Development of an Unbiased Antigen-Mining Approach To Identify Novel Vaccine Antigens and Diagnostic Reagents for Bovine Tuberculosis

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Previous experiments for the identification of novel diagnostic or vaccine candidates for bovine tuberculosis have followed a targeted approach, wherein specific groups of proteins suspected to contain likely candidates are prioritized for immunological assessment (for example, with in silico approaches). However, a disadvantage of this approach is that the sets of proteins analyzed are restricted by the initial selection criteria. In this paper, we describe a series of experiments to evaluate a nonbiased approach to antigen mining by utilizing a Gateway clone set for Mycobacterium tuberculosis, which constitutes a library of clones expressing 3,294 M. tuberculosis proteins. Although whole-blood culture experiments using Mycobacterium bovis-infected animals and M. bovis BCG-vaccinated controls did not reveal proteins capable of differential diagnosis, several novel immunogenic proteins were identified and prioritized for efficacy studies in a murine vaccination/challenge model. These results demonstrate that Rs3329-immunized mice had lower bacterial cell counts in their spleens following challenge with M. bovis. In conclusion, we demonstrate that this nonbiased approach to antigen mining is a useful tool for identifying and prioritizing novel proteins for further assessment as vaccine antigens.

Bovine tuberculosis (TB) caused by the bacterial pathogen Mycobacterium bovis poses a major economic and animal health problem to the farming community. To support the current “test-and-cull” strategy, vaccination of cattle against M. bovis infection is being considered as one of the long-term control options for reducing the risk and incidence of bovine TB in Great Britain. Promising vaccination strategies are based on a heterologous prime-boost approach, in which the immune system is primed by vaccination with Mycobacterium bovis BCG and subsequently boosted by immunization with either DNA (1), protein (2), or viral (3) subunit vaccines. Thus, the identification of immunogenic proteins suitable for use as the boosting subunit vaccine remains a high priority in TB research. Furthermore, given that BCG vaccination compromises the current diagnostic tests for bovine TB that utilize bovine tuberculin (4), it is essential that alternative mycobacterial antigens capable of discriminating between infected animals and uninfected but vaccinated animals are identified, to allow for continuation of test-and-cull control strategies in parallel with BCG vaccination.

Previous antigen-mining experiments for identification of novel diagnostic or vaccine candidates for bovine TB followed a targeted approach, wherein specific groups of proteins suspected to contain likely candidates were identified and then individual members of those groups were evaluated for immunogenicity; these included proteins identified by comparative genomics (5), differential transcription rates (6), or gene expression profiles associated with latent mycobacterial infection (7,8), members of the PE/PPE family (9), and potentially secreted proteins, including members of the 6-kDa early secretory antigenic target of Mycobacterium tuberculosis (ESAT-6) family (10). Although such targeted studies have identified several immunogenic proteins, a disadvantage is that they are restricted to a relatively small set of proteins biased by the initial selection criteria. To overcome this, we have developed a nonbiased approach to antigen mining by utilizing an extensive library of clones expressing mycobacterial proteins. Experiments evaluating this approach, including the generation of proteins, the development of screening experiments in cattle, and the assessment of responses in a murine vaccination/challenge model, are described herein.

MATERIALS AND METHODS

Cattle. All animals were housed at the Animal Health and Veterinary Laboratories Agency (AHVLA) at the time of blood sampling, and procedures were conducted within the limits of a United Kingdom Home Office license under the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee. Heparinized blood samples were obtained from a total of 57 naturally infected, single intradermal cervical comparative tuberculin test-positive reactors (TB reactors), from herds known to have bovine tuberculosis. These animals also tested positive for M. bovis infection using the gamma interferon (IFN-γ) Bovigam test (Prionics AG, Switzerland) (data not shown). Blood samples were also obtained from a total of 50 non-M. bovis-infected animals that were subsequently vaccinated with BCG, as described previously (11). Briefly, calves (~6 months of age) from bovine TB-free herds were vaccinated with BCG Danish (Statens Serum Institute, Copenhagen, Denmark) by subcutaneous injection of 1 × 10^6 CFU into the side of the neck.

