The Journal of Immunology

Control of Chronic *Mycobacterium tuberculosis* Infection by CD4 KLRG1$^-$ IL-2–Secreting Central Memory Cells

Thomas Lindenstrøm, Niels Peter Hell Knudsen, Else Marie Agger and Peter Andersen

J Immunol 2013; 190:6311-6319; Prepublished online 15 May 2013;
doi: 10.4049/jimmunol.1300248
http://www.jimmunol.org/content/190/12/6311

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Control of Chronic *Mycobacterium tuberculosis* Infection by CD4 KLRG1$^-$ IL-2–Secreting Central Memory Cells

Thomas Lindenstrøm, Niels Peter Hell Knudsen, Else Marie Agger, and Peter Andersen

The bacille Calmette–Guérin vaccine provides very efficient protection in standard animal models of *Mycobacterium tuberculosis* challenge. We show in this article that although bacille Calmette–Guérin controlled *M. tuberculosis* growth for 7 wk of infection, the protection was gradually lost as the infection entered the chronic phase. The regrowth of *M. tuberculosis* coincided with an almost complete disappearance of IL-2–producing CD4 T cells. Booster vaccination with a subunit vaccine (Ag85B-ESAT-6+CAF01) expanded IL-2$^+$ CD4$^+$ T cell coexpressing either TNF-α or TNF-α/IFN-γ, and the maintenance of this population in the late stage of infection was associated with enhanced control of bacterial growth. The IL-2$^+$ CD4$^+$ T cell subsets were KLRG1$^-$ (nonterminally differentiated), were found to be CD62L^{high}, and further maintained a pronounced proliferative and cytokine-producing potential in the draining lymph nodes, when the animals were challenged 2 y postvaccination. These results suggest that the CD4$^+$ KLRG1$^-$ IL-2–secreting subsets are central memory T cells with the potential to continuously replenish the T cells at the site of infection and prevent attrition and functional exhaustion. ***The Journal of Immunology*****, 2013, 190: 6311–6319.**

Infection with *Mycobacterium tuberculosis* can persist lifelong and leads to a quiescent latent state of infection in >90% of all individuals infected. In the remaining 10%, clinical disease, tuberculosis (TB), will develop. Annually, 9 million new cases are reported, and it is estimated that TB accounts for ∼1.5 million deaths yearly (1). The only currently available vaccine against TB is the bacille Calmette–Guérin (BCG), which is an attenuated strain of the closely related *M. bovis*. BCG provides protection against severe disseminated childhood TB, including tuberculous meningitis, but shows poor and highly variable efficacy against pulmonary TB in adolescents and adults (2–4). Although BCG clearly has its limitations, it remains the most widely used vaccine in the world, primarily because of its efficacy against the pediatric forms of disseminated TB (5). BCG-induced Th1 immunity has been shown to decline with time from vaccination and is generally thought to last no more than 10–15 y (6–8), although immunity exceeding several decades has been reported (9). One of the prevailing strategies in TB vaccine research is to develop a BCG booster vaccine, and there are several demonstrations in both preclinical (10, 11) and clinical studies (12) that this is indeed a feasible way forward. However, of key importance for our attempts to develop improved TB vaccine strategies is a detailed understanding of BCG's limitations, and that the maintenance of vaccine promoted immunological memory in infected and noninfected individuals is one such issue where our knowledge currently is insufficient. In high-endemic regions such as Sub-Saharan Africa, the incidence of infection increases during childhood (13–15) to reach the staggering level of 60–70% of latent TB infection in some of the most afflicted populations at age >25 y (15). Memory immunity after BCG vaccination can therefore not be studied in isolation in naive animals but needs to be evaluated on a background of ongoing chronic infection to reflect a scenario where people in many cases experience a lifelong continuous exposure to the pathogen and its Ags.

For viral pathogens, persistent or chronic infections often lead to impaired CD8 T cell responses characterized by a stepwise and progressive loss of T cell function (16, 17), which are closely linked to the process of T cell exhaustion. For CD4 T cells, the phenomenon is not nearly as well characterized and understood, but there are clear indications that immune regulation and functional exhaustion take place in a number of chronic infections (18–22). In animal models, ongoing TB infection has recently been reported to drive T cells into a stage of terminal differentiation (23). Data from infected individuals provide the same overall interpretation with progressive impairment of *M. tuberculosis*–specific CD4 T cell responses with increasing mycobacterial load (24–26), a development that could be abrogated by anti-TB treatment (24). Whereas various cross-sectional studies in children have shown that BCG primarily promotes effector/effector memory T cells (similar to the real TB infection) (27, 28), a recent longitudinal study in infants has documented a more complex pattern, with BCG-specific CD4 T cells displaying characteristics of both central and effector memory T cells (29). In mice models, we have previously reported that adoptive transfer of *M. tuberculosis*–specific memory cells (from cleared infection or after subunit vaccination) can confer significant protection to challenge (30, 31), and that central memory T cells (T_{CM}) are the most important subset responsible for this effect (30). Immune regulation exerted by inhibitory receptor signaling such as KLRG1, through the PD-1–PD-L1/2 axis, regulatory T cells, and immunosuppressive cytokines, are all mechanisms developed to avoid excess immunopathology (32); but in TB, there is increasing evidence that these pathways also play an important role in the long-term maintenance of the equilibrium needed to support chronic infection (20, 21). Thus, to be successful, any novel vaccine

Tuberculosis Vaccine Research, Department of Infectious Disease Immunology, Statens Serum Institut, DK-2300 Copenhagen, Denmark

Received for publication January 25, 2013. Accepted for publication April 12, 2013.

This work was supported by European Commission Contract FP7-HEALTH-F3-2009-241745 under the NEWTBVAC consortium and through the ADITEC consortium supported by the European Commission through Contract FP7-HEALTH-2011.1.4-4-280873.

Address correspondence and reprint requests to Dr. Thomas Lindenstrøm or Prof. Peter Andersen, Department of Infectious Disease Immunology, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark. E-mail addresses: THI@ssi.dk (T.L.) or PA@ssi.dk (P.A.)

Abbreviations used in this article: BCG, bacille Calmette–Guérin; PPD, purified protein derivative of tuberculin; TB, tuberculosis; T_{CM}, central memory T cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300248

strategy to boost BCG needs to be able to overcome or counteract all these complex signals in an environment of chronic TB infection and maintain a reservoir of specific self-renewing T cells that can mediate long-term containment.

In this article, we show that BCG-mediated protection is lost as the infection enters the chronic phase, and that this waning of memory immunity is coincident with a loss of IL-2–producing CD4 T cells and an increase of KLRG1$^+$ terminally differentiated T cells. We found that a single boost of BCG with the CAF01-adjuvanted fusion protein Ag85B-ESAT6 (H1) significantly improves the long-term protection compared with BCG alone and shows that this increase in protective efficacy is associated with a selective induction, expansion, and maintenance of CD4 KLRG1$^-$ central memory cells secreting IL-2.

Materials and Methods

Animals and handling

Female C57BL/6 mice aged 6–7 wk were purchased from Harlan Scandinavia (Allerød, Denmark). Animals were kept at the experimental animal facilities at Statens Serum Institut and handled by authorized personnel. All mice were allowed to rest for 1 wk before experimentation and throughout the study were fed irradiation sterilized 2016 Global Rodent Maintenance diet and water ad libitum. All manipulations were conducted in accordance with the regulations of the Danish Ministry of Justice and animal protection committees by Danish Animal Experiments Inspectorate Permit 2009/561-1655, in compliance with European Community Directive 86/609 for the care and use of laboratory animals. Infected animals were housed in cages contained within laminar flow safety enclosures (Scantainer; Scanbur) in a separate biosafety level 3 facility.

Bacteria, Ags, and immunization

M. tuberculosis Erdman was grown at 37˚C on Middlebrook 7H11 (BD Pharmingen) agar or in suspension in Sauton medium (BD Pharmingen) enriched with 0.5% sodium pyruvate, 0.5% glucose, and 0.2% Tween 80. BCG Danish strain 1331 was grown at 37˚C in Middlebrook 7H9 medium (BD Pharmingen). All bacteria were grown to log phase and then stored at −80˚C in growth medium at ~5 × 10^8 CFU/ml. Bacteria were thawed, placed in an ultrasound bath for 5 min, clumps dispersed by forcing bacteria through a syringe, washed, and diluted in PBS before immunizations and infections. The subunit vaccine Ag, a fusion protein of Ag85B and ESAT-6 (H1), was produced as a recombinant Ag as previously described (33). Vaccine Ag was mixed with the adjuvant CAF01 (DDA/TDB; 250/50 μg/dose) before injection. CAF01 was prepared by the film method as described elsewhere (34). Mice primed with BCG were immunized once with 5 × 10^6 CFU BCG Danish 1331 by the s.c. route at the base of the tail. Four weeks later, one group received a single s.c. booster with 2 μg of the heterologous subunit vaccine H1 (Ag85B-ESAT-6)+CAF01 in a total volume of 200 μl.

