Role for Gr-1+ Cells in the Control of High-Dose Mycobacterium bovis Recombinant BCG

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*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is an attractive target for development as a live vaccine vector delivering transgenic antigens from HIV and other pathogens. Most studies aimed at defining the clearance of BCG have been performed at doses between $10^2$ and $10^5$ CFU. Interestingly, however, recombinant BCG (rBCG) administered at doses of $>10^6$ CFU effectively generates antigen-specific T-cell responses and primes for heterologous boost responses. Thus, defining clearance at high doses might aid in the optimization of rBCG as a vector. In this study, we used bioluminescence imaging to examine the kinetics of rBCG transgene expression and clearance in mice immunized with $5 \times 10^7$ CFU rBCG expressing luciferase. Similar to studies using low-dose rBCG, our results demonstrate that the adaptive immune response is necessary for long-term control of rBCG beginning 9 days after immunizing mice. However, in contrast to these reports, we observed that the majority of mycobacterial antigen was eliminated prior to day 9. By examining knockout and antibody-mediated depletion mouse models, we demonstrate that the rapid clearance of rBCG occurs in the first 24 h and is mediated by Gr-1+ cells. As Gr-1+ granulocytes have been described as having no impact on BCG clearance at low doses, our results reveal an unappreciated role for Gr-1+ neutrophils and inflammatory monocytes in the clearance of high-dose rBCG. This work demonstrates the potential of applying bioluminescence imaging to rBCG in order to gain an understanding of the immune response and increase the efficacy of rBCG as a vaccine vector.

*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is an attenuated mycobacterial vaccine administered to newborns for the prevention of childhood miliary tuberculosis. BCG possesses several attributes that make it a highly favorable candidate for development as a recombinant vaccine vector. These include its ability (i) to express transgenic antigens from pathogens, such as HIV, (ii) to induce strong T-cell responses associated with the release of gamma interferon (IFN-γ) and other Th1 cytokines, and (iii) to generate T-cell responses specific for transgenic antigens, which can ultimately be increased by heterologous boost vaccination (1–8). The aforementioned responses are generated with high doses of recombinant BCG (rBCG). Specifically, rBCG administered to mice at a dose equal to $10^6$ CFU rapidly induces the development of transgene product-specific T cells, a response not observed using lower doses of $10^4$ to $10^5$ CFU (9–11). Furthermore, rBCG doses of $>10^7$ CFU induce detectable primary T-cell responses directed against the foreign transgenic epitope in rhesus macaque studies, responses that are not observed at lower doses (5, 12).

Despite the fact that transgene-product specific T cells are generated in response to high doses of rBCG, very few experiments have been performed to determine the clearance of high-dose BCG. Instead, experiments determining the clearance of BCG have been done using lower doses of the vaccine, primarily in an effort to model the pathogenic transmission of *Mycobacterium tuberculosis* and *M. bovis*. In these studies, the BCG burdens in the spleen and liver increased 10-fold from the time of inoculation until day 21, at which point a noticeable decline occurred in the number of colonies recovered (3, 13–16). In addition, the innate immune response has been observed to play a minimal role in the early clearance of BCG in these studies, and the clearance that does occur after day 21 coincides with the generation of CD4+ and CD8+ T-cell responses capable of producing IFN-γ and directly lysing infected cells. The lack of innate clearance is most apparent in that neutrophils, which are known to control the growth of fast-growing species of bacteria, are ineffective at clearing mycobacteria in this immunization model despite being recruited to the site of inoculation and phagocytosing BCG (3, 15, 17).