Preparation of antigens. (i) Gateway antigens. M. tuberculosis proteins were prepared using the Gateway complete clone set for M. tuberculosis (J. Craig Venter Institute), representing the genes from 3,294 M. tuberculosis open reading frames (ORFs). DH10B cells containing the M. tuberculosis H37Rv Gateway entry clone were grown overnight at 37°C,
with shaking, in glucose-supplemented LB (LB + G) broth containing kanamycin, following which the entry clone plasmid was isolated using the QIAprep Spin miniprep kit (Qiagen, United Kingdom). The M. tuberculosis gene of interest was transferred from the entry clone plasmid into the His-tag-containing petDest42 expression vector (Invitrogen, United Kingdom) using LR Clonase II (Invitrogen) and then was used to transform Library Efficiency DH5α cells (Invitrogen). DH5α transformants containing the petDest42 expression clones were selected by growth on ampicillin-supplemented LB + G (LB + G/amp") agar plates and cultured overnight at 37°C in LB + G/amp" broth to increase the yield of the expression clone, which was subsequently isolated using the QIAprep Spin miniprep kit and used to transform BL21 (DE3) cells (Invitrogen). Similarly, BL21 (DE3) cells containing the expression clone were selected and expanded by overnight culture, and protein expression was induced using Overnight Express Autoinduction System 1 (Novagen, United Kingdom), following the manufacturer’s protocol. Sequencing of the inserts within the expression clones confirmed that they contained the M. tuberculosis gene of interest.

Induced BL21 (DE3) cells were pelleted by centrifugation, resuspended in binding buffer (8 M urea, 20 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, 1 mM β-mercaptoethanol [pH 7.4]), and mechanically disrupted using a FastPrep homogenizer (Thermo, United Kingdom) following the addition of lysis matrix. Samples were clarified by centrifugation, and the supernatants were applied to His Spin Trap columns (GE Healthcare, United Kingdom) following the manufacturer’s protocol. Sequencing of the inserts within the expression clones confirmed that they contained the M. tuberculosis gene of interest.

Mouse immunization experiments. (i) Animals. Female BALB/c mice (Charles River Ltd., United Kingdom) were used at 8 weeks of age and were housed in appropriate biosafety level 3 containment facilities at the AHVLA. All animal work was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986, under appropriate personal and project licenses. The study protocol was approved by the AHVLA animal ethics committee (United Kingdom Home Office PCD no. 70/6905).

(ii) Immunization and mycobacterial challenge. Mice were immunized subcutaneously three times (2 weeks apart) with 100-µl volumes containing 10 µg of either Rv0878c, Rv3329, or Rv3432c proteins emulsified in adjuvant consisting of 25 µg of monophosphoryl lipid A (MPL) detoxified from Salmomella minnesota (Avanti Polar Lipids, United States), dissolved in 0.2% triethylamine (Sigma, United Kingdom) at 65°C with sonication, combined with 250 µg of dimethyldioctadecyl ammonium bromide (DDA) (Sigma, United Kingdom). Control mice were immunized with MPL-DDA adjuvant alone. A separate group of mice were immunized with a single intradermal injection, at the base of the tail, with 2 x 10^6 CFU of the human vaccine M. bovis BCG Danish 1331, prepared according to the manufacturer’s instruction (Statens Serum Institute, Denmark). Six weeks after the first immunization, 6 animals per group were euthanized and their spleens were processed for immunological assays (detail below). M. bovis isolate AFB122/97 was grown and stored as described previously (12). Six weeks following immunization, mice were challenged intravenously with approximately 677 CFU of virulent M. bovis; they were euthanized 4 weeks later, their lungs and spleens were removed and homogenized, the homogenates were serially diluted, and the dilutions were plated on modified Middlebrook 7H11 agar medium. Bacterial colonies were enumerated 4 weeks later, following incubation at 37°C.

