly5.11) host mice and 1 day post-transfer, mice were infected s.c. with 1 × 10^6 CFU BCG:Ct-OVA or BCG:GM-CSF-OVA. On day 3 or 7 postvaccination, the activation of transgenic T cells in the DLNs, spleen, and NDLNs were analyzed by flow cytometry. Cells were stained with Abs for the following markers using appropriate fluorochromes and concentrations (BD Pharmingen): CD4, CD44, CD62L, CD45.1, and CD45.2. Before acquisition of the samples, 4′,6-diamidino-2-phenylindole dihydrochloride was added to exclude dead cells. The CFSE profile of dividing cells was analyzed as described (32, 33).

Statistical analysis

Statistical analysis of the results from immunological and log-transformed bacterial counts were conducted using ANOVA. Fisher’s protected least significant difference ANOVA post hoc test was used for pair-wise comparison of multi-grouped data sets. Differences with p < 0.05 were considered significant.

Results

Biologically active GM-CSF secreted by BCG differentiates APC in vitro

The central role of APCs in the control of mycobacterial infection (8, 9), coupled with the well-established ability of GM-CSF to influence the differentiation and activation of APC subsets, suggested GM-CSF may serve to improve the efficacy of anti-Mycobacterium vaccines. To explore this hypothesis, we made use of BCG:GM-CSF (25). We first determined whether GM-CSF secreted by BCG was able to influence the differentiation of APCs in established in vitro systems. Secretion of GM-CSF was only detected in the culture supernatant of BCG:GM-CSF and not with BCG:Ct (Fig. 1A). Culture of bone marrow progenitor cells with supernatants from BCG:GM-CSF for 5 days resulted in the generation of CD11c+CD11b+ cells, which were observed at low numbers in cultures supplemented with BCG:Ct supernatants or medium alone (Fig. 1B). APCs differentiated with BCG-derived GM-CSF were potent producers of IL-12p40 after BCG infection, whereas no IL-12 was detected in infected cells generated by BCG:Ct supernatants (Fig. 1C). In addition, progenitor cells infected with BCG were capable of presenting Ag to an M. tuberculosis Ag85B p25.240–258-specific CD4+ T cell hybridoma (T. M. Wozniak and W. J. Britton, manuscript in preparation) as
Biologically active GM-CSF produced by BCG leads to the differentiation of bone marrow progenitors. The production of GM-CSF from supernatants of 2-wk broth cultures was determined by ELISA (A). Concentrated supernatants from BCG:GM-CSF or BCG:Ct cultures were added to bone marrow progenitor cells and the generation of CD11c+CD11b+ cells examined (B). The release of IL-12 by CD11c+CD11b+ cells generated by BCG-derived GM-CSF was determined by ELISA (C). The ability of the same cells to present the p25 peptide from the M. tuberculosis Ag85B protein to a p25-specific CD4+ T cell hybridoma was assessed by the release of IL-2 after 72 h of coculture (D). The significances of differences between the groups were analyzed by ANOVA (**, p < 0.05). Data are the mean ± SEM for triplicate wells and are representative of two independent experiments. ND; Not detected.

FIGURE 1. Biologically active GM-CSF produced by BCG leads to the differentiation of bone marrow progenitors. The production of GM-CSF from supernatants of 2-wk broth cultures was determined by ELISA (A). Concentrated supernatants from BCG:GM-CSF or BCG:Ct cultures were added to bone marrow progenitor cells and the generation of CD11c+CD11b+ cells examined (B). The release of IL-12 by CD11c+CD11b+ cells generated by BCG-derived GM-CSF was determined by ELISA (C). The ability of the same cells to present the p25 peptide from the M. tuberculosis Ag85B protein to a p25-specific CD4+ T cell hybridoma was assessed by the release of IL-2 after 72 h of coculture (D). The significances of differences between the groups were analyzed by ANOVA (**, p < 0.05). Data are the mean ± SEM for triplicate wells and are representative of two independent experiments. ND; Not detected.

Expression of GM-CSF by BCG increases APC numbers in the DLNs following immunization

As GM-CSF secreted by BCG was able to promote the in vitro differentiation of APCs, we next determined whether BCG:GM-CSF influenced the recruitment and/or differentiation of APCs following vaccination. After s.c immunization of mice with rBCG strains, the number of CD11c+MHCII+ cells in the DLN increased over the course of BCG infection when compared with naive control mice (Fig. 2A). Further, the influence of GM-CSF secreted by BCG led to an increase in CD11c+MHCII+ cells compared with mice vaccinated with the BCG:Ct, with the most significant increase occurring at day 7 postinfection. Examination of DC numbers in the spleens and NDLNs of mice vaccinated with either rBCG strain revealed no significant increase in DC numbers when compared with naive mice (Fig. 2, B and C).

