

## CD4<sup>+</sup> T-Cell Subsets That Mediate Immunological Memory to *Mycobacterium tuberculosis* Infection in Mice

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**We have studied CD4<sup>+</sup> T cells that mediate immunological memory to an intravenous infection with *Mycobacterium tuberculosis*. The studies were conducted with a mouse model of memory immunity in which mice are rendered immune by a primary infection followed by antibiotic treatment and rest. Shortly after reinfection, tuberculosis-specific memory cells were recruited from the recirculating pool, leading to rapidly increasing precursor frequencies in the liver and a simultaneous decrease in the blood. A small subset of the infiltrating T cells was rapidly activated (<20 h) and expressed high levels of intracellular gamma interferon and the T-cell activation markers CD69 and CD25. These memory effector T cells expressed intermediate levels of CD45RB and were heterogeneous with regard to the L-selectin and CD44 markers. By adoptive transfer into nude mice, the highest level of resistance to a challenge with *M. tuberculosis* was mediated by CD45RB<sup>high</sup>, L-selectin<sup>high</sup>, CD44<sup>low</sup> cells. Taken together, these two lines of evidence support an important role for memory cells which have reverted to a naive phenotype in the long-term protection against *M. tuberculosis*.**

Immunological memory is defined as the ability to generate a highly accelerated and intense immune response during the secondary encounter with a pathogen. There are broad areas of agreement on the nature of B-cell memory, but T-cell memory is still not fully understood. Although this is still an area of ongoing debate, some of the features which have been generally accepted as associated with T-cell memory are increased antigen-specific precursor frequencies as well as changed functional abilities of the individual cells (22, 32).

Memory T cells have for years been considered to differ from naive lymphocytes by changes in cell surface marker expression, such as the downregulation of CD45RB and L-selectin and upregulation of CD44 (1, 11). Antigen priming of T cells also results in the upregulation of various adhesion molecules, and Mackay advanced the concept that memory cells migrate preferentially through peripheral tissues, in agreement with the function of this subset in immune surveillance (23). However, in recent years there has been an intensified debate as to whether these changes of surface molecules represent irreversible changes or merely identify a subset of recently activated T cells (7, 27). In this regard, evidence has been provided which demonstrates that a subset within the memory population returns to a nondividing state and regains the phenotype of naive cells (35, 39). Recently, it was demonstrated that when depleted of antigen, memory cells specific for dinitrochlorobenzene reverted completely to the CD45R<sup>high</sup> state, whereas only a minimal quantity of antigen was sufficient to retain a significant number of T cells in the CD45<sup>low</sup> state (12). Based on these findings, Bell and colleagues suggested the term memory revertants for such quiescent memory cells, which in most respects resemble naive cells but with an increased frequency of antigen-specific precursors (8).

Precursor frequencies and phenotypic changes are only indirect parameters for monitoring immunological memory to various pathogens. Cellular dynamics as well as effector functions in the CD8<sup>+</sup> T-cell memory subset have been studied

with various models of viral infections, such as infection with lymphocytic choriomeningitis virus and influenza virus (17, 42).

Immunological memory in the CD4<sup>+</sup> T-cell subset is less well understood. *Mycobacterium tuberculosis* represents an intracellular pathogen for which the CD4<sup>+</sup> T cells are the main mediators of protective immunity and for which a better understanding of immunological memory is essential in ongoing attempts to develop a new and more efficient vaccine against the disease (18, 30). In a number of classical studies with rodent models, it was found that memory cells are generated in animals cured from a primary infection with *M. tuberculosis* (or BCG) by chemotherapy. These memory cells could be isolated from the thoracic duct (21) and were reported to be long-lived and noncycling CD4<sup>+</sup> T cells (29) which rapidly accumulate in the infected organs, resulting in increased numbers of CD45RB<sup>low</sup>, CD44<sup>high</sup> cells (2,15).

The present study investigated the involvement of different memory T-cell subsets in protective immunity to *M. tuberculosis*. We have studied the recruitment and activation of antigen-specific CD4<sup>+</sup> memory T cells during the recall of immunity. Combined with the results of adoptive transfer experiments with purified CD4<sup>+</sup> T-cell subsets expressing different levels of the surface markers CD45RB, CD44, and L-selectin, our data suggest an important role for memory cells which have reverted to a naive phenotype in long-lived immunity to *M. tuberculosis*.