Experimental infections and CFU enumeration

Six weeks after the H1-booster (10 wk after BCG priming), mice were aerosol challenged with *M. tuberculosis* Erdman using an inhalation exposure system (Biaera Aero MP aerosol system) calibrated to deliver ~100 CFU *M. tuberculosis* Erdman/mouse as previously described (35). Mice (six mice/group/time point) were subsequently euthanized at weeks 7, 26, and 50 into the infection, and CFU levels were determined in BCG, BCG_{prime}-H1+CAF01$_{boost}$, as well as nonimmunized mice. Bacterial levels were determined by plating serial dilutions of individual lung homogenates onto Middlebrook 7H11 agar supplemented with 2 μg 2-thiophenecarboxylic acid hydrazide/ml (Becton Dickinson, Oxford, U.K.) to selectively inhibit the growth of any residual BCG bacteria in organs from BCG immunized mice. Colonies were enumerated after 2–3 wk of incubation at 37˚C, and protective efficacy was expressed as the log10 values of the geometric mean for six mice/group. To assess the degree of KLRG1 expression, mice were immunized and challenged as described earlier, and immunological readouts were carried out 10 wk into infection. For the long-term memory experiment shown in Fig. 5, mice were immunized as described earlier, but aerosol challenged ~2 y after last immunization. Three weeks into the infection, mice were sacrificed and immune responses in lung-draining tracheobronchial lymph nodes were analyzed. Mice were administered BrdU (Sigma; 0.8 mg/ml) in drinking water 4 d before euthanization to determine the degree of proliferation.

Cell preparations

Lung, splenic, and lymph node cell preparations were obtained by homogenization through a 100-μm nylon cell strainer (BD Pharmingen) and washed twice in RPMI 1640 (Life Technologies Invitrogen, Taastrup, Denmark). All cell cultures were performed in Nuclon V-bottom microtiter plates (96-well plates; Nunc, Roskilde, Denmark) containing 1–2 × 10^6 cells/well in a volume of 200 μl RPMI 1640 supplemented with 5 × 10^{-5} M 2-ME, 1 mM glutamine, 1% pyruvate, 1% penicillin-streptomycin, 1% HEPES, and 10% FCS (all Life Technologies Invitrogen, Taastrup, Denmark). Based on preceding dose–response assays, protein Ags (purified protein derivative of tuberculin [PPD] and rAg85B) were used at a concentration of 5 μg/ml, whereas peptide mixes of 18-mer overlapping peptides spanning mycobacterial Ags ESAT-6 and TB10.4 were used at a concentration of 2 μg/ml (each peptide).

Flow-cytometric staining and analysis

Intracellular staining was essentially done as previously described (36). Cells were stimulated for 1 h in the presence of specific Ags and 1 μg/ml anti-CD28 (clone 37.51) and anti-CD49d (clone 9C10[MFR4.B]; both BD Pharmingen), and subsequently incubated for 5–6 h at 37˚C after addition of 10 μg/ml Brefeldin A (Sigma-Aldrich, Brøndby, Denmark) and 0.7 μl/ml Monensin/Golgi-stop (BD Pharmingen). After overnight storage at 4˚C, cells were washed in FACS buffer (PBS containing 0.1% sodium azide and 1% FCS), and subsequently surface stained with 1:200 dilutions (unless otherwise stated) of anti-CD4 (clone GK1.5) conjugated to either allophycocyanin-Cy7, allophycocyanin-eF780 (1:600; eBiosciences), FITC or PerCp-Cy5.5, anti–CD8-PerCp-Cy5.5 (clone 53-6.7), anti–CD44-FITC (clone IM7; all BD Pharmingen, San Diego, CA), anti–PD-1(CD279)-PE-Cy7 (data not shown; clone RMP1-30; 1:150; BioLegend), anti–KLRG1-allophycocyanin (clone 2F1; eBiosciences) and anti–CD62L-allophycocyanin-eF780 (1:100; clone MEL-14; eBiosciences). After permeabilization (Cytofix/Cytoperm kit; BD Pharmingen), intracellular staining was performed using 1:200 dilutions of anti–IFN-γ–PE–Cy7 or FITC (clone XMG1.2; eBiosciences), anti–TNF-α–PE (MP6-XT22; BD Pharmingen) or PerCp-eF710 (eBiosciences), and anti–IL-2–allophycocyanin or –allophycocyanin-Cy7 (clone JES6-5h4; BD Pharmingen) mAbs. BrdU staining was performed as in Elvang et al. (37). Samples were analyzed using a six-color BD FACSCanto flow cytometer (BD Biosciences). Responses were analyzed using FlowJo software version 8.8 (Mac; Tree Star) followed by Pestle and Spice software (38) by Boolean gating of IFN-γ, TNF-α, and IL-2$^+$ CD4/CD8 CD44^{high} (gating: Singlets, lymphocytes, CD4 versus CD8, CD44 versus cytokine). In the *Results*, the percentage of total responding CD4/CD8 CD44^{high} T cells was determined by summing the percentage of cells producing IFN-γ, TNF-α, or IL-2 in any combination ("any cytokine"). Using Spice, the percentage of IL-2–producing cells relative to the total cytokine-producing CD4$^+$ CD44^{high} was determined, and the degree of multifunctionality was finally established by separating the CD4$^+$CD44^{high} T cell population into seven distinct subpopulations based on their production of IFN-γ, IL-2, or TNF-α in any combination and reported both in terms of frequency and the representation of each of these subpopulations relative to total responders (Fig. 1B). Regarding exhaustion markers, "any cytokine"–producing cells were examined for their expression of KLRG1. Further, the percentage of cytokine-responsive cells expressing KLRG1 was determined, and the distribution of cells producing IFN-γ, TNF-α, and IL-2 in any combination among KLRG1$^+$ and KLRG1$^-$ cells was finally established. In assays staining for BrdU incorporation, BrdU +ve CD4 T cells were included in the Boolean gating strategy, and the cytokine-producing subsets incorporating BrdU were determined.

Statistics

Significant differences between vaccination groups and nonimmunized mice were tested by ANOVA using Tukey posttest for multiple comparisons. A p value <0.05 was considered significant.

Results

Boosting BCG with the H1 subunit vaccine results in increased control with chronic M. tuberculosis *infection*

We compared the protective efficacy provided by BCG and BCG boosted with the H1/CAF01 subunit vaccine short term (7 wk) and at late time points (26 and 50 wk) into a chronic TB infection. Seven weeks after challenge with *M. tuberculosis*, BCG and the prime-boost group provided comparable and significant levels of protection relative to nonimmunized controls corresponding to an ~1 log reduction in lung CFU levels ($p < 0.001$; Fig. 1A). Later