(iii) Cell isolation and immunological assays. Spleen cells were prepared from mice at 6 weeks postimmunization, as described previously (13). A total of 5 x 10^6 splenocytes were incubated for 16 h in 96-well filter plates (MSI/S4510; Millipore, Ireland) in duplicate, with or without antigen (10 µg/ml), and the frequency of IFN-γ-secretors was detected by an enzyme-linked immunosorbent spot (ELISPOT) assay (Mabtech, Sweden), according to the manufacturer’s instruction. In addition, 5 x 10^6 splenocytes were cultured for 72 h with or without antigen (10 µg/ml), following which supernatants were harvested and stored at −70°C until required. Simultaneous production of interleukin 2 (IL-2), IL-4, IL-6, IL-10, IL-12, IFN-γ, and tumor necrosis factor alpha (TNF-α) was measured using a cytokine multiplex assay, according to the manufacturer’s instruction (Mesoscale Discovery, Gaithersburg, MD).

Statistical analysis. Receiver operating characteristic (ROC) curve analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc.), while one-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison test and nonparametric ANOVA with the Dunn multiple-comparison test were performed using GraphPad InStat 3 software (GraphPad Software, Inc.).

RESULTS
In order to evaluate the efficiency of antigen preparation through the Gateway process, a training set of 49 M. tuberculosis genes was initially selected, including genes encoding the known immunogenic antigens Rv2873 (MPB83), Rv2875 (MPB70), Rv3873c (PPE family member), and Rv3875 (ESAT-6). Both the cloning of the genes into the petDest42 expression vector and the transformation of BL21 (DE3) cells were efficient processes, with an overall success rate of 96%. In comparison, induction of protein expression was less efficient, with 31 (63%) of the total of 49 samples being induced. However, the majority of these induced proteins were successfully purified, resulting in overall efficiency in antigen preparation of 59%.

To investigate whether the immunogenicity of proteins is preserved through the Gateway process, we initially isolated the pro-
ing whole-blood cultures from BCG-vaccinated animals. Again, all animals recognized the positive controls PPD-B and SEB (data not shown). None of the BCG-vaccinated animals recognized the ESAT-6/CFP-10 peptide cocktail (Fig. 1A), which is as expected, given that these two proteins are absent in BCG, while protein antigens Rv2873, Rv3873c, and Rv2875 induced responses in 78%, 89%, and 67% of BCG-vaccinated animals, respectively (Fig. 1A). Unexpectedly, and in contrast to the peptide cocktail, the Rv3875 (ESAT-6) protein (which is deleted from the BCG vaccine strain) produced through the Gateway process induced IFN-γ production in 3 (33%) of 9 BCG-vaccinated animals, suggesting that recognition of the Gateway proteins may in part be nonspecific and due to contaminants. To address this issue, we decided to define a more-stringent cutoff for assessing IFN-γ production in whole-blood cultures. Receiver operating characteristic (ROC) curve analysis was performed on the Gateway Rv3875 protein-induced IFN-γ responses using the data from the TB reactor and BCG-vaccinated animals (Fig. 1B). For a specificity set at 100%, the ROC curve analysis (AUC, 0.810; P = 0.014) defined a positive response as an ΔOD450 Value of >0.338. Using this more stringent cutoff (ΔOD450 values of >0.338 for both of the duplicate wells), the number of TB reactor animals producing IFN-γ in response to Rv3875 protein stimulation was reduced from 12 (86%) of 14 to 8 (57%) of 14, while no BCG-vaccinated animals mounted a positive response (Fig. 1C). In addition, applying this more-stringent cutoff reduced the proportions of TB reactor animals responding to Rv2873, Rv3873c, and Rv2875 to 29%, 21%, and 29%, respectively (Fig. 1C). Use of this stringent cutoff also resulted in lower frequencies of responses to Rv3873c and Rv2875 in BCG-vaccinated animals (44% and 33%, respectively), while responses to Rv2873 were largely unaffected (67%).

