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NOTES

Memory T Lymphocytes Generated by *Mycobacterium bovis* BCG Vaccination Reside within a CD4 $CD44^{lo}$ CD62 $Ligand^{hi}$ Population

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In the lungs of mice vaccinated with *Mycobacterium bovis* BCG, there was an accumulation of CD4 cells expressing the activated effector phenotype $CD44^{hi}$ CD62 $ligand^{lo}$ ($CD62L^{lo}$) which were capable of secreting gamma interferon. Upon cell transfer, however, cells expressing a resting/naïve phenotype ($CD44^{lo}$ $CD62L^{hi}$) were capable of protecting the recipients from a virulent challenge infection, suggesting the emergence of T-cell memory from within this subset.

The emergence of the acquired immune response to *Mycobacterium tuberculosis* is mediated by the production of the cytokines interleukin-12 (IL-12) and IL-23 from infected macrophages, which in turn triggers antigen-specific T cells to secrete gamma interferon (IFN-γ) (4–6, 9, 10). These events result in the control and containment of the infection in the lungs, giving rise to a chronic disease state (15). When T cells are harvested from the lungs during this time, the great majority of both CD4 and CD8 cells express a so-called activated effector phenotype ($CD44^{hi}$ CD62 $ligand^{lo}$ [$CD62L^{lo}$]) and secrete IFN-γ when restimulated in vitro with antigen (12).

Because the infected lung in the chronic stage contains in excess of 10^5 viable bacteria, as often as not suppression of this system by steroids or infusion of neutralizing antibodies to T-cell markers or cytokines results in fatal progressive growth of the infection (7, 8). It is reasonable to believe that the persistence of antigen continues to drive an activated T-cell population and does not permit the emergence of memory immunity.

To try to achieve the latter, several laboratories have used extended chemotherapy to completely clear surviving viable bacteria from the infected host (1, 14). This probably still does not completely remove mycobacterial antigens, but it certainly prevents any further production of proteins made only by living bacteria that are the predominant targets of the initial immune response (16).

A further tactic is to inject a live vaccine such as the current tuberculosis vaccine *Mycobacterium bovis* BCG into a subcutaneous site where its dissemination and growth are very limited. After such inoculation, some bacteria reach the draining nodes and small numbers can reach the spleen, but none can be found in other sites, including the lungs. In the present study we show that in mice given an immunizing dose of BCG followed by chemotherapy to destroy the remaining bacilli, there was a significant rise in numbers of CD4 cells expressing an activated effector phenotype that could be found residing in the lungs, probably in lymphoid tissue surrounding the larger airways. If the mouse was then challenged by aerosol infection with *Mycobacterium tuberculosis*, there was a further sharp rise in the number of cells expressing this phenotype and also capable of secreting IFN-γ. Based upon these observations, therefore, we could conclude that memory immunity in such mice resides in lymphoid tissues, has an effector phenotype, and is IFN-γ positive.

That this hypothesis was almost certainly incorrect, however, was suggested by further studies in which effector or resting/naïve cells were harvested from the lungs or spleens of the BCG memory-immune animals and passively transferred into $Rag^{-/-}$ recipient mice. Both sets of cells found their way back to the lungs to certain degrees, but in mice injected with $CD44^{lo}$ $CD62L^{hi}$ CD4 T cells, there was a very substantial homeostatic proliferation of cells, including protective IFN-γ-secreting cells, as well as a switch to the effector cell phenotype. These data imply that that true memory T cells in BCG-immunized mice reside within a population of IFN-γ-negative cells that do not express an effector phenotype until they are exposed to the challenge infection.