### MATERIALS AND METHODS

**Animals.** These studies were performed with female C57BL/6J mice and nude mice on the C57BL/6J background. All mice were purchased at 8 to 12 weeks of age from Bomholtgaard, Ry, Denmark. The animals were housed in cages contained within a laminar flow safety enclosure during the infection experiments.

**Bacteria.** *M. tuberculosis* H37Rv was grown on Middlebrook 7H11 medium or in suspension in modified Sauton medium enriched with 0.5% sodium pyruvate, 0.5% glucose, and 0.02% Tween 80. The liquid cultures were aliquoted and stored at –80°C for use in experimental infections as previously described (4).

**Experimental infections.** Intravenous infections were administered via the lateral tail vein with an inoculum of  $5 \times 10^4$  *M. tuberculosis* organisms suspended in phosphate-buffered saline in a volume of 0.1 ml. Memory immune mice were obtained by treating infected mice (after 1 month of infection) with isoniazid (Merck) (100 mg/liter) and rifabutin (Farmatolia Carlo Erba) (100 mg/liter) in their drinking water for 2 months. After this treatment, no live bacteria could be detected in the organs of these mice by whole-organ culture. The mice were rested for 4 to 6 months before being used for experiments (5). For the study of

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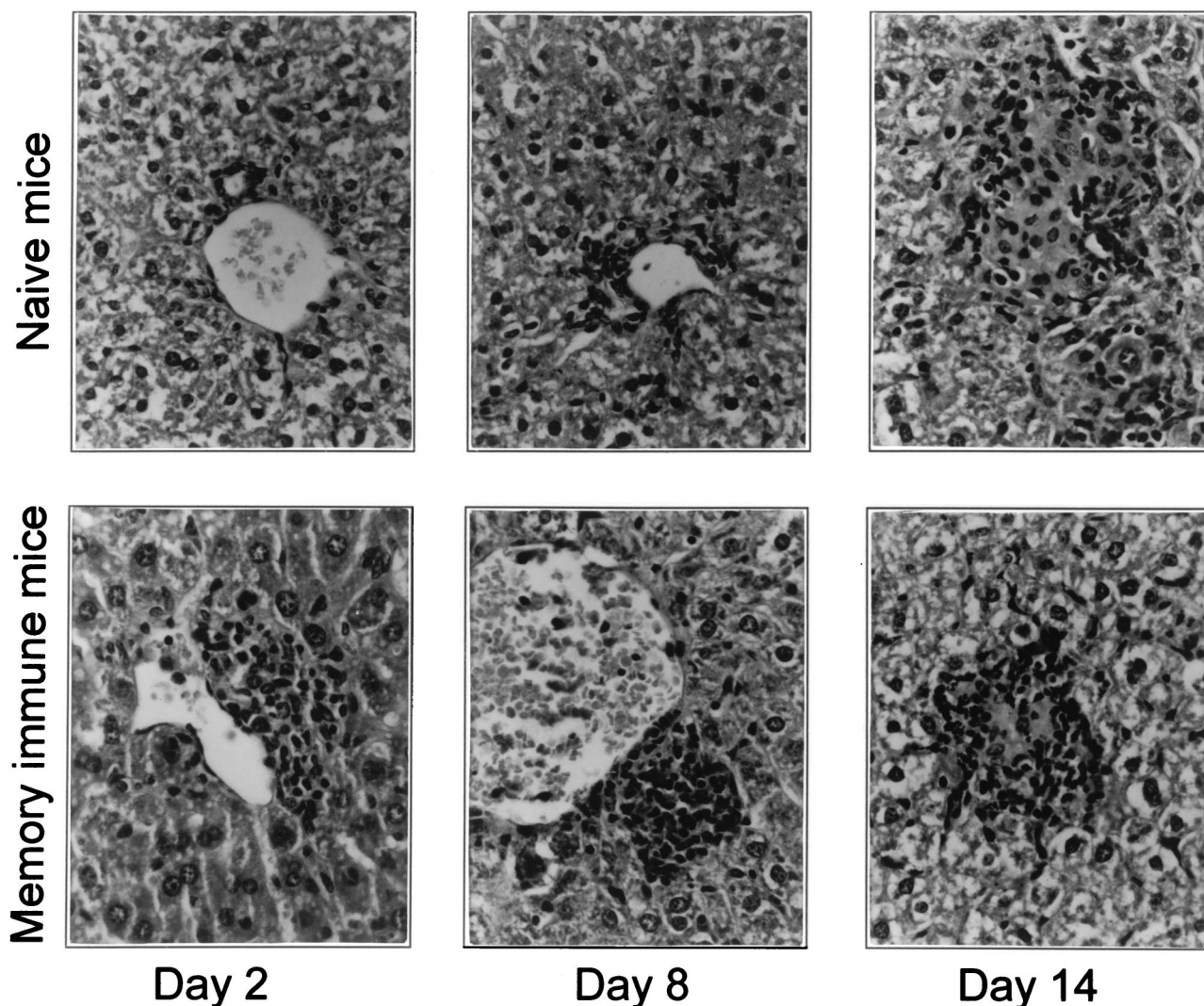