We reasoned that, if the responses to the Gateway proteins were in part driven by nonspecific reactions to contaminating products found in the Gateway protein preparations, then these responses would be absent when pools of overlapping synthetic peptides were used to stimulate whole-blood cultures. To explore this further, we selected 8 Gateway proteins (Rv3548c, Rv0831c, Rv1580c, Rv3846, Rv1093, Rv1916, Rv2952, and Rv2820c) from the training set that were frequently recognized by TB reactor animals. Using the standard cutoff for positivity (ΔOD450 >0.1), all eight proteins induced IFN-γ responses in at least one-half of the TB reactor animals studied (Fig. 2, white bars). When these data were reanalyzed using the more-stringent cutoff for positivity (ΔOD450 >0.338) (Fig. 2, black bars), the proportions of animals classified as responding to the different Gateway proteins were substantially reduced. When pools of synthetic overlapping peptides were used to stimulate whole-blood cultures from TB reactor animals (Fig. 2, gray bars), the proportions of animals mounting positive IFN-γ responses were, on the whole, similar to those seen by using the stringent cutoff for Gateway protein responses, although we observed higher peptide-inducing responder frequencies for Rv2952 and Rv2820c than when we applied the more stringent cutoff to Gateway protein responses.

To identify novel vaccine antigens and/or diagnostic reagents, we performed unbiased antigen-mining experiments using the Gateway complete clone set for M. tuberculosis. In total, 513 individual gene-cloning attempts have been performed, resulting in the successful preparation of 266 proteins (52% overall efficiency). To prioritize these proteins for further screening experiments, we first identified immunogenic proteins recognized by TB

FIG 1 Recognition of known immunogenic antigens produced through the Gateway process. (A) Screening of the indicated proteins or peptide cocktail for their ability to stimulate IFN-γ production in whole-blood cultures from 14 TB reactor and 9 BCG-vaccinated animals. An IFN-γ result was considered positive if the ELISA optical density at 450 nm (OD450) with antigen minus the OD450 without antigen (ΔOD450) was ≥0.1, and results are represented as responder frequencies. (B) ROC curve showing the accuracy of Gateway Rv3875 protein-induced IFN-γ responses in discriminating between TB reactor and BCG-vaccinated animals. Arrow, ΔOD450 cutoff of 0.338. CI, confidence interval. (C) Reanalysis of the data shown in panel A using the ΔOD450 cutoff of 0.338 for determination of positive IFN-γ responses. E/C, ESAT-6/CFP-10.
reactor animals using the standard cutoff (ΔOD_{450} > 0.1). Although we acknowledged that this would identify some nonspecific responses, we considered the need to be more "inclusive" early on, to maximize the number of potential antigens prioritized for further screening experiments. Of the 266 Gateway proteins, 33 novel proteins that were recognized by the majority (i.e., ≥ 50%) of the TB reactor animals studied (data not shown) were identified, and these proteins were then rescreened in BCG-vaccinated animals. The proportions of TB reactor and BCG-vaccinated animals mounting specific IFN-γ responses, as defined by an ΔOD_{450} cutoff of > 0.338, are shown in Fig. 3. Even using this more-stringent cutoff, all 33 novel proteins were recognized by at least one TB reactor animal, with responder frequencies ranging from 7% to 33%. With the exception of Rv3846, which was not recognized by any BCG-vaccinated animal studied, all of the Gateway proteins induced IFN-γ production in greater proportions of BCG-vaccinated animals, with responder frequencies ranging from 11% to 60%.

Having identified novel mycobacterial proteins recognized by the immune systems of M. bovis-infected or BCG-vaccinated cattle, we next investigated their potential as subunit vaccines using a mouse vaccination/challenge model. The mycobacterial proteins Rv0878c, Rv3432c, and Rv3329 were initially selected for these studies, because they were most frequently recognized in the cattle system (Fig. 3). As purified protein antigens are poorly immunogenic when administered by themselves, protein immunizations were delivered with MPL-DDA adjuvant. As controls, a group of mice were immunized with either adjuvant only or BCG. Following vaccination with Rv3432, a 6-fold increase in the frequency of Rv3432c-specific IFN-γ-secreting splenocytes was observed, compared to mice vaccinated with adjuvant alone (Fig. 4A). Similarly, a 3-fold increase in the frequency of Rv3329-specific IFN-γ-secreting splenocytes was observed in mice vaccinated with Rv3329, compared to mice vaccinated with adjuvant alone (Fig. 4B). In contrast, vaccination with Rv0878c did not induce greater levels of Rv0878c-specific IFN-γ-secreting splenocytes than did the adjuvant alone (Fig. 4C).