Six- to 8-week-old C57BL/6 female mice and $Rag^{-/-}$ gene-disrupted mice with the same background were purchased from the Jackson Laboratories, Bar Harbor, ME. They were kept under barrier conditions in a level III biosafety laboratory and fed sterile water and chow. Mice were immunized with 10^6 CFU *M. bovis* BCG Pasteur via the subcutaneous route. Four weeks following immunization, mice were fed isoniazid in their drinking water (100 mg/liter) for 30 days. Four weeks following the cessation of isoniazid treatment, animals were infected with *M. tuberculosis* H37Rv by aerosol exposure using a Glas-

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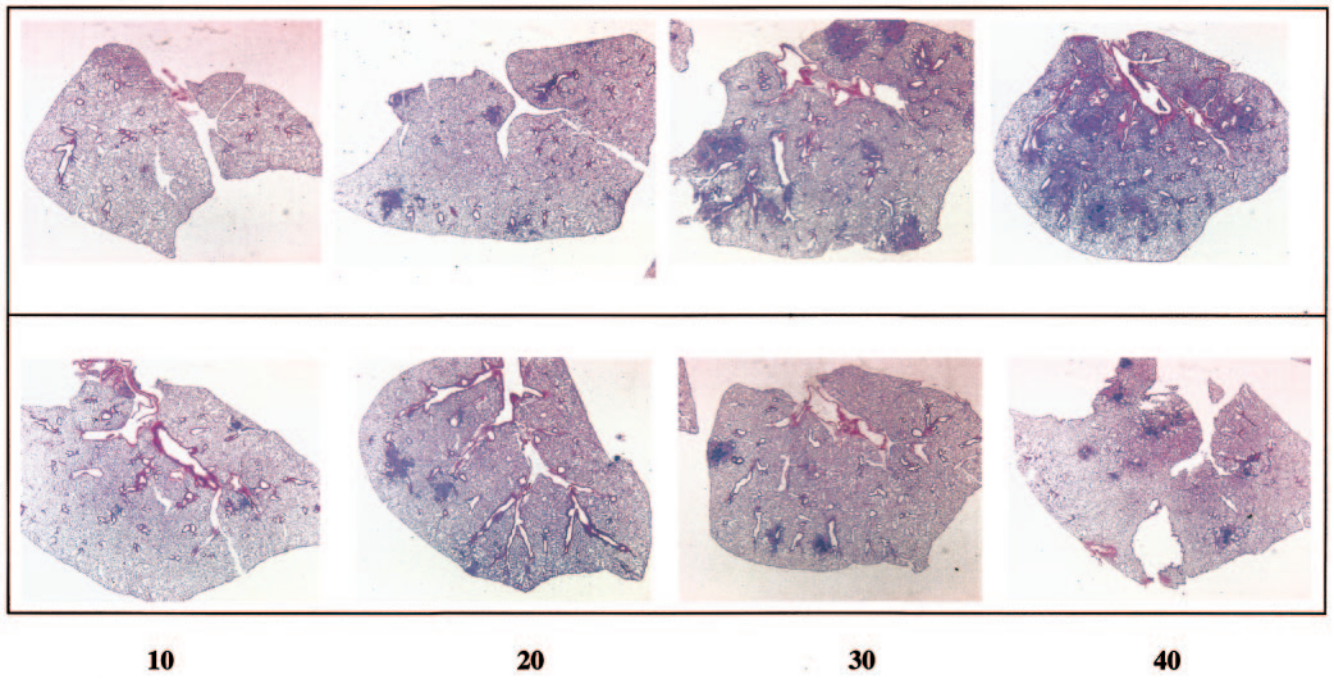

FIG. 1. Representative low-power photomicrographs of sections of whole lung taken at the indicated days from control mice (top panel) and mice vaccinated with BCG and treated with isoniazid (bottom panel) after exposure to an aerosol infection with *M. tuberculosis* H37Rv. Control animals developed large multifocal coalescing granulomas by day 40, whereas lesions in the vaccinated mice remained small and lymphocytic. Data are representative of four to five mice per group. Sections are stained with hematoxylin and eosin.

Col aerosol generator (Glas-Col, Terre Haute, IN) so that ~100 viable bacteria were deposited in the lungs of each animal.