We rationalized that the kinetics of BCG clearance may be different when administered at a high dose compared to a low dose, and because antigen dose and clearance critically impact the development of T-cell responses, knowledge of these factors might aid in optimization of the dosing, delivery, and design of mycobacterial vaccine vectors (18, 19). In this study, we sought to quantify the clearance of high-dose rBCG *in vivo* using bioluminescence imaging, a technique that has only recently been applied to BCG (20). To this end, we created a recombinant strain of BCG expressing luciferase and analyzed transgene expression and stability *in vivo* following the inoculation of mice with $5 \times 10^7$ CFU. We observed a rapid decrease in whole-body luminescence that was delineated into two phases, rBCG clearance and control, when analyzed using knockout mice and antibody-mediated depletion models. We found that long-term control was absent in recombination activating gene 1-deficient (RAG−/−) mice, consistent with previous work demonstrating that CD4+ and CD8+ T cells are critical for the long-term control of BCG. Most importantly, however, an initial phase of rapid clearance in the 14 days following inoculation was apparent, in which the majority of rBCG was...
eliminated. This clearance was noticeably absent in the first 24 h after inoculation in mice depleted of cells carrying the granulocyte differentiation antigen 1 (Gr-1) marker, indicating a critical role for Gr-1− neutrophils and inflammatory monocytes. By defining a role for Gr-1− cells in the early clearance of high-dose rBCG, our work reveals a previously unappreciated contribution of neutrophils and inflammatory monocytes to vaccine-related mycobacterial clearance and underscores the need for a better understanding of the immune response to recombinant mycobacteria in order to enhance their efficacy as vaccine vectors.

**MATERIALS AND METHODS**

**Creation of recombinant Mycobacterium smegmatis-Luc and rBCG-Luc.** The firefly (Photinus pyralis) luciferase gene luc2 was cloned by PCR from plasmid pGL4 (Promega), digested with NdeI and PstI, and ligated into the mycobacterial expression plasmid pH222 under the control of the α-antigen promoter to make pMYB1, and correct cloning was confirmed by sequencing. Plasmid pMYB1 contains the kanamycin resistance gene aph. pMYB1 was amplified in Escherichia coli strain DH5α and transformed into M. smegmatis strain mc²155 and BCG strain Pasteur (courtesy of William Jacobs, Jr., Albert Einstein College of Medicine).

**In vitro luminescence assessment.** rBCG-Luc cultures were grown to an optical density at 600 nm (OD₆₀₀) of 1. Two milliliters of culture, normalized by OD₆₀₀, was pelleted at 3,000 rpm, lysed in Bright-Glo lysis buffer with luciferin substrate, and assayed on a luminometer.

**Bioluminescence imaging.** Mice anesthetized with ketamine-xylazine were injected with 100 µl of an isotonic salt solution containing 33 mg/ml α-luciferin (Xenogen). Twelve minutes later, *in vivo* imaging was performed for 60 s using an IVIS-110 imaging system (Xenogen). The luminescence intensity images were overlaid on gray-scale images of the mice. The Living Image software was then used to determine luminescence in a constant-size region of interest (ROI) for all mice. Some mice, despite receiving rBCG-Luc, did not demonstrate any luminescence at the initial 15-min time point and were excluded from the analysis.

**Ex vivo colony assessment.** Mice inoculated with 5 × 10⁷ CFU of rBCG-Luc were sacrificed at 2, 4, 6, and 8 weeks. Inoculum was plated at serial dilutions to determine the CFU dose. The spleens were isolated, homogenized, and plated at serial dilutions on 7H10 plates with kanamycin (20 µg/ml) or without antibiotic. pMYB1 retention was calculated by comparing the ratio of colonies growing on plates with kanamycin to colonies growing on plates without antibiotic.

**Mice.** Sex- and age-matched adult recombination activation gene 1-deficient (RAG−/−) (B6;129S7-Rag1tm1Mom/J), beige mice (C57BL/6-Lyst-bb/J), and C57BL/6 mice were obtained from The Jackson Laboratory. BALB/c mice were obtained from Charles River Laboratories. The mice were housed in the Harvard Institute of Medicine Animal Research Facility; all mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committees (IACUC 04119) of Beth Israel Deaconess Medical Center (BIDMC), Harvard Institutes of Medicine, and Harvard Medical School.

**Antibody depletion.** Depletion was done by injecting 1,600 µg anti-asialo GM1 antibody (NK cells), 250 µg RB6-8C5 (Gr-1− cells), or equivalent amounts of isotype control antibody 2 days prior to BCG-Luc inoculation. A complete blood count (CBC) was used on the day of rBCG inoculation to assess NK depletion from peripheral blood following anti-asialo GM1 treatment and neutrophil depletion following RB6-8C5 treatment. Depletion using anti-asialo GM1 was 72.4% effective, and depletion using RB6-8C5 was 74.2% effective, when assessed by CBC.