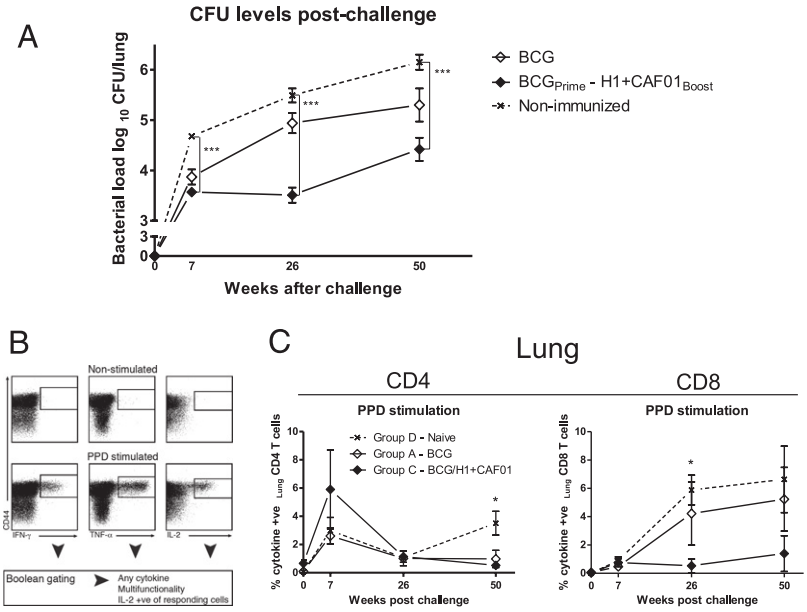

FIGURE 1. Protection and PPD responses in BCG and BCG/H1+CAF01 boosted mice in a long-term model of *M. tuberculosis* infection. (**A**) Bacterial load in lungs of mice chronically infected for 50 wk. Mice (C57BL/6) were immunized once with 5×10^6 BCG Danish by the s.c. route at the base of the tail (0.2 ml 2.5×10^7 CFU/ml BCG Danish). Four weeks later, one group received a single s.c. booster with 2 μg of the heterologous subunit vaccine H1 (Ag85B-ESAT-6)+CAF01 (250/50). Six weeks after the booster, mice were aerosol challenged with *M. tuberculosis* Erdman. Mice (6 mice/group/time point) were subsequently euthanized at weeks 7, 26, and 50 into the infection, and CFU levels were determined in BCG, BCG_{Prime}-H1+$CAF01_{boost}$, as well as nonimmunized, challenged mice. Error bars represent SEM. ANOVA with Tukey posttest. Week 7 postinfection: $***p < 0.001$; BCG versus BCG_{Prime}-H1+$CAF01_{boost}$ n.s.; week 26 postinfection: $***p < 0.001$; BCG versus nonimmunized n.s.; week 50 postinfection: $***p < 0.001$; BCG versus nonimmunized n.s.; BCG versus BCG_{Prime}-H1+$CAF01_{boost}$ n.s. Repeated once until week 26 with comparable results. (**B**) Evaluation of CD4 responses to PPD in mice preinfection and postinfection (here lung responses of BCG-H1/CAF01 mice 7 wk postimmunization). As shown, IFN-γ–, TNF-α–, and IL-2–producing $CD44^{high}$ cells were gated and based on Boolean gating, and cytokine coexpression profiles were established (seven subpopulations of cytokine-producing cells in any combination). The frequency of Ag-specific "any cytokine-response" was established, as well as the degree of multifunctionality. Finally, IL-2 +ve cells (in any combination) of the cytokine-responding cells were determined, both in terms of proportion and frequency to determine levels of self-renewing memory T cells induced and maintained into chronicity by the different immunization regimens. (**C**) Time course of PPD-responsive (IFN-γ, TNF-α, IL-2 in any combination) CD4 and CD8 T cells in lungs before and long into a chronic *M. tuberculosis* infection ($n = 4$ mice/group/time point (though 3 mice/group at day 0); error bars indicate SEM. Repeated once until week 26 with comparable results. ANOVA with Tukey posttest; $*p < 0.05$.

during infection, the protective efficacy of BCG clearly declined and no significant protection relative to the nonimmunized control animals were found at weeks 26 and 50 postchallenge (Fig. 1A). From week 7 onward, neither nonimmunized nor BCG-immunized animals were capable of controlling the infection, and bacterial numbers increased to log 6.15 and 5.3, respectively, at week 50. Only the BCG-H1/CAF01 immunized animals provided significant protection at all time points and controlled the late stages of infection much more efficiently than BCG alone, leading to a 1.7–2 log10 reduction in CFU loads at weeks 26 and 50 ($p < 0.001$; Fig. 1A).

The mycobacteria-specific T cell response was analyzed at different time points during infection by restimulation of T cells isolated from perfused lungs followed by intracellular flowcytometry (Fig. 1B). Initially, we focused on describing possible differences among the groups in the frequency of TB-specific T cells during the course of infection. Increased numbers of PPD-specific CD4 cells were seen in the boosted group at the 7-wk time point compared with BCG alone, but the difference was not statistically significant (Fig. 1C). Otherwise, no clear differences on CD4 PPD responses were observed except at the latest time point (50 wk), where nonimmunized mice had the highest number of PPD-specific T cells ($p < 0.05$; Fig. 1C). PPD-specific CD8 responses were at the same level in the early phases of infection (7 wk) but were quite different later during infection where both nonimmunized and BCG-immunized mice were found to have higher numbers of specific CD8 cells compared with the boosted group (week 26; $p < 0.05$). Thus, CD8 responses to PPD seem to correlate with infection levels as previously demonstrated in a similar model (10). Taken together, these data demonstrate that the magnitude of PPD-specific, cytokine-producing cells cannot readily explain the large difference observed in the ability to contain late-stage infection between the BCG immunized group and the group boosted with H1+CAF01.

Boosting with H1/CAF01 maintains IL-2–producing CD4 T cells throughout infection

The cytokine profile of PPD-specific CD4 T cell responses in lung and spleen of vaccinated and nonvaccinated mice was subsequently analyzed at different time points postinfection. BCG-vaccinated mice had a very significant proportion (~30%) of IL-2 +ve cells before infection (time 0, Fig. 2A), but this population was rapidly diminished during infection, and IL-2–producing cells (in any combination) accounted for only 5 and <2% of the cytokine-producing CD4 T cells at weeks 26 and 50, respectively. This cytokine expression level was similar to the unvaccinated infected control animals (Fig. 2A). The subunit boost maintained a higher frequency of IL-2–producing CD4 cells ($TNF\text{-}\alpha^+IL\text{-}2^+$ and $IFN\text{-}\gamma^+TNF\text{-}\alpha^+IL\text{-}2^+$), detectable at all time points of infection (Fig. 2A). These IL-2–producing memory cells were maintained long into chronic infection and still accounted for ~10% of the PPD-responsive population almost a year into the infection. The difference between the BCG-vaccinated and the boost group was most pronounced at week 26, where IL-2–producing cells in the

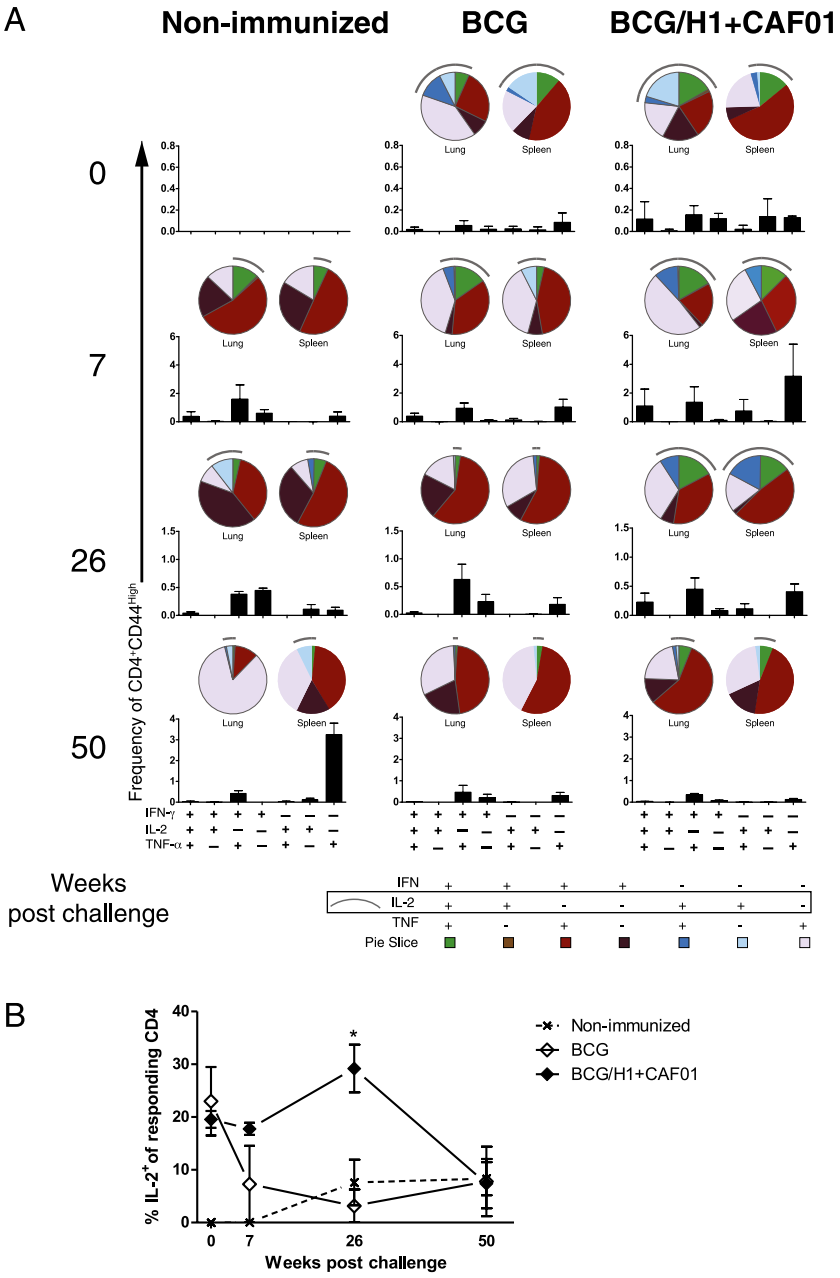

FIGURE 2. Degree of multifunctional PPD responses and IL-2–producing CD4 T cell subsets in BCG and BCG/H1+CAF01 vaccinated mice during chronic *M. tuberculosis* infection. (**A**) Frequency of multifunctional PPD-specific CD4 $CD44^{high}$ T cells in lungs of mice before and at 7, 26, and 50 wk into a chronic *M. tuberculosis* infection. Pies above each bar chart denote the proportion of each cytokine-producing subset of the responding cells from both lung and spleen, whereas the arcs denote IL-2–producing cells (in any combination) of the PPD-responding CD4 T $CD44^{high}$ cells (n = 3–4 mice/group/time point; error bars denote SEM). Repeated once until week 26 with comparable results. (**B**) The time course in the proportion of IL-2 +ve self-renewing memory cells of the PPD-responsive CD4 $CD44^{high}$ T cells in spleen during chronic infection (n = 3–4 mice/group/time point; error bars indicate SEM). Repeated once until week 26 with comparable results. ANOVA with Tukey posttest; $^{*}p < 0.05$.

boosted group equaled ~20% of the PPD-responding CD4 T cells in the lungs, whereas such cells were almost absent in the lung of the BCG group and accounted for only 5% of the responding cells. Similarly, the proportions of IL-2 +ve cells in the spleen of BCG-immunized mice were negligible in all phases of infection (<7% at all times during infection; Fig. 2B). The prime-boost group for comparison maintained a very high proportion of IL-2–producing CD4 cells in the spleen up to 26 wk into the infection. At this time point, the boosted group had a significantly higher proportion of $IL\text{-}2^{+}$ CD4 T cells (30 versus 3%; $p < 0.05$; Fig. 2B).