For flow cytometric analysis and cell sorting, single-cell suspensions of spleens or lungs (12) from BCG-infected mice were resuspended in deficient RPMI medium 1640 (dRPMI; Irvine Scientific, Santa Ana, CA) containing 2% heat-inactivated endotoxin, a low concentration of fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 10 mM HEPES buffer (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 2% minimal essential medium-nonessential amino acids (100×; Sigma-Aldrich), and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Cells were incubated in the dark for 20 min at 4°C with specific antibody (directly conjugated to fluorescein isothiocyanate, phycoerythrin [PE], or PE-cyanine dye 5, at 3 μg/ml), followed by two washes using dRPMI. Cell surface markers were analyzed with the following specific antibodies: fluorescein isothiocyanate anti-CD44 (clone IM7), PE anti-CD62L (clone MEL-14), and PE-cyanine dye 5 anti-CD4 (clone RM4-5). Specific populations were sorted using a MoFlo flow cytometer/cell sorter (DakoCytomation, Fort Collins, CO), and the data were analyzed using Summit software (DakoCytomation). Viable lymphocytes were gated based upon their forward- and side-scatter characteristics, and cell populations were analyzed and sorted based upon expression of fluorochrome-labeled markers. Within the viable lymphocyte gate, spleen-derived cells were sorted for expression of $CD4^+$ $CD44^{lo}$ $CD62L^{hi}$ or $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$, and 10^5 cells in 100 μl phosphate-buffered saline were transferred into the lateral tail vein of $Rag^{-/-}$ mice. Similar sorting was performed using lung digest cells, but due to lower numbers, only 2×10^4 cells in 100 μl phosphate-buffered saline were transferred as described above. The purity of the sorted spleen-derived populations was >80 to 85%, while the purity of sorted lung-derived populations was >90 to 95%. Samples of sorted cell populations were spun onto glass slides using a Cytospin cytocentrifuge (Shandon Instruments, Sewickley, PA) and were stained using Hema 3 stain set (Biochemical Science, Swedesboro, NJ) to verify lymphocyte cell morphology. Approximately 20 mice were used to provide donor cells, and these cells were transferred into groups of five recipients. Mice receiving cells by passive transfer were infected with *M. tuberculosis* as described above and then harvested 30 days later. Numbers of cells with an activated effector phenotype and secreting IFN-γ were determined by flow cytometry as described previously (12). Bacterial loads in the right lobe were determined by plating homogenates on nutrient agar and counting the colony formation 21 days after incubation at 37°C. Whole lungs from infected mice were removed and fixed in a 10% formal saline solution. They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

The BCG vaccine protects mice by generating an immunity capable of recognizing the presence of infection more quickly, leading to slowing in the growth of the bacterial load in the lungs by approximately 1 $\log_{10}$ by 30 to 40 days after the aerosol challenge with *M. tuberculosis* (17). As shown in Fig. 1, this can also be visualized by a reduction in the size of granulomatous lesions as the infection is more quickly contained and inflammation is lessened in comparison to what occurs in unprotected controls. At day 20 of the infection, lesions were similar in size and distribution, but whereas these continued to