**Statistical analysis.** The data are expressed as the mean ± standard error of the mean (SEM). Statistical tests were performed at each time point using Prism 5, Student’s t test for experiments comparing two cohorts of mice, and a one-way analysis of variance (ANOVA) with a Tukey multiple comparison posttest for experiments comparing three cohorts of mice. A P value of <0.05 was considered significant.

**RESULTS**

*In vivo* bioluminescence imaging can be used to detect transgene expression following recombinant mycobacterial inoculation. To assess transgene production *in vivo*, we created recombinant *M. smegmatis* mc²155 and rBCG strains expressing luciferase (recombinant *M. smegmatis*-Luc and rBCG-Luc) that could be used for *in vivo* bioluminescence imaging. Initial optimization of the IVIS-110 machine and Living Image software was performed using mice that received 1 × 10⁶ CFU recombinant *M. smegmatis*-Luc intraperitoneally (i.p.) (Fig. 1A). These mice were then imaged using the IVIS-110 charge-coupled device (CCD) for 60 s to generate luminescence intensity images overlaid on gray-scale images. The mice inoculated with 10⁶ CFU of recombinant *M. smegmatis*-Luc showed intense luminescence that had disseminated from the point of injection. In a mouse inoculated with recombinant *M. smegmatis*-Luc and assessed at 4 h, 5 × 10⁷ relative light units (RLU) was detected in the region of interest (ROI). A background level of 5 × 10⁵ RLU was detected from a mouse inoculated with an equal dose of *M. smegmatis* not expressing luciferase. Thus, luminescence from mycobacterium-encoded luciferase can be detected in mice and used to quantify transgene expression *in vivo*.

Luminescence decreases rapidly following high-dose rBCG-Luc inoculation. We then sought to use this system to assess the *in vivo* clearance of luciferase produced by rBCG. *In vivo* luminescence was assessed in C57BL/6 mice inoculated with 5 × 10⁷ CFU rBCG-Luc (Fig. 1B). Thirty minutes after inoculation, a mean luminescence level of 3.51 × 10⁷ RLU was detected from each mouse. This level of luminescence decreased 84% by day three to a mean luminescence of 5.5 × 10⁶ RLU. By day seven postinoculation, luminescence decreased 96.3% to a mean value of 1.3 × 10⁶ RLU. Luminescence further decreased to levels near background over the 8 weeks that these mice were monitored (Fig. 1C).

The significant decrease in luminescence apparent in the first week following inoculation was inconsistent with previously published low-dose models, in which burdens of slow-growing mycobacteria increased 10-fold (9, 21). Two possible explanations for the decrease in luminescence included a loss of bacterial transgene expression and the clearance of rBCG by the immune system.

Decreased luminescence is due to mycobacterial clearance and not to plasmid loss or transgene mutation. To determine whether the loss of luminescence was a consequence of bacterial clearance by the immune system or a consequence of the loss of luciferase expression by rBCG-Luc, we performed *ex vivo* splenic colony counts at 2-week intervals following high-dose BCG vaccination and assessed both CFU and transgene expression from the CFU. Homogenized spleens were plated at serial dilutions on plates with or without kanamycin (Fig. 2A). Two weeks after inoculation, 2.6% of the input bacteria were recovered from the spleen (1.3 × 10⁶ CFU/spleen on plates without drug compared to an input of 5 × 10⁷ CFU), while during this same time period, luminescence decreased to 2.59% of that at the 30-min reading (3.51 × 10⁷ RLU at 30 min compared to 9.08 × 10⁶ RLU at 14 days) in a separate cohort of mice. At 4 weeks postinoculation, splenic CFU had further decreased to 0.48%, and luminescence had further decreased to 1.2%. By 6 weeks, splenic CFU was 0.1% of the input inoculation and luminescence was 0.8%. Thus, in performing *ex vivo* CFU counts, we obtained results that sup-
ported the hypothesis that reduced luminescence levels were a result of rBCG being cleared.