FIG. 1. Cellular infiltration and granuloma formation in the livers of naive and memory immune mice challenged with *M. tuberculosis*. Photomicrographs of livers from naive (top panel) and memory immune (bottom panel) mice at days 2 to 14 of infection with *M. tuberculosis* are shown. The granulomas are often initially situated in the perivascular regions around portal veins and at later time points are scattered throughout the liver parenchyme. Hematoxylin-eosin staining was used. Magnification,  $\times 300$ .

the recall reaction, immune animals were infected with an inoculum of  $10^6$  bacteria.

**Purification of liver and blood lymphocytes.** Mice were anesthetized, and blood from the orbital veins was collected in heparin tubes. The liver was perfused by opening the vena cava caudalis and injecting 8 to 10 ml of phosphate-buffered saline-heparin (3%) until a color change of the organ indicated an efficient removal of circulating blood. The liver was cut into pieces and carefully forced through a metal mesh. After disruption, the large particles were allowed to settle and the supernatant was centrifuged. The cell suspension was treated with 0.84% (wt/vol) ammonium chloride to lyse residual erythrocytes, and the liver lymphocytes were separated from the rest of the tissue by density gradient centrifugation (Lympholyte mammal; Cedarlane, Ontario, Canada). Blood lymphocytes were also purified on a density gradient.

**Flow cytometric analysis.** Blood and liver cells were incubated on ice with a panel of antibodies against the surface molecules CD45RB (clone 16A), CD44 (clone Pgp-1), CD25 (clone 7D4), L-selectin (clone Mel-14), and CD69 (clone H1-2F3), followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat immunoglobulin G (IgG) (Zymed, South San Francisco, Calif.). The cells were coupled with biotinylated anti-CD4 (clone RM4-5; Pharmingen, San Diego, Calif.), followed by streptavidin PerCP (Becton Dickinson, Mountain View, Calif.). This step was done in the presence of 5% normal rat serum. For the detection of intracellular gamma interferon (IFN- $\gamma$ ), cells were initially incubated with brefeldin A (Sigma) (5  $\mu\text{g/ml}$ ) at 37°C for 2.5 h, and after staining of the surface molecules, the cells were permeabilized with 0.1% (wt/vol) saponin

and stained with phycoerythrin-conjugated anti-IFN- $\gamma$  (clone XMG1.2; Pharmingen). Finally, cells were suspended in paraformaldehyde (4%) and analyzed in a FACSCalibur instrument (Becton Dickinson). The data were analyzed by using Cell Quest software (Becton Dickinson).

**ELISPOT technique.** The ELISPOT assay was done as described by Brandt et al. (10). Briefly, cells stimulated with short-term culture filtrate (ST-CF) from *M. tuberculosis* (20  $\mu\text{g/ml}$ ) (3) for 18 to 22 h were subsequently cultured without antigen for 7 h directly in the ELISPOT plates. For each group of cultured cells serial twofold dilutions were prepared, with a starting concentration of  $2 \times 10^5$  cells. The cells were removed by washing, and the site of cytokine secretion was detected with biotin-labeled rat anti-murine IFN- $\gamma$  monoclonal antibody (clone XMG1.2; Pharmingen).