IL-2–producing CD4 T cells are specific for the H1 vaccine

The observed differences in T cell quality can be interpreted either as a subset promoted by the booster vaccine or a consequence of the efficient control with bacterial replication in the BCG boost group. In the last case, low CFU levels would be expected to result in a lower degree of transition to terminally differentiated effectors, and thus to increased levels of $IL\text{-}2^{+}$ memory cells directed to mycobacterial Ags in general. To distinguish these two possibilities, we analyzed whether the frequency and quality of CD4 T cells specific for Ags contained within the subunit booster vaccine (Ag85B and ESAT-6) differed from Ags primed by BCG (TB10.4) that did not control the chronic stage of infection. Clear and significantly higher CD4 T cell responses to the two subunit-derived Ags (Ag85B and ESAT-6) could be observed in the BCG-H1/CAF01 group 7 wk postinfection ($p < 0.05$ any cytokine response; Fig. 3A), whereas responses to TB10.4 were comparable among the groups. In terms of magnitude of the response, no other significant differences between the groups could be observed except for CD4 responses to TB10.4 in the nonimmunized group at 50 wk postinfection ($p < 0.01$; Fig. 3A). At week 26, where the largest difference in bacterial numbers was found (Fig. 1A), no significant differences in the magnitude of CD4 responses to any of the three Ags were found. However, when focusing on the cytokine expression of responses, the BCG-H1/CAF01 booster group was found to promote a vaccine-specific response of a clearly different quality than BCG alone. The booster group was characterized by a large proportion of IL-2 +ve T cells directed to

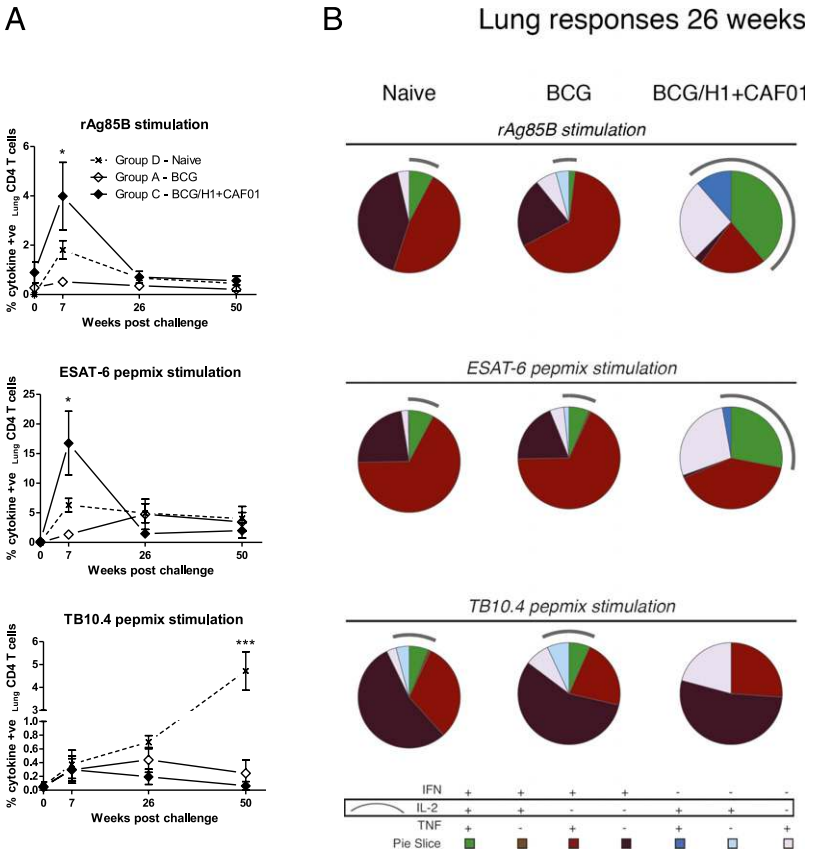

FIGURE 3. The maintenance of IL-2–producing memory cell differs among Ags primed by subunit vaccination and BCG and/or infection. (**A**) Time course of rAg85B- (top), ESAT-6 pepmix- (middle), and TB10.4 pepmix-responsive (bottom) CD4 CD44^{high} T cells (IFN-γ, TNF-α, IL-2 in any combination) in lungs before and long into a chronic *M. tuberculosis* infection. Mean ± SEM; n = 3–4 mice/group/time point. ANOVA with Tukey posttest; $*p < 0.05$, $***p < 0.001$. Repeated once until week 26 with comparable results. (**B**) Pies charts denote the proportion of each cytokine-producing subset of the responding cells from the lungs of infected mice 26 wk into a *M. tuberculosis* infection, whereas the arcs denote IL-2–producing cells (in any combination) of the rAg85B– (top), ESAT-6 pepmix– (middle), and TB10.4 pepmix–responding (bottom) CD4 T CD44^{high} cells. Note that although Ag85B and ESAT-6 recall responses in the BCG-H1/CAF01 group are characterized by a high proportion of IL-2–producing cells (in any combination) even 26 wk into infection, TB10.4 recall responses (thus primed by the BCG vaccination and boosted/maintained by infection) in this group are completely devoid of these memory cells. n = 4 mice/group; representative of two independent experiments.

Ag85B and ESAT-6 (both contained in the subunit booster vaccine) and no IL-2+ve cells directed to TB10.4 (expressed by BCG). In the BCG group, only a few IL-2$^+$ cells were found and primarily directed to TB10.4. This demonstrates that the ability to maintain an IL-2$^+$ memory population during infection is specifically related to Ags contained in and promoted by the subunit vaccine and not a consequence of a general impact on the phenotypic profile of all T cells primed during infection.

Boosting with H1/CAF01 prevents CD4 T cell exhaustion

We continued our characterization of immune responses in the vaccinated groups by analyzing the differentiation marker/inhibitory receptor KLGR1. This marker has previously been demonstrated to identify CD4 T cell populations at different stages of differentiation during persistent infections, including TB infection (23, 39). In *M. tuberculosis* infection, KLRG1^{high}PD-1^{low} expression defines a population of terminally differentiated T cells with capacity for production of effector cytokines, but limited proliferative potential (23). We examined the expression of the KLRG1 receptor on cytokine-producing CD4 T cells in the lungs of nonimmunized, BCG-immunized, and BCG-H1/CAF01 boosted mice 10 wk into an infection (Fig. 4). We focused our analysis on ESAT-6 responses, which would either be primed by the subunit boost (BCG-H1/CAF01) or driven by the infection (nonimmunized and BCG). Among ESAT-6–specific CD4 T cells producing "any cytokine," we observed a pronounced and highly significant reduction in the proportion of terminally differentiated KLRG1-expressing cells in both the BCG group (24.6%) and the boosted group (6.1%), compared with the nonimmunized controls (56.5%; Fig. 4A, 4B). Interestingly, we also observed a large difference in the expression of KLRG1 among PD-1$^+$ cells in the different groups (with KLRG1 +ve cells constituting ∼50, 20, and 8% of the PD-1–expressing cells in the nonimmunized, BCG, and boosted groups, respectively; data not shown). We next asked how the T cell cytokine expression phenotypes were distributed among KLRG1 −ve and KLRG1 +ve subsets. To address this, we split ESAT-6–responsive cells into two groups based on KLRG1 expression, and the relative contribution of cytokine-coproducing subsets within these two groups was determined (Fig. 4C). In the nonimmunized and the BCG group, no clear differences could be observed between cells expressing KLRG1 or not in terms of their composition of cytokine-coproducing subsets. However, in the BCG-H1/CAF01 group, the majority of KLRG1$^-$ ESAT-6–specific cells were IL-2 producers, and the IL-2$^+$TNF-α$^+$ population accounted for half of the KLRG1$^-$ population. This was in contrast with the KLRG1$^+$ subset, which comprised T cells producing cytokines in any combination (Fig. 4C). Interestingly, the classical effector T cells characterized by IFN-γ single producers or in combination with TNF-α were selectively underrepresented among the KLRG1$^-$ cells in the boosted group compared with nonvaccinated or the BCG group (<10 versus ∼50%, respectively).