To examine whether the loss of the luciferase expression plasmid from rBCG was a contributing factor to the decreased luminescence, we determined the plasmid retention ratio of ex vivo colonies, calculated as the ratio of colonies growing on kanamycin-containing plates to colonies growing on plates without antibiotic (Fig. 2B). The colony counts on plates containing kanamycin versus those on plates with no drug differed by <4% for the input bacteria as well as for the bacteria at 2 weeks, suggesting that at both of these time points, plasmid loss was not affecting in vivo bioluminescence imaging. Compared to the retention at 2 weeks, we observed decreased retention at later points, with colony counts at 4, 6, and 8 weeks indicating that between 20 and 40% of the mycobacteria had lost the plasmid.

The expression of a transgenic protein may exert a negative selective pressure on bacteria, driving selection for null mutations of the luciferase gene within the host. We assessed 15 to 18 colonies isolated from each of three mice 8 weeks after inoculation to determine whether the mycobacteria containing plasmid retained the ability to express luciferase. We observed that all isolated ex vivo colonies tested were able to express luciferase in an in vitro setting (Fig. 2C). Therefore, mutation of the transgene that would have led to the loss of transgene expression was not a driving force behind the decreased luminescence observed in bioluminescence imaging.

Bacterial clearance before day 9 occurs independently of the adaptive immune system, but clearance after day 9 requires the adaptive immune system. Traditional RAG-dependent adaptive immune responses, including CD4+ and CD8+ T-cell responses, require weeks to develop (22). However, there exist RAG-dependent innate/adaptive cell types, such as γδ T cells, which demonstrate the ability to respond immediately to mycobacteria and produce IFN-γ (23, 24). To assess the contribution of the adaptive immune response to rBCG clearance, we examined wild-type and RAG-1-deficient (RAG−/−) mice inoculated with rBCG-Luc. The clearance of luminescence was not significantly different during the first week following inoculation in RAG−/− and wild-type mice (Fig. 3A). However, at day 9, a noticeable divergence appeared in these two cohorts of mice, and the imaging of RAG−/− mice showed significantly higher luminescence than that from the imaging of wild-type mice. From week 2 until week 8, rBCG-Luc luminescence increased from 6.4 × 105 RLU to 1.2 × 106 RLU in RAG−/− mice, while luminescence did not increase in wild-type mice during the same time frame.

We simultaneously examined ex vivo colony counts from the spleens of additional cohorts of RAG−/− and wild-type mice. At 2 weeks, 2.6% of the inoculated mycobacteria were recovered from the spleens of wild-type mice, while 7.0% of the inoculated mycobacteria were recovered from the spleens of RAG−/− mice. These ex vivo CFU counts were in agreement with the bioluminescence imaging data, demonstrating that the mycobacterial burden in RAG−/− mice increased after day 9, while their wild-type counterparts controlled mycobacterial burden (Fig. 3B). Ex vivo colony counts from the spleens of RAG−/− mice increased 69% from week 2 to 8, from 3.5 × 106 to 5.1 × 106 CFU/spleen, while the colony counts in the wild-type mice decreased 95% during the same time frame, from 1.3 × 106 to 6.4 × 104 CFU/spleen.

FIG 1 Luminescence in mice decreases rapidly following high-dose rBCG-Luc administration. (A) Conditions for IVIS imaging were determined using a dose of 10⁶ CFU recombinant M. smegmatis-Luc. Photon emission is quantified in a constant region of interest (ROI) and is displayed as a heat map overlaid on a gray-scale image of a mouse that received recombinant M. smegmatis-Luc (top) and a mouse that received M. smegmatis (bottom). (B) A dose of 5 × 10⁷ CFU rBCG-Luc administered i.p. generated luminescence in C57BL/6 mice that decreased over time. One mouse, representative of four, is displayed. (C) Intense luminescence was detected during the first week following rBCG-Luc administration to a cohort of four C57BL/6 mice. Luminescence decreased 96.3% by the end of the first week to levels that were near background. Error bars represent standard errors of the mean (SEM).
Together, these data clearly demonstrate that the adaptive immune response does not play a role in the clearance of high-dose immunization of rBCG before day 9. However, beginning at day 9, the control of rBCG requires the adaptive immune response, and in the absence of T and B cells, bacterial burdens increase.