**Adoptive transfer of resistance to *M. tuberculosis* with purified CD4<sup>+</sup> T-cell subsets.** T lymphocytes were isolated from the spleens and blood of naive and memory immune animals 4 to 6 months after the clearance of the primary infection. The mice were anesthetized, and blood (0.5 to 0.7 ml) was drawn before the mice were killed by cervical dislocation. The spleens were removed, and lymphocytes were obtained as described previously (2, 4). The spleen and blood lymphocytes were pooled, and CD4<sup>+</sup> T cells were purified by passage through CD4 columns (MCD4C-1000; RD Systems, Minneapolis, Minn.). The remaining B cells were removed by incubation with anti-B220 on ice (clone RA3-6B2), washing, and coupling to goat anti-rat IgG MACS beads followed by passage through magnetic columns (VS columns; Miltenyi Biotech, Bergisch Gladbach, Germany). The resulting preparation was >90% pure CD4<sup>+</sup> T cells.

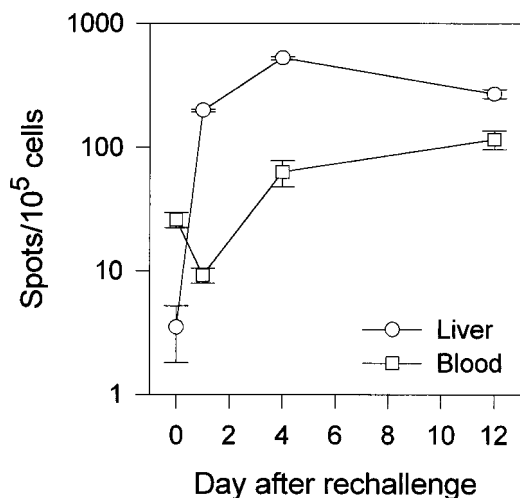


FIG. 2. Recruitment of recirculating *M. tuberculosis*-specific memory cells to the liver during the course of tuberculosis infection. Lymphocytes were purified from the blood and liver and used for ELISPOT analysis of *M. tuberculosis* ST-CF-specific precursors. Each point represents the mean and standard error of the mean of data from five individual mice. Results at all timepoints throughout infection were significantly different from those at day zero. The experiment was repeated with similar results.

This preparation was incubated with anti-CD45RB on ice for 15 min, washed, and incubated with goat anti-rat MACS beads for 10 min. The cells were eluted from the column in a stepwise manner according to the instructions of the manufacturer. For fluorescence-activated cell sorter evaluation of the purified subsets, the procedure was finished by 5 min of incubation with FITC-conjugated rabbit anti-rat IgG (Zymed). The intensity of the CD45RB marker appears to be slightly lower on separated cells as a consequence of the staining procedure, where the MACS- and FITC-conjugated secondary antibodies, both directed against the rat IgG (anti-CD45RB), are used consecutively. The subsets were transferred into nude recipient mice which were subsequently (<2 h) challenged with *M. tuberculosis* ( $5 \times 10^4$  organisms) by the intravenous route. The mice were killed 2 weeks later, and organs were removed and homogenized in phosphate-buffered saline. The homogenates were plated in serial threefold dilutions on Middlebrook 7H11 medium. After 2 to 3 weeks of incubation, the numbers of bacteria were determined and subtracted from those obtained in control nude mice not reconstituted with T cells. The resulting values are referred to as  $\log_{10}$  resistance. The levels of protection mediated by the different subsets were compared by one way analysis of variance of  $\log_{10}$  CFU.

## RESULTS

**Recruitment of CD4<sup>+</sup> memory T cells during the recall of immunity to *M. tuberculosis*.** Memory immune mice, cured of their primary infection with *M. tuberculosis*, were rested for 4 to 6 months after antibiotic treatment to ensure the resolution of granulomas from the primary infection and the establishment of immunological memory. As previously reported, protective immunity to *M. tuberculosis* was efficiently expressed in the organs of memory immune animals, with around 10 to 50 times fewer bacteria than in naive animals after the first week of infection (2, 29). Biopsies from livers of infected naive and memory immune animals were fixed and processed for histological examination. Before challenge, the livers of the memory immune animals had a normal appearance, with rare cases (<1/liver cross section) of small remnants of the granulomas generated during the primary infection. The memory immune mice were characterized by a rapid cellular infiltration consisting of scattered lymphocyte and macrophage accumulations often situated adjacent to small veins (Fig. 1). The first changes were recognizable within 24 to 48 h after infection as discrete accumulations of lymphocytes focused as perivascular cuffs around the portal veins. By day 8, these lesions had developed

into small, dense, well-circumscribed granulomas with abundant lymphocytes and few macrophages. A liver cross section typically contained about 50 granulomas.