We also observed changes in the number of cells with a macrophage phenotype (CD11c+CD11b+F480+) after vaccination with BCG:GM-CSF. At both 7 and 14 days postvaccination, significantly greater CD11c+CD11b+F480+ cells were present in the DLN compared with mice immunized with the BCG control strain (Fig. 2D). This increase was less apparent in the spleen and NDLN (Figs. 2, E and F). Changes in cell number after BCG:GM-CSF were not observed in other cells of the innate immune response, as we observed no significant difference in the number of NK1.1+ cells after vaccination with BCG:GM-CSF or BCG:Ct (data not shown). These data indicate that GM-CSF secreted by BCG increases APC numbers at sites draining the site of immunization but not at distant sites.

Influence of GM-CSF secretion by BCG on the activation state of DCs

To determine whether secretion of GM-CSF by BCG could influence the early activation of DCs in the DLN, expression of costimulatory molecules was analyzed on DCs. The two major DC subsets detected in murine lymph nodes were CD11c+MHCII+ (Fig. 3, A and C) and CD11c+MHCII+ populations (Fig. 3, B and E). The CD11c+MHCII+ DC subset is tissue-derived with DCs migrating into the lymph node from the dermis and epidermis (35). Changes in the expression of the costimulatory molecules CD80 and CD86 on these DC populations were analyzed at day 3 postvaccination. Migratory DCs had a greater expression of both CD80 (Fig. 3C) and CD86 (Fig. 3E) in the DLN upon immunization with BCG compared with naive controls, which was further increased in mice immunized with BCG:GM-CSF. No significant increase in CD80 and CD86 was observed in this subset in NDLN from mice immunized with either rBCG strain (Fig. 3, D and F). Therefore GM-CSF altered the local activation of DCs after vaccination with rBCG.

Expression of GM-CSF by BCG leads to enhanced activation of Ag-specific T cells in vivo

The increased capacity of BCG:GM-CSF to influence DC numbers and activation state may influence the resulting T cell response after vaccination. To examine this, we developed rBCG strains that expressed both GM-CSF and a truncated portion of the OVA protein. The expression of the OVA gene by rBCG was confirmed by Western blotting of BCG:GM-CSF-OVA and BCG:Ct-OVA cell lysates and revealed similar expression levels of the truncated OVA protein (Fig. 4A). This allowed direct in vivo comparison of the immunogenicity of the strains.

FIGURE 2. Immunization with GM-CSF-secreting BCG leads to increased APC numbers in the DLNs. C57BL/6 mice were immunized by s.c. injection with 1 × 106 CFU of rBCG strains, and at 7, 14, and 56 days postimmunization, mice were sacrificed and the number of CD11c+MHCII+ (A–C) or CD11c+CD11b+F480+ (D–F) cells in the DLNs (A and D), spleen (B and E), or NDLNs (C and F) determined by flow cytometry. Data are the mean ± SEM and are representative of one of two independent experiments. (●), BCG:GM-CSF; (□), BCG:Ct; (○), unvaccinated. The significances of differences were determined by ANOVA between BCG:Ct and BCG:GM-CSF (*, p < 0.05) and between BCG:GM-CSF and naive controls (†, p < 0.01).
The frequency of Ag-specific IFN-γ-secreting CD4+ T cells in the DLNs. This difference correlated with an increase in the proportion of divided cells with a CD62Llow phenotype, suggesting the enhanced generation of effector memory T cells (Fig. 4C). Therefore the secretion of GM-CSF improved the capacity of BCG to control M. tuberculosis growth at a site of bacterial dissemination but not the primary site of infection.

**Discussion**

A variety of approaches have been used to influence immune responses to vaccines (36). In this study, a rBCG strain secreting murine GM-CSF was used to determine whether modulating APC function would enhance the vaccine efficacy of BCG. GM-CSF was secreted by BCG in a functional form with the capacity to stimulate the generation of APCs from bone marrow progenitors in vitro. These APCs were capable of secreting IL-12p40 and activating naive CD4+ T cells (Fig. 1). This effect was also observed in vivo with BCG:GM-CSF stimulating early increases in the number of macrophages and DCs in lymph nodes draining the site of vaccination (Fig. 2). Interestingly, a similar increase in macrophage-like cells (F4/80+CD11b+) in the peritoneal exudate was observed by Burger et al. (14) following i.p. injection of an adenovirus encoding murine GM-CSF. Secretion of GM-CSF by BCG did not have a selective effect on any particular DC subpopulation defined by the expression of CD8, CD11b, and B220 (not shown). This parallels observations made by Vremec et al. (37) whereby over-expression of GM-CSF in transgenic mice increased the total number of DCs; however, the cytokine had no effect on the proportions of DC subtypes. Other studies, however, have demonstrated a preferential expansion of CD11bhiCD8α+ DC subsets