**Early mycobacterial clearance is not mediated by NK cells or IFN-γ.** We examined NK cells and IFN-γ for their possible contribution to the clearance of rBCG occurring between the time of inoculation and the initiation of adaptive immune responses. NK cells are reported to play a minor role in the control of mycobacteria, unless T and B cells are absent (25). However, because they are capable of responding rapidly to other pathogens, and a large rise in NK cell numbers is observed in the peritoneal cavity of mice following i.p. infection with M. tuberculosis (26) and in the lungs of mice infected with M. tuberculosis (27), we considered the possibility that NK cells might have a role in the early clearance of high-dose rBCG immunization.

We inoculated mice harboring a spontaneous mutation affecting NK cell production and function (bgj mice), as well as mice depleted of NK cells by the anti-asialo GM1 antibody, with high-dose rBCG. In both cases, no significant difference was seen in the clearance of rBCG-Luc in the first 5 days postinoculation compared to that in wild-type mice (Fig. 4A and B).

Studies using mice deficient in IFN-γ production have demonstrated the critical nature of IFN-γ in the control of low-dose M. tuberculosis inoculation (28, 29). Using the same IFN-γ deficient (IFN-γ−−) mice, we observed no significant difference in the clearance of rBCG compared to that in wild-type mice in the first 5 days following inoculation (Fig. 4C).

**Rapid clearance of high-dose mycobacteria is mediated by Gr-1+ cells.** In the absence of a role for RAG-1-dependent cells, NK cells, and IFN-γ in the immediate clearance of high-dose rBCG, we hypothesized that neutrophils or inflammatory monocytes may be active, despite the fact that the depletion by the RB6-8C5 antibody of Gr-1+ cells performed by Seiler et al. (15) failed to demonstrate a difference in the control of rBCG at a dose of 10⁴ CFU compared to that in wild-type mice (15). We depleted mice of neutrophils and inflammatory monocytes using the same RB6-8C5 anti-Gr-1 antibody and inoculated them with 5 × 10⁷ CFU rBCG i.p. In mice depleted using RB6-8C5, rBCG burden increased in the first 24 h following inoculation (Fig. 5). This was a significant increase (P < 0.001) in rBCG burden compared to that in mice that received a dose of nonspecific isotype control antibody or in wild-type mice, in which the rBCG burden decreased in the first 24 h postinoculation. At 2 h postinoculation, the RLU emitted from Gr-1+ depleted mice was 88% higher than that in mice receiving the control antibody. Between 2 and 8 h, the RLU from the Gr-1−− depleted mice further increased another 2.2-fold, while the RLU from control mice further decreased 2-fold, resulting in a total difference in RLU between the Gr-1+−− depleted mice and control mice of >8-fold at 8 h (P < 0.001) postinoculation. Thereafter, the RLU decreased in both cohorts of mice until day 3, at which point there was no discernible difference.

**DISCUSSION**

We have used bioluminescence imaging to monitor rBCG burden in real time and to examine the contribution of different arms of the immune response to the clearance of high-dose rBCG vaccination. The use of bioluminescence as a surrogate for mycobacterial burden has allowed us to clearly distinguish two phases of clearance following high-dose rBCG inoculation: a phase of rapid bacterial elimination spanning 14 days, followed by a phase of...
control in which bacterial burdens neither increase nor are completely eliminated. In the first phase of high-dose rBCG clearance, we observed a 97.41% decrease in rBCG luminescence and a 97.4% decrease in ex vivo CFU counts between days 1 and 14, a finding that was novel considering that previous publications have demonstrated a rise in BCG CFU following low-dose inoculation (15, 16).

Our data suggest that this decrease in luminescence is not attributable to a loss in the ability of the mycobacteria to produce transgenic protein, as every ex vivo-isolated mycobacterium containing the plasmid was capable of producing luciferase. However, our analysis does not preclude the fact that bacteria may decrease their luciferase production in vivo.