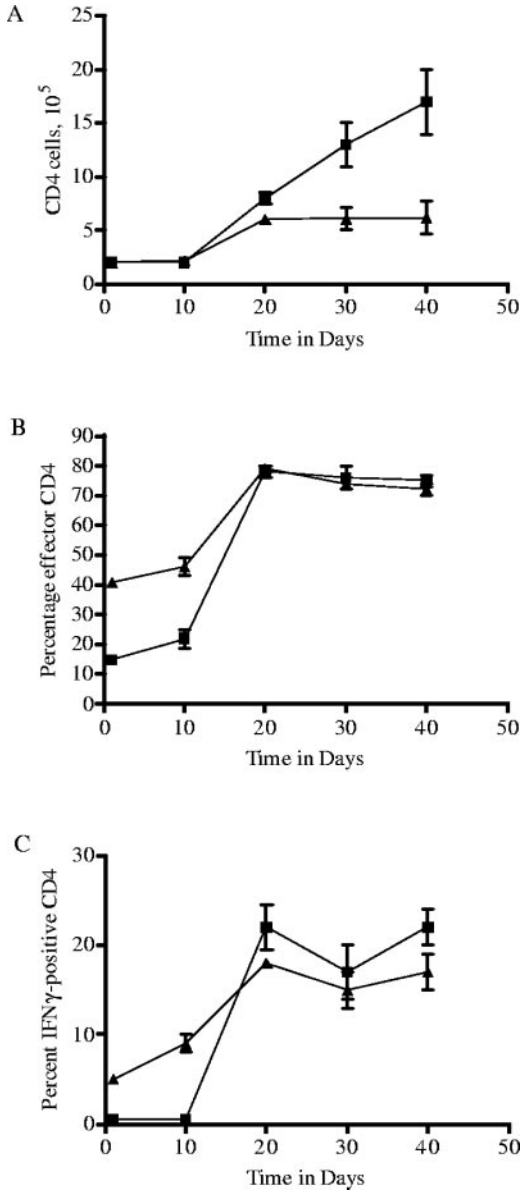

FIG. 2. Total CD4 cell numbers in the lungs of control mice (squares) or BCG-vaccinated mice (triangles) after aerosol challenge with *M. tuberculosis* (A), activated $CD44^{hi}$ $CD62L^{lo}$ cells (B), and cells positive for IFN-γ (C). Values are means ± standard errors of the means for four to five animals.

grow in size in control mice, they remained small in vaccinated mice.

As anticipated, because of the rapid control of the infection in the memory immune BCG-vaccinated mice, CD4 cell numbers in the lung tissues increased but quickly slowed, whereas in control mice, numbers of CD4 cells continued to increase (Fig. 2). What was noticeable, however, were the baseline values of the mice removed for analysis prior to exposure of the rest to infection. In these mice, 40% of CD4 cells expressed the activated/effector $CD44^{hi}$ $CD62L^{lo}$ phenotype even prior to aerosol challenge (first measured 14 days after the isoniazid treatment was stopped). Similarly, whereas the number of CD4 cells staining positive for IFN-γ were negligible in the control group, 5% of cells in the BCG-vaccinated group stained positive. After exposure to *M. tuberculosis*, in both groups, the percentage of CD4 cells expressing the activated phenotype rose to 80% and the percentage staining positive for IFN-γ was 20%.

The observation that both activated CD4 cells and IFN-γ-positive cells were present in the lungs of the vaccinated mice was unexpected, given the fact that BCG inoculated subcutaneously does not reach the lungs. Also, any residual BCG surviving the host response to the vaccination should have been very effectively cleared by the isoniazid treatment. To study this further, mice were vaccinated as described above but not challenged. Instead, lung cells were digested over time and the presence of activated CD4 cells in the lungs measured. As shown in Fig. 3, to our surprise cells with this phenotype could be detected up to 170 days later.

Given the apparently continuous presence of activated effector cells in the lungs, it was reasonable to hypothesize that this population represented a relatively long-lived subset of CD4 cells that mediated memory immunity to the challenge infection with *M. tuberculosis*. To test this further, cells with this phenotype, as well as CD4 cells expressing a naïve/resting phenotype as comparison controls, were collected from the spleens and lungs of BCG-vaccinated mice by high-speed cell sorting and transferred to $Rag^{-/-}$ mice, 24 h after which these recipients were aerosol infected with *M. tuberculosis*. Representative cell plots after sorting are shown in Fig. 4. Cells were also observed after cytospinning to verify their morphology as lymphocytes.