IL-2–producing CD4 T cells possess high proliferative capacity

In this study, we have observed a correlation between efficient protection (in the boosted group) and the presence of IL-2$^+$ TNF-α$^+$, KLGR1$^-$ CD4 T cells, a subset that most likely represent long-lived, proliferating, and self-renewing central memory cells (23, 36, 40). We therefore continued by a rigorous assessment of the memory potential of this population. First, we examined whether the IL-2–producing cells promoted by the H1+CAF01 subunit vaccine are phenotypically characteristic of T_{CM}. To this end, splenocytes from mice 11 wk after three immunizations with H1+CAF01 were examined for coexpression of IL-2 and CD62L. We found that the IL-2$^+$ T cells induced by the H1+CAF01 vaccine were almost exclusively confined to the CD62L^{high} population, and

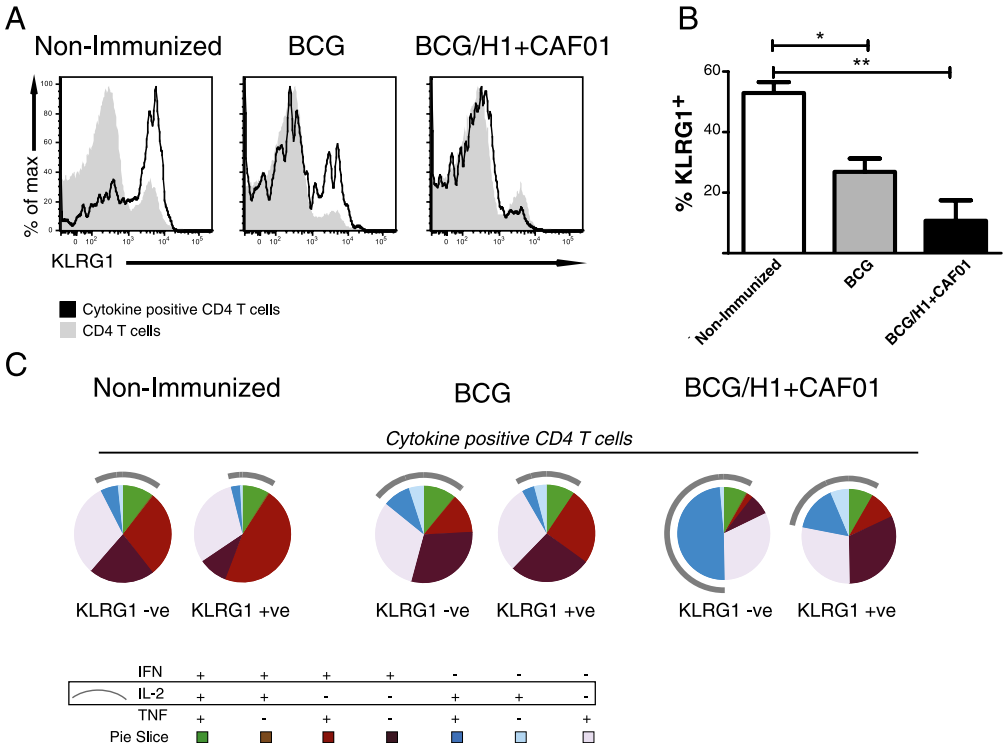

FIGURE 4. The expression of exhaustion/differentiation marker KLRG1 is influenced by vaccination status. In a separate experiment, four mice/group either nonimmunized, BCG immunized, or boosted once with H1/CAF01 were aerosol challenged 6 wk after last immunization. Ten weeks into the infection, mice were sacrificed and responses in the lung determined. Cytokine-producing cells ("any cytokine") were subsequently examined for their KLRG1 expression. Representative of two independent experiments. (**A**) Representative FACS histograms showing KLRG1 expression for ESAT-6–specific CD4 T cells (producing "any cytokine") 10 wk postinfection in mice either nonimmunized (*left panel*), BCG (*middle panel*), or BCG-H1/CAF01 (*right panel*) immunized. Histogram overlays show KLRG1 expression in ESAT-6–specific CD4 T cells (producing "any cytokine") relative to all CD4 T cells in each group. (**B**) The proportion of ESAT-6–responsive cells being KLRG1$^+$ in nonimmunized, BCG, or BCG/H1+CAF01 boosted mice was subsequently established. ANOVA with Tukey posttest; $*p < 0.05$, $**p < 0.01$. (**C**) ESAT-6–responsive cells were subdivided based on their KLRG1 expression, and the distribution of cytokine coexpressing subsets within KLRG1 −ve versus KLRG1 +ve cells was subsequently determined. The arcs above each pie chart denote IL-2–producing cells (in any combination) of the ESAT-6–responding CD4 T cells.

that a large proportion of these cells therefore can be characterized as central memory cells (Supplemental Fig. 1).We next investigated T cells, which are recruited to and proliferate in the lymph nodes draining the TB-infected lung during the recall of a long-term memory response. To this end, animals that were either nonimmunized, BCG, or BCG boosted with H1/CAF01 were immunized and rested for almost 2 y to ensure the establishment of a truly resting memory population. The mice were then aerosol challenged and sacrificed 3 wk into the infection. Mice were administered BrdU in their drinking water 4 d before termination, and cytokine-producing subsets incorporating BrdU were determined in pooled (3–4 mice/group), lung-draining tracheobronchial lymph nodes (Fig. 5). A few mice from each group received BrdU in drinking water for 4 d and were euthanized before infection. At this time point before challenge (2 y after immunization), no detectable T cell proliferation driven by the immunization could be detected (data not shown). As we paid special attention to the IL-2–producing CD4 T cell subset(s) specifically induced by the subunit boost, we started by looking at BrdU incorporation in the IL-2$^+$ ESAT-6–specific CD4 T cells (Fig. 5A). ESAT-6–specific IL-2 production was negligible in the lung-draining lymph nodes of the nonimmunized group 3 wk into the infection regardless of whether the CD4 T cells had incorporated BrdU (Fig. 5A). This was clearly different in the BCG-H1/CAF01 group, where the ESAT-6–specific IL-2 response was pronounced and the majority had incorporated BrdU (Fig. 5A). IL-2–producing cells were also found in the BCG vaccinated mice but at a much lower level and without noteworthy BrdU incorporation (Fig. 5A). We next analyzed the distribution of cytokine coproducing subsets among the proliferating cells. We identified the TNF-α^+IL-2$^+$, but also the IFN-γ^+TNF-α^+IL-2$^+$, subsets to dominate the BrdU$^+$ CD4 T cell population in the prime-boost group, although TNF-α single-positive cells were also identified within this proliferating pool (Fig. 5B). The BrdU$^-$ population was dominated by IL-2 single-positive T cells, but this population was also found in the BCG alone group (Fig. 5B). Thus, the expansion of TNF-α^+IL-2$^+$ CD4 T cells (with or without concomitant production capacity for IFN-γ) seen in the lung-draining lymph nodes of the boosted animals represents a long-lived reservoir of specific central memory cells with proliferative potential that can be mobilized from secondary lymphoid tissues upon challenge.

Discussion

A notable characteristic of infection with *M. tuberculosis* is that adaptive immune responses can arrest progression of the disease, but rarely (if ever) eradicate the bacteria resulting in sterile immunity. This is a consequence of the fact that *M. tuberculosis* possesses highly effective mechanisms for evading effector T cell responses and establishes an often lifelong coexistence with the infected human host. To be efficient, TB vaccines need to be able to overcome or counteract the complex regulatory signals encountered in such an environment of ongoing TB infection and maintain a strong memory immunity resulting in long-term containment of infection. In agreement with previous observations

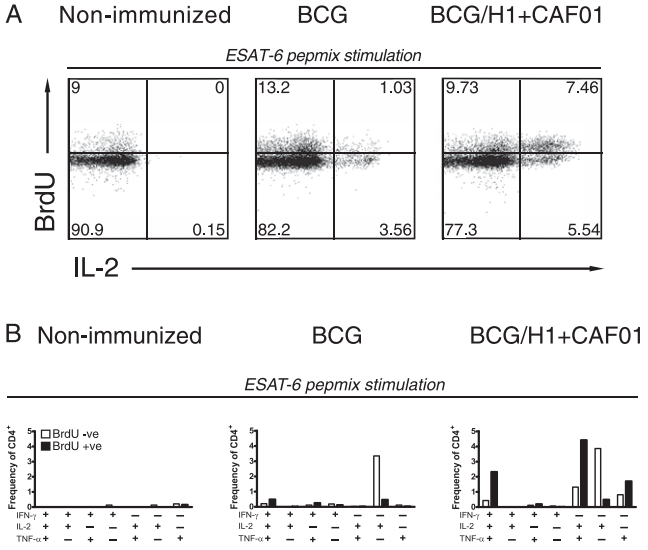

FIGURE 5. IL-2 production and BrdU incorporation in the lung-draining lymph nodes of long-term memory mice after challenge. (**A** and **B**) In a separate experiment, 3–4 mice/group were aerosol challenged ~2 y after last immunization. Three weeks into the infection, mice were sacrificed and responses in the lung-draining, tracheobronchial lymph nodes were analyzed (pooled from 3–4 mice). Mice were given BrdU in drinking water 4 d before termination. (A) FACS plots showing BrdU incorporation relative to Ag-specific IL-2 production after ESAT-6 pepmix recall in lung-draining lymph nodes. (B) Cytokine-producing subsets with (black bars) or without (white bars) BrdU incorporation after stimulation with ESAT-6 pepmix.