Very few promoters are expressed at a constant level in mycobacteria, especially because the pathogen radically changes its growth rate following infection. There is some modulation in the expression of antigen 85b (Ag85b), but we believe that it does not change the interpretation of our data, as the magnitude of mRNA change is small compared to the several-log change in luciferase expression we saw within the first few days. Within the first 24 h, Fontán et al. (30) found that the Ag85b promoter is minimally changed in human monocyte THP1 cells infected with M. tuberculosis strain H37Rv. Fontán et al. (30) documented changes ranging from −0.15- to +1.33-fold in Ag85b mRNA at 4 and 24 h.

Rogerson et al. (31) further defined a time course of Ag85b expression, showing an increase in Ag85b mRNA until day 20 and then a decrease from day 20 and on once the infection hit the stationary phase. As Rogerson et al. demonstrated increased Ag85b expression through day 20, and we saw our reduction in luciferase occurring in the time frame from day 1 to day 20, this indicates that the reduction in luminescence from days 1 to 20 is a reduction in CFU and not a result of decreased Ag85b mRNA expression.

Furthermore, in RAG−/− mice, we saw luminescence increase as splenic CFU levels increased from day 14 to day 28. This does not support a hypothesis that the Ag85b promoter activity at later times is decreased to such a level that luciferase expression cannot be detected by bioimaging.

The loss of the plasmid itself did not appear to account for the dramatic loss in bioluminescence. Plasmid loss was calculated to be 0% at 2 weeks after in vivo inoculation, increasing to only approximately 40% after the 2-week time point. Similar results were reported by Méderlé et al. (32), in which the loss of plasmid from rBCG occurred 2 weeks after the mycobacteria were injected in vivo and no longer under antibiotic selection for the kanamycin marker. Thus, the fact that we observed such high plasmid retention in the first 2 weeks indicates that bioluminescence remains tightly correlated with mycobacterial burden for this time frame; however, the drop in plasmid retention between weeks two and four suggests that luminescence may underestimate mycobacterial burden by as much as 40% at later time points.

The clearance of rBCG following high-dose inoculation involved distinct branches of the immune response. Our findings indicate that Gr−1 cells are, in part, responsible for the clearance of rBCG immediately following inoculation, as there was a significant increase in luminescence in Gr−1−-depleted mice compared to that in wild-type animals. Gr−1 cells, primarily neutrophils and inflammatory monocytes, are rapid responders that are not
traditionally associated with the clearance of slow-growing mycobacteria. In contrast, the second phase of control, mediated by the adaptive immune response, was evident from the increase in bacterial burdens beginning at day 14 in RAG\(^{-/-}\)/H11002 mice compared to the response in wild-type mice. This finding further indicates that T cells are dispensable for early clearance of rBCG and supports an important role for neutrophils or inflammatory monocytes in this early response.

Our use of the RB6-8C5 anti-GR-1 antibody in this work simultaneously depletes neutrophils and inflammatory monocytes. These cell types share effector mechanisms, and both are recruited to sites of infection, but they differ in their mechanisms of activation and recruitment; recently, they have been shown to make differential contributions to the control of *Listeria monocytogenes* and herpes simplex virus 1 (33, 34). The antibody depletion of Gr-1\(^+\) cells is unable to distinguish the contribution of each cell type given the affinity of RB6-8C5 for Ly-6C and Ly-6G, with Ly-6G being found selectively on neutrophils and Ly-6C being...

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**FIG 4** Early clearance of rBCG-Luc is not mediated by NK cells or IFN-\(\gamma\). Groups of four NK cell-deficient beige mice (A) and BALB/c mice depleted of NK cells by infusion of anti-asialo GM-1 antibody (B) were injected with \(5 \times 10^7\) CFU rBCG-Luc. Neither NK cell-deficient nor NK cell-depleted mice had statistically different kinetics of mycobacterial clearance during the first week. The data are representative of the results from two independent experiments. (C) Groups of four IFN-\(\gamma\)-deficient and control mice had comparable early kinetics of rBCG-Luc clearance. Error bars represent SEM. No statistical differences between the results in the control and experimental group were observed. Statistical analyses were performed using Student’s \(t\) test.