Thirty days later, mice were harvested and analyzed for lung cell influx as well as any evidence of protection measured as a reduction in bacterial load. As shown in Table 1, activated phenotype cells ($CD44^{hi}$ $CD62L^{lo}$) were able to return to the lungs, but virtually none were found to be secreting IFN-γ. This was also reflected in the complete lack of protection against the infection in terms of bacterial numbers. In contrast, large numbers of cells expressing this activated phenotype were found in the lungs of mice that had been infused with splenic CD4 cells expressing the naïve/resting phenotype, with a 10-fold increase in cells positive for IFN-γ. In addition, there was also a significant 10-fold reduction in the bacterial load in these mice. A similar trend was seen in mice infused with lung cells, but these cells were probably not transferred in sufficient numbers to result in protection.

These data suggest, therefore, that BCG vaccination induces two separate CD4 T-cell populations. The first expresses an activated effector phenotype and becomes established in peripheral lymphoid tissues, including the lungs, soon after the animal has been vaccinated. This population is either relatively long-lived or is continuously replaced by new cells coming from the circulation, at least for a finite period of time. The second population assumes a nonactivated phenotype, but can very rapidly and efficiently expand into an effector population upon exposure to the challenge infection. It is reasonable to suggest, therefore, that this second population represents a TH1 memory T-cell pool, but this idea obviously warrants further examination.

There is some support for our findings in the recent literature. Wu and colleagues (20) polarized CD4 responses in

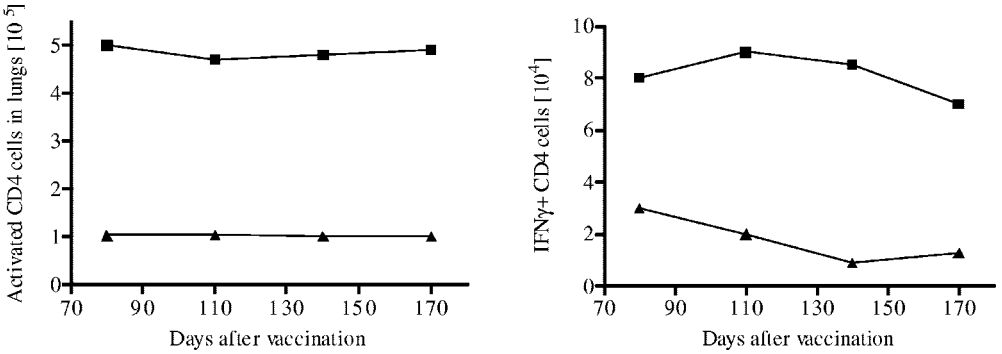

FIG. 3. Numbers of activated and IFN-γ-positive CD4 cells in BCG-vaccinated mice. BCG-immunized mice or saline controls were treated by chemotherapy as described in the text, and then the numbers of cells in the lungs were determined using flow cytometry. Data are expressed as means for four to five mice for each time point. Standard errors of the means did not exceed 15%.

ovalbumin transgenic mice towards a TH1 lineage using IL-12. This resulted in the generation of short-lived effector cells secreting IFN-γ when stimulated with antigen. At the same time, however, they also generated a second population of CD4 cells that were IFN-γ negative and that were responsible for recall immunity after transfer to naïve recipients. Based upon these observations, they concluded that memory TH1 T cells arose from a separate lineage of antigen-reactive cells.

In a similar infection model in which TH1 immunity is required, Zaph and colleagues (21) recently showed in a mouse model of *Leishmania* infection that effector CD4 cells were lost in the absence of antigen, but upon secondary challenge, cen-