Lastly, activation of antigen-specific IFN-γ production in response to all three proteins was evident in BCG-vaccinated mice (Fig. 4A to C). The immune profiles of protein vaccine-induced responses were further characterized by quantifying the production of numerous cytokines following in vitro restimulation of splenocytes with the different candidate proteins. Significantly greater levels of Rv3329-induced IFN-γ, IL-12, IL-17, IL-2, and IL-4 production were observed in mice vaccinated with Rv3329 than in those immunized with the adjuvant alone (Table 1). With the exception of IL-12 production, similar results were observed for Rv3432c-specific responses in mice vaccinated with this protein, compared to those immunized with adjuvant alone. In contrast, no significant differences in the levels of Rv0878c-induced cytokine production were observed for mice immunized with Rv0878c protein versus adjuvant alone (data not shown).

Having demonstrated the potential of Rv3432c and Rv3329 subunit vaccines in priming antigen-specific immune activation, we next investigated whether these responses could protect mice from challenge with virulent M. bovis. As expected, control mice immunized with BCG exhibited significant reductions in bacterial burdens in both the lung (Fig. 5A) and the spleen (Fig. 5B).
The aim of this study was to develop an unbiased screening procedure that would identify potentially immunogenic mycobacterial proteins, without preselection of particular gene products based on their known function. Initial data revealed a set of 33 gene products that were recognized by the majority of TB reactor animals studied. Analysis of these using the TubercuList database (14) revealed that only four had been characterized previously, while the remaining 29 gene products were described as having probable or possible functions (mainly enzymatic functions) or indeed were classified only as hypothetical proteins. Given the unlikelihood that many of these gene products would have fallen into categories specifically targeted for screening experiments, our results highlight the requirement for continued unbiased approaches to the identification of novel immunogenic proteins.

One area of concern in using recombinant proteins generated through Escherichia coli expression systems in experiments measuring immunological responses is the level of contamination with lipopolysaccharide (LPS) or E. coli proteins and the potential for generation of nonspecific responses. Several approaches can be used to reduce LPS contamination, including the use of affinity adsorbents such as polymyxin B (15), Triton X-114 phase separation (16), ion exchange or gel filtration chromatography (17), ultrafiltration (18), and sucrose gradient centrifugation. However, the success of these approaches in removing LPS is strongly dependent on the properties of the target protein and can result in protein damage and/or decreased yields in some cases (19). For these reasons and to maintain the high-throughput nature of the protein generation process, we decided not to include additional laboratory steps to remove potential endotoxin contamination. Instead, we addressed the issue of induction of nonspecific responses by generating appropriate cutoff values to classify IFN-γ ELISA responses as positive. To this end, recombinant ESAT-6 protein produced through the Gateway system was used to stimulate whole-blood cultures from BCG-vaccinated cattle. Given that BCG Danish does not express ESAT-6 (20), any IFN-γ response detected in these animals would be due to nonspecific activation; therefore, using ROC analysis, we were able to derive a more-stringent cutoff value for analyzing IFN-γ ELISA data that accounted for possible LPS contamination of the recombinant protein preparations. Also, by using synthetic peptide cocktails that contained undetectable levels of endotoxin contamination, we were able to estimate the proportions of M. bovis-infected animals testing positive for various gene products. In general, similar responder frequencies were observed when responses to the recombinant proteins for these gene products were analyzed using the stringent cutoff, thus supporting the concept that this more-stringent cutoff for analyzing Gateway protein responses reduced the detection of potential nonspecific responses.