(41, 42), we show that BCG in an animal infection model fails to promote a T cell response with the ability to contain the later stages of infection. The protection we observe is transient and lost as the infection progresses into the chronic stage. In this study, we show that this loss of immunity and regrowth of *M. tuberculosis* is coincident with a change of T cell quality and almost complete disappearance of IL-2–producing CD4 T cells. Importantly, we demonstrate that a booster vaccination expand and maintain CD4 cells characterized by their IL-2 production, low levels of KLGR1 expression, and with proliferative potential up to 2 y after vaccination. Furthermore, we show that the IL-2^+ T cells imprinted by the subunit vaccine are $CD62L^{high}$ typical of a T_{CM} phenotype. The overrepresentation of the IL-2^+TNF-α^+ subset among KLRG1 −ve cells supports the notion that this population represents a less differentiated cell type (40) and suggests that these cells are intrinsically more resistant to functional exhaustion. This subset therefore fulfills important criteria for being the key memory pool that mediates both long-term protection after vaccination and provides the reservoir of central memory cells that continuously replenish and prevent exhaustion of the T cell reservoir during ongoing infection. The relative importance of T_{CM} and T_{EM} for protective immunity against TB is not fully settled. However, our group has earlier reported that adoptive transfer of memory T cells (primary *M. tuberculosis* infection cleared by antibiotic treatment) into nude mice conferred protection and further documented that the highest level of resistance to challenge with *M. tuberculosis* was mediated by cells with a central memory phenotype ($CD45RB^{high}$, $CD62L^{high}$, $CD44^{low}$) (30).

That ongoing mycobacterial infection has a detrimental influence on the quality of the T cell response is in accordance with data from both animal models (36, 43) and infected individuals (24–26). Progressive impairment of *M. tuberculosis*–specific T cell responses in TB patients has been reported to be associated with increasing mycobacterial load (24–26), leading to a decrease in polyfunctional and IL-2–producing cells (24). As the infection was brought to a stop by anti-TB treatment, T cell quality and the proportion of IL-2–producing subsets was again partly normalized (24). Ag persistency is thus one important factor responsible for T cell exhaustion, and in an elegant transgenic model of Ag clearance by molecular switch, it has been shown that persistent Ag by itself rapidly induces a dysfunctional state of the CD4 T cells in terms of proliferative capacity and IL-2 production, which is only partially reversible after Ag removal (44). Henao-Tamayo et al. (45) recently used a murine model to show that clearance of *M. tuberculosis* by chemotherapy led to expansion of both T_{EM} and T_{CM} accompanied by a rapid but transient containment of a secondary infection. In this study, PD-1 expression dropped dramatically to low levels coincident with the breakdown of immunity (45). Thus, Ag persistency definitely plays an important role for T cell exhaustion and attrition, but the functional abilities of T cells are also controlled by other mechanisms such as the expression and combination of various inhibitory receptors, including CTLA-4, KLRG1, and PD-1.

Our study demonstrates that a single H1/CAF01 booster immunization after BCG significantly enhances the protection in the later stages of infection in mice. This increased protection was most likely not related to the number of specific T cells in the lung, as no significant difference in CD4 frequency between the BCG and the BCG-H1/CAF01 group was found. For CD8 responses, even higher numbers were found in the BCG and nonimmunized groups; data that are in line with reports suggesting that infection-driven CD8 responses primarily reflect CFU levels (10, 24, 43, 46, 47), rather than mediating a protective response to *M. tuberculosis* infection (48–51). Our data demonstrate that the main function of the subunit booster seems to be the prevention of the accelerated decay of the IL-2^+ memory CD4 T cell pool found after BCG vaccination. Interestingly, not only live vaccines such as BCG and *Leishmania* (36, 52), but also viral vectors seem inferior at promoting, in particular, IL-2/TNF-α^+ $CD4^+$ T cells (53) (R. Billeskov, C. Aagaard, J.P. Christensen, P. Andersen, and J. Dietrich, unpublished observations). By boosting with H1/CAF01, BCG responses can be supplemented with a pool of TNF-α^+IL-2^+ central memory cells possessing high proliferative potential, a reservoir of memory cells we suggest is pivotal for the late-stage containment of ongoing TB infection. Notably, we excluded the possibility that the influence was indirect and simply a consequence of improved bacterial control in the boosted groups, as the maintenance of IL-2–producing memory cells were not uniform across Ags and could be directly attributed to Ags promoted by the subunit vaccine. In a murine model of chronic *M. tuberculosis* infection, Reiley et al. (23) recently reported that CD4 T cells transitioned into terminally differentiated KLRG1-expressing effector cells with low proliferative capacity, a transition associated with loss of efficient immune surveillance in the chronic stages of infection. Accordingly, we observed that nonimmunized mice 10 wk into infection harbored a large population of KLRG1-expressing CD4 T cells, a trait consistently seen for all TB restimulation Ags used. Prior BCG vaccination, and to an even larger extent the H1/CAF01 booster, significantly diminished the emergence of this KLRG1-expressing subset, and thus blunted and delayed the exhaustion process. We further observed that the TNF-α^+IL-2^+ population was overrepresented in the $KLRG1^-$ compared with $KLRG1^+$ subset, which supports the conclusion that this phenotype is indeed not an exhausted T cell population. In agreement with this conclusion, the IL-2–producing T cells were primarily confined to a $CD62L^{high}$ TCM population and exhibited an impressive proliferative potential as demonstrated in mice

challenged with *M. tuberculosis* 2 y after vaccination. Responses in the lung-draining lymph nodes from these memory mice 3 wk into infection revealed that the H1/CAF01 boosted animals had a large population of vaccine-specific BrdU and IL-2$^+$ T cells (mostly as TNF-α^+IL-2$^+$ and IFN-γ^+TNF-α^+IL-2$^+$). Through eliciting a population of KLRG1$^-$ IL-2–producing CD4 cells, the improved containment of infection mediated by the H1/CAF01 boost most likely rely on this pool of long-lived, proliferating central memory cells for replenishing and maintaining Ag-specific effector T cells during the chronic stages of *M. tuberculosis* infection. Our interpretation of these data, however, does not exclude other contributing mechanisms not measured in this study, such as differences in the expression of additional cytokines, functional avidity, or cytotoxic potential of the different CD4 T cell subsets.

So why is the subunit boost so much more efficient than BCG in maintaining a stable and robust memory population resistant to infection-driven terminal differentiation and exhaustion? As discussed earlier, it seems not to be a simple consequence of a larger memory pool in the boosted group. Clearly, the specificity of the response is different, as boosting with H1 results in the selective expansion of ESAT-6–specific T cells not found after BCG vaccination and this obviously represent one difference. However, the most important difference probably relates to the quality of the primary T cell response promoted by the subunit vaccine and by BCG. It has previously been reported that BCG is unable to maintain a large population of high-quality memory cells (10, 36, 54). BCG-promoted responses are (like the real TB infection) dominated by effector-like T cell subsets secreting IFN-γ and/or TNF-α (41, 54, 55), and the numbers of IL-2$^+$ T cells are much lower than after a H1/CAF01 subunit vaccination (36). The recent identification of BCG-specific CD4 T cells with a central memory phenotype (based on CCR7 expression) in a longitudinal study of infants (29) seems at odds with the observations in the mouse model and led Soares et al. (29) to suggest that BCG might be cleared more rapidly in humans than in mice. The fate and longevity of BCG in humans is not known with certainty (and for obvious reasons is difficult to assess), but reports of BCG lymphadenitis and BCG dissemination years after BCG vaccination (56–60) have been used to support a relatively long life span of this vaccine in humans. For this discussion, it is important to note that in the study by Soares et al. (29), the BCG-specific CD4 T cells presented functional attributes more akin to T_{EM} (characterized by IFN-γ), which was clearly different from the IL-2$^+$ CD4 T_{CM}-specific for tetanus toxoid. Based on functional criteria, the subset induced by BCG in humans is therefore quite similar to the observations from the mouse model. It is, however, important to bear in mind that in addition to Ag persistence, the observed difference in vaccine responses between BCG and an adjuvanted subunit vaccine may relate to the different environment that such two fundamentally different vaccines represent during priming. This viewpoint is further reinforced by other recent data from our laboratory, where a TB vaccine given in the context of an adjuvanted subunit vaccine promotes higher levels of IL-2–producing T cells leading to better containment of a chronic *M. tuberculosis* infection compared with the same Ags given in a live viral vector (R. Billeskov, C. Aagaard, J.P. Christensen, P. Andersen, and J. Dietrich, unpublished observation).