In the naive animals only very limited changes were found in the first few days postinfection. The first detectable changes were typically found at day 6 to 8. These changes had the appearance of sparse cellular infiltrates adjacent to veins, but without an organized granulomatous structure. At day 14, these lesions were markedly enlarged, but compared to those in the memory immune animals, these granulomas were less organized and contained only a few lymphocytes and numerous epithelioid macrophages with markedly enlarged cytoplasm.

At various time points after infection, lymphocytes were purified from the blood and livers of the memory immune mice, and the frequencies of *M. tuberculosis* ST-CF-reactive precursors in the two populations were analyzed by an ELISPOT analysis. Before infection, the memory immune animals were found to have a high frequency (1/4,000) of recirculating ST-CF-specific precursors in the blood, whereas a much lower frequency of precursors (1/28,000) was found in the liver (Fig. 2). Only 24 h after challenge, a marked influx resulting in an almost 60-fold increase in the frequency of reactive T cells in the liver was found (frequency of 1/500). This recruitment of T cells to the liver was accompanied by a significant drop in the frequency of mycobacterium-reactive cells in the blood. These initial changes were followed by a period of 11 days where the frequency in the blood gradually increased to a level ( $\sim 1/1,500$ ) slightly above the preinfection level, whereas the frequency in the liver remained at a constant high level of 1/200 to 1/400. The frequency of ST-CF-reactive T cells in the livers of mice during primary infection was always more than 100 times below the level in memory immune animals throughout the study period.

**Phenotypic changes in CD4<sup>+</sup> memory T cells during recall of immunity.** The dynamic development of the CD4<sup>+</sup> T-cell population recruited to the liver in the first phase of the infection was monitored by two-color flow cytometry. The expression of surface molecules normally used to distinguish memory cells (CD45RB, CD44, and L-selectin), as well as activation markers (CD69 and CD25) and cell size, was monitored. Compared to the heterogeneous expression of most markers in the blood, the lymphocyte population trafficking through the uninfected livers of memory immune mice (day 0) exhibited a predominant memory phenotype, with low levels of CD45RB and L-selectin and high levels of CD44 (Fig. 3). This profile was not different for cells obtained from the livers of naive mice (results not shown). The cells expressed low levels of activation markers and had the same average size (mean FSC, 333) as cells in the blood (mean FSC, 341). At day 1 of infection, a significant proportion of the CD4<sup>+</sup> T cells isolated from the liver expressed the activation markers CD69 and CD25 (19 and 7%, respectively) and had a markedly increased cell size (mean FSC, 436), indicating blast formation. Compared to those at day 0, an increased proportion (30%) of the CD4<sup>+</sup> T cells recruited to the infected liver had high expression of CD45RB. The CD44 level also was slightly upregulated, whereas L-selectin levels from day 1 on were low, with <10% L-selectin<sup>high</sup> cells. The population expressing high levels of CD45RB diminished at later time points during the infection. At day 5, the isolated cells expressed a classical memory phenotype, with low levels of CD45RB and L-selectin and high levels of CD44 (Fig. 3). The expression of activation markers was transient and was not found after day 2. The blast formation was more prolonged and an increased cell size (mean FSC,