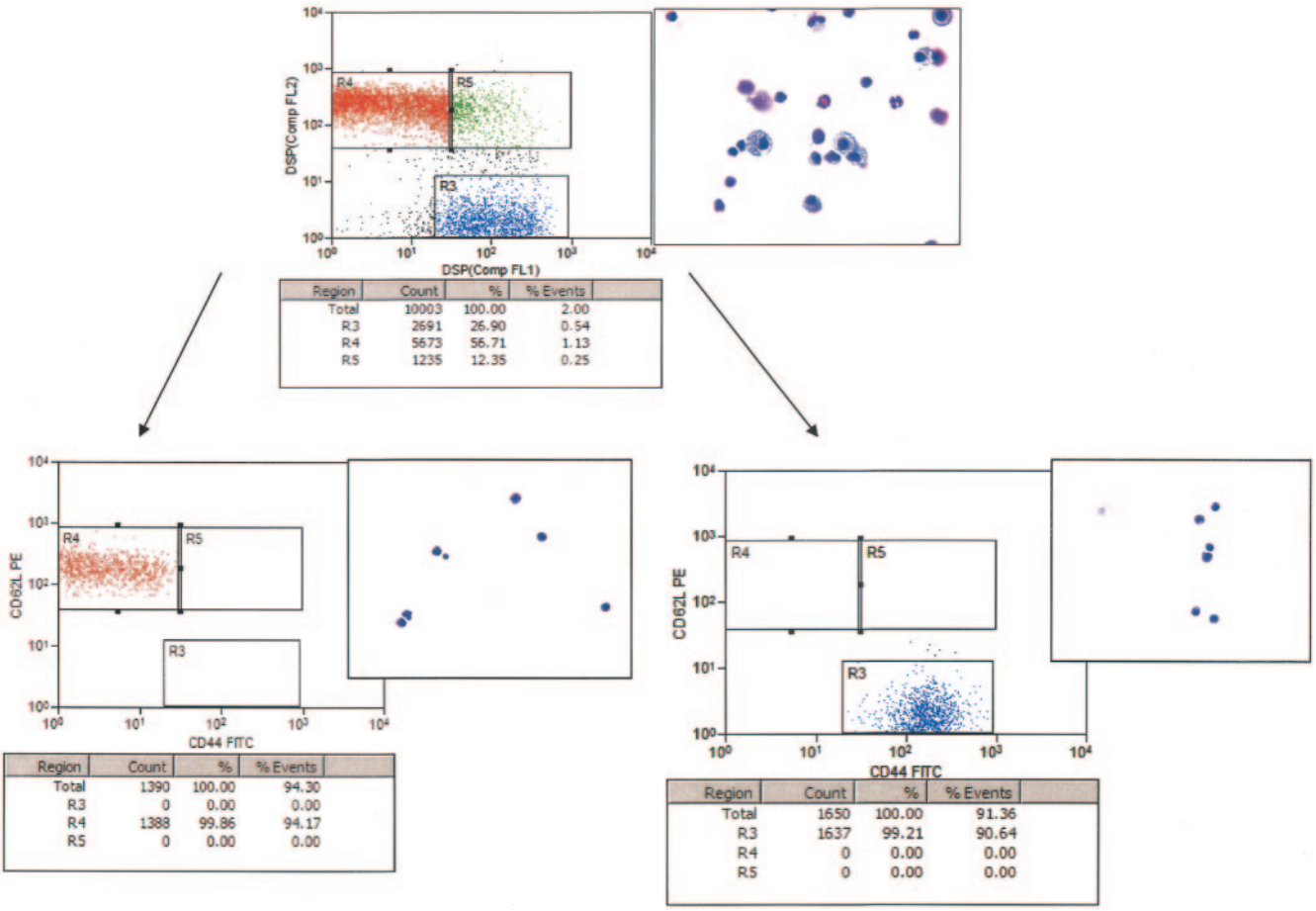

FIG. 4. Representative cell sorting data showing the separation of activated effector and naïve/resting cell populations prior to transfer. Cell morphology consistent with lymphocytes was further confirmed by cytospinning.

TABLE 1. Lung cellular influx after transfer and challenge

Phenotype transferred	Source	No. of cells transferred	No. of $CD44^{hi}$ $CD62L^{lo}$ cells recovered in lungs[a]	No. of IFN^+ cells	Protection (log)[b]
$CD44^{hi}$ $CD62L^{lo}$	Spleen	10^5	7×10^4	4×10^3	None
$CD44^{hi}$ $CD62L^{lo}$	Lung	2×10^4	3×10^4	1.3×10^2	None
$CD44^{lo}$ $CD62L^{hi}$	Spleen	10^5	4.6×10^5	2.8×10^4	0.95
$CD44^{lo}$ $CD62L^{hi}$	Lung	2×10^4	1×10^5	5.3×10^3	None