It is a well-established fact that BCG provides the lowest level of protection against TB in the regions with the highest prevalence of latent infection (4). Adult pulmonary TB in these populations would in a large number of cases be expected to represent reactivation of latent TB (13, 61, 62). Understanding the underlying mechanism(s) for this loss of protection by BCG during ongoing latent infection is key for the development of new TB vaccine strategies, including prime-boost scenarios that will succeed in the efforts to control TB. We suggest that TNF-α^+IL-2$^+$ central memory CD4 T cells, characterized by high proliferative capacity and low KLRG1 expression levels, identify the key memory pool responsible for late-stage containment of TB infection, and ultimately for preventing reactivation of latent TB.

Acknowledgments

We thank Linda Christensen, Rune Fledelius Jensen, Sandra Isling, and Merete Henriksen for excellent technical help, as well as the staff at the experimental animal facilities at Statens Serum Institut. We specifically thank Joshua Woodworth for establishing PD-1/KLRG1 staining panels and for several fruitful discussions on T cell exhaustion and BCG memory.

Disclosures

E.M.A. and P.A. are coinventors of patents relating to cationic liposomes as vaccine adjuvants. P.A. is coinventor of patents relating to TB fusion protein Ag85B-ESAT-6. All rights have been assigned to the Statens Serum Institut. The other authors have no financial conflicts of interest.

References

1. WHO. 2011. Global Tuberculosis Control. WHO report 2011. World Health Organization, Geneva, Switzerland.
2. Colditz, G. A., C. S. Berkey, F. Mosteller, T. F. Brewer, M. E. Wilson, E. Burdick, and H. V. Fineberg. 1995. The efficacy of bacillus Calmette-Guérin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature. *Pediatrics* 96: 29–35.
3. Colditz, G. A., T. F. Brewer, C. S. Berkey, M. E. Wilson, E. Burdick, H. V. Fineberg, and F. Mosteller. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *JAMA* 271: 698–702.
4. Fine, P. E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 346: 1339–1345.
5. Trunz, B. B., P. E. M. Fine, and C. Dye. 2006. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 367: 1173–1180.
6. Brooks, J. V., A. A. Frank, M. A. Keen, J. T. Bellisle, and I. M. Orme. 2001. Boosting vaccine for tuberculosis. *Infect. Immun.* 69: 2714–2717.
7. Kipnis, A., S. Irwin, A. A. Izzo, R. J. Basaraba, and I. M. Orme. 2005. Memory T lymphocytes generated by Mycobacterium bovis BCG vaccination reside within a CD4 CD44lo CD62 ligand(hi) population. *Infect. Immun.* 73: 7759–7764.
8. Sterne, J. A. C., L. C. Rodrigues, and I. N. Guedes. 1998. Does the efficacy of BCG decline with time since vaccination? *Int. J. Tuberc. Lung Dis.* 2: 200–207.
9. Aronson, N. E., M. Santosham, G. W. Comstock, R. S. Howard, L. H. Moulton, E. R. Rhoades, and L. H. Harrison. 2004. Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: A 60-year follow-up study. *JAMA* 291: 2086–2091.
10. Billeskov, R., T. T. Elvang, P. L. Andersen, and J. Dietrich. 2012. The HyVac4 subunit vaccine efficiently boosts BCG-primed anti-mycobacterial protective immunity. *PLoS ONE* 7: e39909.
11. Brennan, M. J., B. Clagett, H. Fitzgerald, V. Chen, A. Williams, A. A. Izzo, and L. F. Barker. 2012. Preclinical evidence for implementing a prime-boost vaccine strategy for tuberculosis. *Vaccine* 30: 2811–2823.
12. Rowland, R., and H. McShane. 2011. Tuberculosis vaccines in clinical trials. *Expert Rev. Vaccines* 10: 645–658.
13. Crampin, A. C., J. R. Glynn, and P. E. M. Fine. 2009. What has Karonga taught us? Tuberculosis studied over three decades. *Int. J. Tuberc. Lung Dis.* 13: 153–164.
14. Donald, P. R. 2004. Childhood tuberculosis: the hidden epidemic. *Int. J. Tuberc. Lung Dis.* 8: 627–629.
15. Gallant, C. J., A. Cobat, L. Simkin, G. F. Black, K. Stanley, J. Hughes, T. M. Doherty, W. A. Hanekom, B. Eley, N. Beyers, et al. 2010. Impact of age and sex on mycobacterial immunity in an area of high tuberculosis incidence. *Int. J. Tuberc. Lung Dis.* 14: 952–959.
16. Akbar, A. N., and S. M. Henson. 2011. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat. Rev. Immunol.* 11: 289–295.
17. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J. Virol.* 77: 4911–4927.
18. Argüello, R. J., M. C. Albareda, M. G. Alvarez, G. Bertocchi, A. H. Armenti, C. Vigliano, P. C. Meckert, R. L. Tarleton, and S. A. Laucella. 2012. Inhibitory receptors are expressed by *Trypanosoma cruzi*-specific effector T cells and in hearts of subjects with chronic Chagas disease. *PLoS ONE* 7: e35966.
19. Jurado, J. O., I. B. Alvarez, V. Pasquinelli, G. J. Martínez, M. F. Quiroga, E. Abbate, R. M. Musella, H. E. Chuluyan, and V. E. García. 2008. Programmed