**Expression of GM-CSF by BCG increases the protective effect of the vaccine against disseminated M. tuberculosis infection**

To determine whether the influence of BCG-derived GM-CSF on APC number, DC activation, and T cell activation translated into increased protective efficacy, C57BL/6 mice were immunized with rBCG strains and aerosol challenged with M. tuberculosis. BCG:GM-CSF afforded ~1-log reduction in M. tuberculosis load in the lungs compared with unvaccinated mice (p < 0.01) (Fig. 5C). The level of protection afforded by BCG:GM-CSF was consistently greater than that following control BCG, however this difference did not reach statistical significance (Fig. 5C). Control BCG reduced the bacterial load in the spleen by ~1.5 log compared with unvaccinated mice (p < 0.01) (Fig. 5D). BCG:GM-CSF further increased protection in the spleen by ~1 log compared with control BCG, and this difference was statistically significant (p < 0.05) (Fig. 5D). Therefore the secretion of GM-CSF improved the capacity of BCG to control M. tuberculosis growth at a site of bacterial dissemination but not the primary site of infection.
following adenoviral production of GM-CSF or delivery of polyethylene glycol-modified GM-CSF (15, 17). The reasons for these differences are unclear but may reflect differences in the level of cytokine produced by vaccine vectors, and the mode of delivery and localization of GM-CSF-secreting constructs. Interestingly, the concentration of GM-CSF detected in culture supernatants of rBCG:GM-CSF is comparable to GM-CSF levels produced by murine or human cells infected with BCG (26, 38), and human PBMCs infected with BCG produce GM-CSF at relatively late time-points postinfection (38). Therefore the capacity of BCG:GM-CSF to deliver the cytokine early after vaccination may account for its potent effect on immunogenicity. We also observed increased activation of DCs in the DLN after BCG:GM-CSF activation compared with DCs obtained from unvaccinated or BCG:Ct vaccinated mice (Fig. 3). This further supports the potential of BCG-derived murine mGM-CSF to influence anti-mycobacterial immunity and is consistent with previous studies which demonstrated that delivery of GM-CSF to mice or humans by viral vectors or tumor cells increased the activation of DCs (17, 39, 40).

BCG:GM-CSF was able to significantly increase antimycobacterial T cell responses even though the recombinant strain secreted relatively low levels of murine GM-CSF (Fig. 1). In addition, we observed no gross pathological changes in mice vaccinated with BCG:GM-CSF compared with those vaccinated with control BCG (data not shown). By contrast, transgenic mice over-expressing GM-CSF demonstrated enlarged and histologically abnormal livers and spleens as well as increased number of macrophages and granulocytes in the peripheral blood (41). Delivery of polyethylene glycol-conjugated GM-CSF to mice impaired resistance against M. tuberculosis infection (42), and transgenic mice over-expressing GM-CSF were unable to control infection with M. tuberculosis at extended time-points postchallenge because of an inability of these mice to develop a normal granulomatous response (43). In humans, however, the use of tumor cells engineered to over-express GM-CSF have extensively been tested in clinical trials, and for the most part the regime is well tolerated (44). Further, recombinant human GM-CSF has been tested in combination with rifampin/isoniazid to treat patients with active tuberculosis with no adverse effects (45). Thus the capacity to deliver transiently low levels of immunostimulatory cytokines via BCG may be advantageous in invoking protective immune responses without negatively influencing pathology.

Secretion of GM-CSF by BCG had a potent effect on T cell immunity with early expansion of T cells after vaccination (Fig. 4), enhanced and sustained priming of anti-mycobacterial IFN-γ-secreting T cells (Fig. 5), and improved protection against disseminated M. tuberculosis after aerosol challenge (Fig. 5). Therefore the effects of rBCG-derived GM-CSF on APC function translated into the generation of protective T cell immunity. The increase in early activation of T cell responses afforded by BCG:GM-CSF may also relate to the capacity of the cytokine to stimulate neutrophil phagocytosis, and neutrophils have been implicated in the transfer of BCG from the site of vaccination to the DLNs for presentation by professional APCs (46, 47). Other cytokines are secreted by rBCG in a functional form and also increase antimycobacterial T cell immunity after vaccination of mice (24–26).
against disseminated *M. tuberculosis* infection. Murine and human GM-CSF display limited biological cross-reactivity (49), which suggests modification of the recombinant strain would be required for use in humans. However the potent activity of human GM-CSF on APC function (44), coupled with the favorable safety profile of studies employing human GM-CSF in clinical trials (44, 45), suggests further study of this vaccine as a candidate to protect against tuberculosis in human populations is warranted.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