Of the 33 gene products that induced IFN-γ production in whole-blood cultures from TB-reactor animals, all except one also induced responses in BCG-vaccinated animals (Fig. 3), precluding their use as diagnostic reagents for discriminating between infected animals and uninfected but vaccinated animals. Given that these genes are located in the genomes of both M. bovis and BCG, these results are not unexpected. Greater responder frequencies were observed for BCG-vaccinated animals than TB reactor animals. The exact repertoire and hierarchy of immunogenic mycobacterial antigens, and how these differ between M. bovis-infected animals and those vaccinated with the M. bovis BCG vaccine strain, remain unclear. It is possible that the immune response in naturally infected cattle may be skewed toward a set of immunodominant mycobacterial antigens, at the expense of other poten-

![FIG 4 Induction of Rv3432c- and Rv3329-specific IFN-γ production following immunization with selected Gateway proteins. Groups of mice received three immunizations (at 2-week intervals) with Rv3432c, Rv3329, Rv0878c, or adjuvant alone, as described in Materials and Methods. An additional group of mice were immunized with BCG as controls. At week 6, mice were sacrificed; splenocytes were restimulated with 10 μg/ml Rv3432c (A), Rv3329 (B), or Rv0878c (C) for 16 h, and the frequency of IFN-γ-secretors was detected by an ELISPOT assay. The results are expressed as mean ± standard error of the mean (SEM) spot-forming units (SFU) per million cells, for 5 or 6 mice per group. *, P < 0.05; **, P < 0.01, one-way ANOVA with Bonferroni multiple-comparison test (versus adjuvant-only group).](cvi.asm.org/1679)
TABLE 1 Antigen-induced cytokine production in vaccinated mice

| Cytokine | Rv3329 specific | Rv3432c specific |
|---------|-----------------|-----------------|
|         | Adjuvant        | Rv3329          | Adjuvant        | Rv3432c |
| IFN-γ   | 22.87 (−80 to 240) | 1,178.14 (580–1,770) | 42.41 (−87 to 340) | 1,182.87 (537–1,943) |
| IL-12   | 6.35 (−4 to 27)  | 45.76 (25–68)    | 40.00 (4–85)     | 62.30 (6–127) |
| IL-17   | 8.03 (−7 to 31)  | 1,295.45 (524–1,989) | 17.02 (−0.6 to 34) | 2,567.36 (1,096–3,438) |
| IL-2    | 32.05 (10–80)   | 681.48 (449–1,065) | 132.12 (88–165)  | 1,097.47 (498–1,752) |
| IL-4    | 0.11 (−2 to 0.4) | 4.08 (2–5)       | 0.60 (−0.08 to 1.4) | 5.70 (3–10) |
| IL-10   | 127.06 (81–228) | 308.13 (168–402) | 362.33 (200–498) | 390.71 (245–511) |
| IL-6    | 568.26 (250–993) | 1,001.48 (623–1,312) | 905.23 (419–1,874) | 959.13 (437–1,840) |
| TNF-α   | 24.30 (4–56)    | 56.21 (25–101)   | 56.68 (22–112)   | 62.06 (22–102) |

\(^a P < 0.01, \) nonparametric ANOVA with the Dunn multiple-comparison test (versus adjuvant-only control mice).

\(^b P < 0.05.\)

 tolling immunogenic proteins. Some of these proteins, including but not restricted to ESAT-6 and CFP-10, are deleted from BCG, allowing us to speculate that other “less immunodominant” antigens may be recognized to a greater extent in BCG-vaccinated animals. The in vitro screening experiments revealed novel gene products that appeared to be targets of the immune system during natural infection and following BCG vaccination, thus prioritizing these proteins for further evaluation in vaccination studies. Due to financial and logistical constraints, it is not possible to test all potential subunit vaccine candidates in a cattle challenge model. Thus, we have utilized a standardized murine challenge model for the initial screening and prioritization of novel subunit vaccines. Immunization with either Rv3329 or Rv3432c protein primed antigen-specific immune activation, as evidenced by (i) increases in the rates of IFN-γ-producing cells in comparison with the adjuvant-only group and (ii) the production of numerous cytokines, including IL-2, IL-12, IL-17, and IL-4. Despite being immunogenic in whole-blood cultures from M. bovis-infected cattle, immunization with Rv0878c failed to prime recall responses in the murine model. Although it is beyond the scope of this study, we speculate that inherent differences in antigen processing, the availability of suitable major histocompatibility complex (MHC) class II molecules for presentation of peptide epitopes, and the T cell receptor repertoire may explain the discrepancy between the two animal systems.