**FIG 5** Mice depleted of Gr-1\(^+\) cells have increased bacterial burdens 24 h after rBCG-Luc inoculation. C57BL/6 mice depleted of neutrophils and inflammatory monocytes using the anti-Gr-1 antibody RB6-8C5 have increased bacterial burdens 24 h after inoculation with \(5 \times 10^7\) rBCG-Luc. Cohorts of 4 mice were used, and the data are representative of the results from two independent experiments. Error bars represent SEM. Statistical analysis was performed using a one-way ANOVA with a Tukey multiple comparison posttest. ***, \(P < 0.001\).
found on neutrophils, subsets of CCR2+ inflammatory monocytes, myeloid suppressor cells, and memory subsets of CD8+ T cells (35, 36). Further research with Ly-6C and Ly-6G antibodies may thus be warranted to dissect the contributions of these cell types to high-dose mycobacterial immunization.

Notwithstanding the above findings, previous reports demonstrated mycobactericidal activity of neutrophils, albeit less pronounced for this slow-growing bacterium compared to the critical role that neutrophils play in clearing fast-growing bacteria, such as Salmonella enterica. Neutrophils can phagocytose BCG, they are capable of being activated by mycobacterial peptides, they produce antimycobacterial peptides, and the depletion of neutrophils in human blood samples decreases the ability of whole blood to restrict M. tuberculosis growth (15, 37, 38). Indeed, even at low doses of BCG, the influx of neutrophils to the site of inoculation has been observed. Despite these observations, neutrophil depletion in mouse models using the same RB6-BCS antibody used here has failed to show any differences in BCG growth or clearance following inoculation with 10⁴ CFU BCG (15). In contrast to these previous studies that used low doses of mycobacteria, our data indicate that Gr-1+ cells play a crucial role in the early clearance of high-dose BCG by rapidly reducing mycobacterial burden by 2 logs following high-dose inoculation. One possible explanation for the discrepancy may lie in the low growth rate of these mycobacteria, which have a doubling time of nearly 30 h. The low mycobacterial burdens initiated in previous studies may be insufficient to trigger sufficient recruitment and activation of Gr-1+ cells, whereas high-dose inoculation rapidly recruits this population (39).

The 24-h increase in BCG burdens following RB6-BCS treatment demonstrates that in the absence of Gr-1+ cells, mycobacteria may replicate uncontained in the host until an adaptive response is formed. Thus, Gr-1+ cells provide one part of a bridge for the host to survive until the development of an adaptive immune response. Our depletion of Gr-1+ cells led to an increase in bacterial burden only in the first 24 h, potentially a result of the rapid repopulation of the neutrophil compartment following depletion, which has been observed by others after anti-Gr-1 antibody administration (15). Furthermore, depletion via intravenous injection has greater impact on circulating cells than tissue-resident cells, and so it is possible that a rise in bacteria following a more complete depletion would be even more profound in length or magnitude than what was observed here.

By playing a significant role in the clearance of BCG, Gr-1+ cells also play a role in trafficking antigens to the lymph nodes for presentation to T cells and can affect the initial antigen programming of T cells (40, 41). Depletion using the anti-Ly-6G and Ly-6C antibody NIMP-R14 has been shown to decrease T-cell priming in response to BCG (42). This function should be considered in the design and delivery of rBCG vectors in order to maximize antigen presentation and the generation of antigen-specific T-cell responses.

In summary, we found that high doses of rBCG administered as part of a vaccine regimen did not replicate the natural course of mycobacterial infection and did not display the published kinetics of clearance that is observed following infection with only a few mycobacteria. In order to contain the large numbers of mycobacteria associated with vaccination, the host mobilizes additional arms of the immune response, including Gr-1+ cells. Together, our findings underscore the need for further research into the role of Gr-1+ cells in rBCG antigen clearance in order to aid in the design of future rBCG vaccines that will maximize antigen delivery and the generation of stronger antigen-specific T-cell responses.

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