death (PD)-1:PD-ligand 1/PD-ligand 2 pathway inhibits T cell effector functions during human tuberculosis. *J. Immunol.* 181: 116–125.
20. Sakai, S., I. Kawamura, T. Okazaki, K. Tsuchiya, R. Uchiyama, and M. Mitsuyama. 2010. PD-1-PD-L1 pathway impairs $T_{(h)}1$ immune response in the late stage of infection with *Mycobacterium bovis bacillus* Calmette-Guérin. *Int. Immunol.* 22: 915–925.
21. Schreiber, H. A., P. D. Hulseberg, J. Lee, J. Prechl, P. Barta, N. Szlavik, J. S. Harding, Z. Fabry, and M. Sandor. 2010. Dendritic cells in chronic mycobacterial granulomas restrict local anti-bacterial T cell response in a murine model. *PLoS ONE* 5: e11453.
22. Smith, P., C. M. Walsh, N. E. Mangan, R. E. Fallon, J. R. Sayers, A. N. J. McKenzie, and P. G. Fallon. 2004. *Schistosoma mansoni* worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. *J. Immunol.* 173: 1240–1248.
23. Reiley, W. W., S. Shafiani, S. T. Wittmer, G. Tucker-Heard, J. J. Moon, M. K. Jenkins, K. B. Urdahl, G. M. Winslow, and D. L. Woodland. 2010. Distinct functions of antigen-specific CD4 T cells during murine *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* 107: 19408–19413.
24. Day, C. L., D. A. Abrahams, L. Lerumo, E. Janse van Rensburg, L. Stone, T. O'rie, B. Pienaar, M. de Kock, G. Kaplan, H. Mahomed, et al. 2011. Functional capacity of *Mycobacterium tuberculosis*-specific T cell responses in humans is associated with mycobacterial load. *J. Immunol.* 187: 2222–2232.
25. Millington, K. A., J. A. Innes, S. Hackforth, T. S. C. Hinks, J. J. Deeks, D. P. S. Dosanjh, V. Guyot-Revol, R. Gunatheesan, P. Klenerman, and A. Lalvani. 2007. Dynamic relationship between IFN-γ and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. *J. Immunol.* 178: 5217–5226.
26. Sester, U., M. Fousse, J. Dirks, U. Mack, A. Prasse, M. Singh, A. Lalvani, and M. Sester. 2011. Whole-blood flow-cytometric analysis of antigen-specific CD4 T-cell cytokine profiles distinguishes active tuberculosis from non-active states. *PLoS ONE* 6: e17813.
27. Kagina, B. M. N., B. Abel, M. Bowmaker, T. J. Scriba, S. Gelderbloem, E. Smit, M. Erasmus, N. Nene, G. Walzl, G. Black, et al. 2009. Delaying BCG vaccination from birth to 10 weeks of age may result in an enhanced memory CD4 T cell response. *Vaccine* 27: 5488–5495.
28. Soares, A. P., T. J. Scriba, S. Joseph, R. Harbacheuski, R. A. Murray, S. J. Gelderbloem, A. Hawkridge, G. D. Hussey, H. Maecker, G. Kaplan, and W. A. Hanekom. 2008. *Bacillus* Calmette-Guérin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J. Immunol.* 180: 3569–3577.
29. Soares, A. P., C. K. Kwong Chung, T. Choice, E. J. Hughes, G. Jacobs, E. J. van Rensburg, G. Khomba, M. de Kock, L. Lerumo, L. Makhethe, et al. 2013. Longitudinal changes in $CD4(^{+})$ T-cell memory responses induced by BCG vaccination of newborns. *J. Infect. Dis.* 207: 1084–1094.
30. Andersen, P., and B. Smedegaard. 2000. $CD4(^{+})$ T-cell subsets that mediate immunological memory to *Mycobacterium tuberculosis* infection in mice. *Infect. Immun.* 68: 621–629.
31. Duffy, D., A. Dawoodji, E. M. Agger, P. Andersen, J. Westermann, and E. B. Bell. 2009. Immunological memory transferred with CD4 T cells specific for tuberculosis antigens Ag85B-TB10.4: persisting antigen enhances protection. *PLoS ONE* 4: e8272.
32. Lázár-Molnár, E., B. Chen, K. A. Sweeney, E. J. Wang, W. J. Liu, J. Lin, S. A. Porcelli, S. C. Almo, S. G. Nathenson, and W. R. Jacobs, Jr. 2010. Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis. *Proc. Natl. Acad. Sci. USA* 107: 13402–13407.
33. Weinrich Olsen, A., L. A. H. van Pinxteren, L. Meng Okkels, P. Birk Rasmussen, and P. Andersen. 2001. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6. *Infect. Immun.* 69: 2773–2778.
34. Davidsen, J., I. Rosenkrands, D. Christensen, A. Vangala, D. Kirby, Y. Perrie, E. M. Agger, and P. Andersen. 2005. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses. *Biochim. Biophys. Acta* 1718: 22–31.
35. Dietrich, J., C. Aagaard, R. Leah, A. W. Olsen, A. Stryhn, T. M. Doherty, and P. Andersen. 2005. Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy. *J. Immunol.* 174: 6332–6339.
36. Lindenstrøm, T., E. M. Agger, K. S. Korsholm, P. A. Darrah, C. Aagaard, R. A. Seder, I. Rosenkrands, and P. Andersen. 2009. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J. Immunol.* 182: 8047–8055.
37. Elvang, T., J. P. Christensen, R. Billeskov, T. Thi Kim Thanh Hoang, P. Holst, A. R. Thomsen, P. Andersen, and J. Dietrich. 2009. CD4 and CD8 T cell responses to the *M. tuberculosis* Ag85B-TB10.4 promoted by adjuvanted subunit, adenovector or heterologous prime boost vaccination. *PLoS ONE* 4: e5139.
38. Roederer, M., J. L. Nozzi, and M. C. Nason. 2011. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A* 79: 167–174.
39. Odorizzi, P. M., and E. J. Wherry. 2012. Inhibitory receptors on lymphocytes: insights from infections. *J. Immunol.* 188: 2957–2965.
40. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* 8: 247–258.
41. Henao-Tamayo, M. I., D. J. Ordway, S. M. Irwin, S. B. Shang, C. Shanley, and I. M. Orme. 2010. Phenotypic definition of effector and memory T-lymphocyte subsets in mice chronically infected with *Mycobacterium tuberculosis*. *Clin. Vaccine Immunol.* 17: 618–625.
42. Orme, I. M. 2010. The Achilles heel of BCG. *Tuberculosis (Edinb.)* 90: 329–332.
43. Aagaard, C., T. Hoang, J. Dietrich, P. J. Cardona, A. Izzo, G. Dolganov, G. K. Schoolnik, J. P. Cassidy, R. Billeskov, and P. Andersen. 2011. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat. Med.* 17: 189–194.
44. Han, S., A. Asoyan, H. Rabenstein, N. Nakano, and R. Obst. 2010. Role of antigen persistence and dose for $CD4^{+}$ T-cell exhaustion and recovery. *Proc. Natl. Acad. Sci. USA* 107: 20453–20458.
45. Henao-Tamayo, M., A. Obregón-Henao, D. J. Ordway, S. B. Shang, C. G. Duncan, and I. M. Orme. 2012. A mouse model of tuberculosis reinfection. *Tuberculosis (Edinb.)* 92: 211–217.
46. Billeskov, R., C. Vingsbo-Lundberg, P. Andersen, and J. Dietrich. 2007. Induction of CD8 T cells against a novel epitope in TB10.4: correlation with mycobacterial virulence and the presence of a functional region of difference-1. *J. Immunol.* 179: 3973–3981.
47. Ryan, A. A., J. K. Nambiar, T. M. Wozniak, B. Roediger, E. Shklovskaya, W. J. Britton, B. Fazekas de St Groth, and J. A. Triccas. 2009. Antigen load governs the differential priming of CD8 T cells in response to the bacille Calmette Guerin vaccine or *Mycobacterium tuberculosis* infection. *J. Immunol.* 182: 7172–7177.
48. Radosevic, K., C. W. Wieland, A. Rodriguez, G. J. Weverling, R. Mintardjo, G. Gillissen, R. Vogels, Y. A. W. Skeiky, D. M. Hone, J. C. Sadoff, et al. 2007. Protective immune responses to a recombinant adenovirus type 35 tuberculosis vaccine in two mouse strains: CD4 and CD8 T-cell epitope mapping and role of gamma interferon. *Infect. Immun.* 75: 4105–4115.
49. van Pinxteren, L. A. H., J. P. Cassidy, B. H. C. Smedegaard, E. M. Agger, and P. Andersen. 2000. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur. J. Immunol.* 30: 3689–3698.
50. Wang, J., M. Santosuosso, P. Ngai, A. Zganiacz, and Z. Xing. 2004. Activation of CD8 T cells by mycobacterial vaccination protects against pulmonary tuberculosis in the absence of CD4 T cells. *J. Immunol.* 173: 4590–4597.
51. Woodworth, J. S., Y. Wu, and S. M. Behar. 2008. *Mycobacterium tuberculosis*-specific $CD8^{+}$ T cells require perforin to kill target cells and provide protection in vivo. *J. Immunol.* 181: 8595–8603.
52. Wille-Reece, U., B. J. Flynn, K. Loré, R. A. Koup, A. P. Miles, A. Saul, R. M. Kedl, J. J. Mattapallil, W. R. Weiss, M. Roederer, and R. A. Seder. 2006. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J. Exp. Med.* 203: 1249–1258.
53. Beveridge, N. E. R., D. A. Price, J. P. Casazza, A. A. Pathan, C. R. Sander, T. E. Asher, D. R. Ambrozak, M. L. Precopio, P. Scheinberg, N. C. Alder, et al. 2007. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific $CD4^{+}$ memory T lymphocyte populations. *Eur. J. Immunol.* 37: 3089–3100.
54. Kaveh, D. A., V. S. Bachy, R. G. Hewinson, and P. J. Hogarth. 2011. Systemic BCG immunization induces persistent lung mucosal multifunctional CD4 T_{EM} cells which expand following virulent mycobacterial challenge. *PLoS ONE* 6: e21566.
55. Darrah, P. A., D. T. Patel, P. M. De Luca, R. W. B. Lindsay, D. F. Davey, B. J. Flynn, S. T. Hoff, P. Andersen, S. G. Reed, S. L. Morris, et al. 2007. Multifunctional T_H1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* 13: 843–850.
56. Deeks, S. L., M. Clark, D. W. Scheifele, B. J. Law, M. Dawar, N. Ahmadipour, W. Walop, C. E. Ellis, and A. King. 2005. Serious adverse events associated with bacille Calmette-Guérin vaccine in Canada. *Pediatr. Infect. Dis. J.* 24: 538–541.
57. Hesseling, A. C., B. J. Marais, R. P. Gie, H. S. Schaaf, P. E. M. Fine, P. Godfrey-Faussett, and N. Beyers. 2007. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 25: 14–18.
58. Hesseling, A. C., H. Rabie, B. J. Marais, M. Manders, M. Lips, H. S. Schaaf, R. P. Gie, M. F. Cotton, P. D. van Helden, R. M. Warren, and N. Beyers. 2006. Bacille Calmette-Guérin vaccine-induced disease in HIV-infected and HIV-uninfected children. *Clin. Infect. Dis.* 42: 548–558.
59. Hofstadler, G., K. Schmitt, G. Tulzer, L. Binder, and B. Brandstetter. 1998. BCG lymphadenitis in an HIV-infected child 9.5 years after vaccination. *AIDS Patient Care STDS* 12: 677–680.
60. Smith, E., S. Thybo, and J. Bennedsen. 1992. Infection with *Mycobacterium bovis* in a patient with AIDS: a late complication of BCG vaccination. *Scand. J. Infect. Dis.* 24: 109–110.
61. Lillebaek, T., A. Dirksen, E. Vynnycky, I. Baess, V. O. Thomsen, and A. B. Andersen. 2003. Stability of DNA patterns and evidence of *Mycobacterium tuberculosis* reactivation occurring decades after the initial infection. *J. Infect. Dis.* 188: 1032–1039.
62. Sonnenberg, P., J. Murray, J. R. Glynn, S. Shearer, B. Kambashi, and P. Godfrey-Faussett. 2001. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. *Lancet* 358: 1687–1693.