Of particular importance, we have shown that immunization with either Rv3329 or Rv3432c induced immune responses characterized by production of IFN-γ, IL-12, and IL-17. It is generally accepted that protective immunity to mycobacterial infection is largely mediated through CD4⁺ Th1 cells that secrete IFN-γ, although other cell populations may also contribute to production of this cytokine (reviewed in references 21 and 22). IL-12 plays a crucial role in the production of IFN-γ by T cells (23), and both the generation and maintenance of an IFN-γ T cell response during mycobacterial infection are dependent on IL-12 production (24–26). Indeed, disruption of either IFN-γ or IL-12 responses in mice impairs the ability to control mycobacterial cell growth (24, 26–28). Although less studied, there is increasing evidence to support a role for IL-17 in protective immunity to mycobacterial infections. Following intratracheal infection with BCG, mice deficient in IL-17 exhibited impaired (i) lung granuloma formation, (ii) recruitment of cells (e.g., neutrophils, monocytes, and lymphocytes) to the lung, and (iii) antigen-induced IFN-γ production (29), while greater bacterial cell numbers were observed in the lungs of IL-17-deficient mice following intratracheal infection with M. tuberculosis (30). Postchallenge increases in IL-17 gene expression were observed in the lungs of mice vaccinated with BCG or the M. tuberculosis ΔsecA2 mutant strain (31, 32), while immunization with an ESAT-6 peptide subunit vaccine induced IL-17-producing cells in the lungs and their absence compro-

FIG 5 Reduced spleen bacterial burdens following immunization with Rv3329. Groups of mice were immunized as described for Fig. 4 and were challenged at week 6 with M. bovis AF2122/97 administered intravenously. Four weeks later, mice were euthanized, their lungs (A) and spleens (B) were removed and homogenized, and serial dilutions were plated on modified Middlebrook 7H11 agar medium for enumeration of bacterial colonies. Each symbol represents a single animal, and horizontal bars depict mean values. *, P < 0.05; *** , P < 0.001, one-way ANOVA with Bonferroni multiple-comparison test (versus adjuvant-only group).
mised Th1 responses and protection (33). Furthermore, BCG and BCG/viral subunit prime-boost experiments in cattle demonstrated that IL-17 expression was upregulated in protected animals after vaccination but before challenge, therefore defining it as a predictor of protection (3). Given that immunization with either Rv3329 or Rv3432c subunit vaccines induced antigen-specific recall responses accompanied by IFN-γ, IL-12, and IL-17 cytokine production, we investigated whether this would generate a level of protection following *M. bovis* challenge. Reduced bacterial counts were indeed observed in the spleens of Rv3329–immunized mice, demonstrating a level of protection with respect to mycobacterial infection. In contrast, no differences in bacterial cell numbers were observed in lung tissue. Although Rv3329–specific responses were detected in spleen cells postvaccination, it remains unknown whether similar responses occurred in lung tissue and whether this may underlie the absence of protection at this site, since the presence of antigen–specific cells in the lungs has been shown to be strongly associated with the protection afforded by BCG vaccination (13). It is interesting to note that, in contrast to Rv3329, vaccine-induced Rv3432c–specific responses were not associated with reduced bacterial counts in either spleen or lung tissue, highlighting the continual requirement for challenge experiments to confirm the protective efficacy of vaccine-induced immune responses to mycobacterial infections.

In summary, we have shown that an unbiased antigen selection/screening strategy identified Rv3329 as a novel immunogenic protein with the potential to reduce the severity of *M. bovis* infection when used alone as a subunit vaccine. Given that this protein also appeared to be a target of the immune system in BCG vaccination when used alone as a subunit vaccine. Given that this protein may underlie the absence of protection at this site, since the human antituberculosis T cell response. J. Immunol. 189:5867–5876.

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