[a] Mean values for five recipient mice. Standard errors of the means indicated a 2 to 5% variance.
[b] Number of cells protected from infection ($n = 5$). Standard errors of the means did not exceed 0.15.

tral memory T cells homed in to infected tissues and efficiently became protective effectors. These observations, therefore, are very similar to ours. Also, Seder and Ahmed have recently suggested (19) that sustaining TH1 responses in vivo may be difficult without continuous boosting being given, unless a living vaccine is used. In the present study we deliberately gave isoniazid therapy to clear the remaining live BCG vaccine to try to establish a memory-immune animal, but this of course would not remove all the antigen from the system, such as that retained by dendritic cells, for instance. However, given the route of immunization, the activated effector cells seen in the lungs prior to aerosol challenge must have arisen via the circulation from the sites of BCG drainage into lymph nodes and the spleen.

The transferred spleen cells that mediated protection ($CD44^{lo}$ $CD62L^{hi}$) do not appear to fall into the category of central memory cells, which express the $CD44^{hi}$ $CD62L^{hi}$ phenotype (19). Further analysis and experimentation are clearly necessary to define this protective population, but it is reasonable to speculate that they consist of an important reservoir of cells that are the possible source of the $CD44^{hi}$ $CD62L^{lo}$ population resident in the lungs of the vaccinated mice even prior to challenge infection. Given their source prior to transfer, it may be that they reside in the spleen and seed into the blood, where they assume a more activated phenotype and migrate to lymphoid tissues, including the lungs. Moreover, if the cells that we observe in the lungs are being continuously replaced by these cells, then this event happens even when the animal is not subsequently challenged with *M. tuberculosis*.

Current thinking (19) suggests a linear pathway from naïve T cells to effector, to effector memory, and then to central memory cells. Our results suggest that the cells that we observed are arrested in this process at some point at the effector level. This may be a peculiarity of the infection model, perhaps driven by potent adjuvantive materials in the cell wall of BCG, which would persist despite chemotherapy. Alternatively, although the BCG was killed by isoniazid, antigen could still persist, perhaps within dendritic macrophages.

It is unknown if this pathway also exists in chronic tuberculosis, in which activated effector cells are continuously found in the lungs (12). This may reflect a simple naïve cell-to-effector cell pathway, with no generation of immunological memory. Indeed, it has been suggested (2) that true memory T cells cannot exist in chronic diseases such as tuberculosis. This may be so, but we cannot rule out the possibility that some type of memory population is still being generated, as older models suggest (14), albeit after disease resolution by chemotherapy. In fact, it could be that the memory cell population continuously feeds cells into the activated phenotype and that the persistence of bacteria in the chronic state drives this process; this would certainly explain our earlier results (12). A gradual exhaustion of the memory T-cell pool due to this mechanism could eventually allow the reactivation of disease, which eventually occurs in aerosol-infected mice, and could also underlie the loss of efficacy of BCG observed in children given this vaccine as neonates once they enter the teenage years (3). This would support the practical idea (13) that BCG generates long-lived T cells and that this population could be reexpanded by prime-boost strategies. It is known, in fact, that central memory T cells have a substantial capacity for rapid expansion (19), which could be exploited for rational vaccine design.

In the context of vaccination strategies for tuberculosis, our initial screening protocols include the demonstration that a given vaccine candidate can induce an IFN-γ response in the vaccinated mouse (11, 18). However, our current findings indicate that this may not be enough and that we may need to show that an IFN-γ-negative population capable of rapid activation and emergence is also generated and persists in the host so that the vaccine protects even if the vaccine-to-challenge interval is significantly extended. Given the fact that most vaccine screening assays are of the short-term variety, coinciding with the initial peak of IFN-γ-mediated immunity after the vaccine is administered, these new data will certainly complicate matters for rational vaccine design for tuberculosis.

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