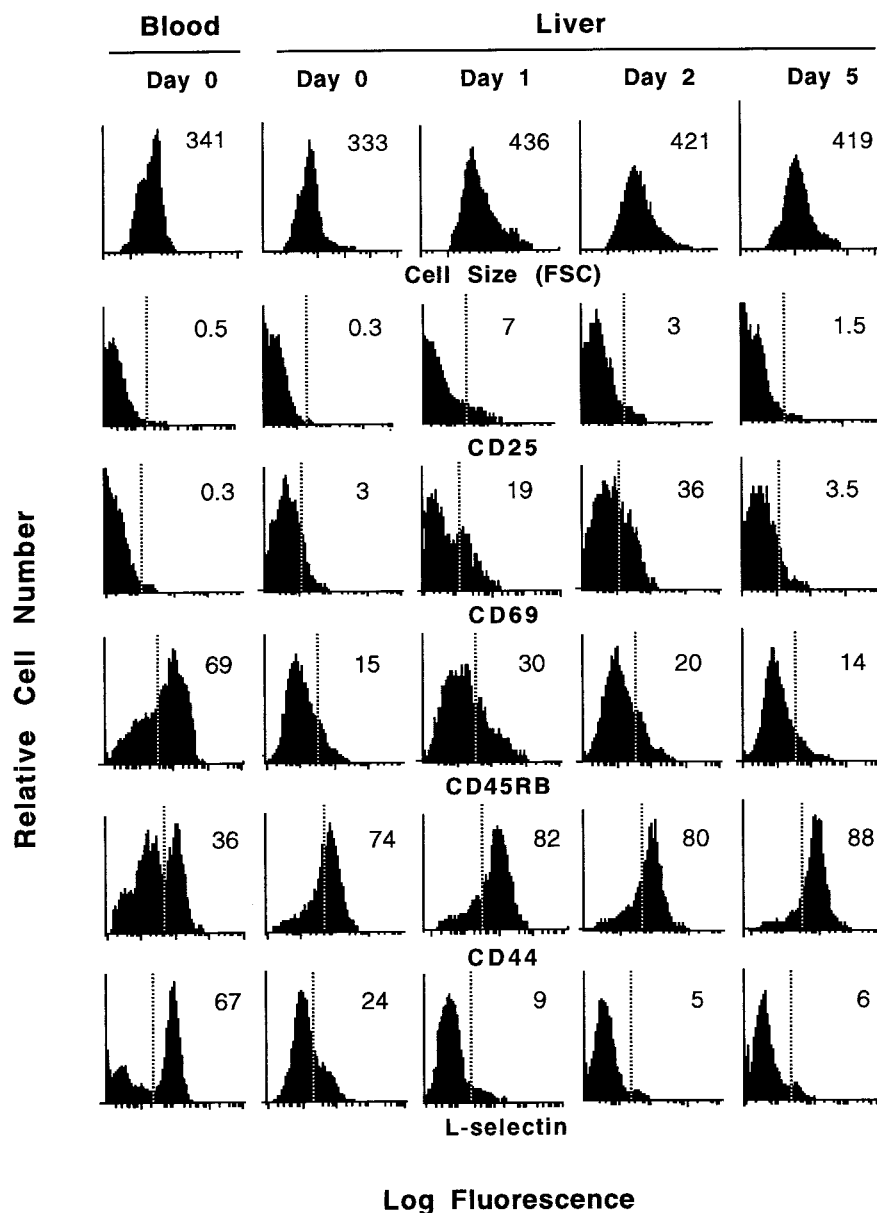


FIG. 3. Two-color flow cytometric analysis of cell size and surface marker expression on  $CD4^+$  cells isolated from the blood and livers of memory immune mice before (day 0) and during the course of infection with *M. tuberculosis*. Lymphocytes were pooled from four to six animals for each time point. The experiment was repeated with the same overall results.

419) was found at day 5. The cell size had returned almost to normal levels by day 12 (mean FSC, 389) (data not shown).

**Identification of  $CD4^+$  memory effector T cells.** To identify the antigen-activated and functionally active memory effector cells, the relationship between the expression of early activation markers (CD69), blast cell formation, and intracellular IFN- $\gamma$  was evaluated at 20 h postinfection. This was done by three-color flow cytometry for the simultaneous monitoring of intracellular cytokines and surface markers. The memory immune animals were characterized by a highly activated  $CD4^+$  subset (18%) which were positive for both CD69 and IFN- $\gamma$  (Fig. 4A, quadrant 2). The activated subset was found to contain blasts with an average cell size (FSC, 479) markedly larger than that of the IFN- $\gamma$ - and CD69-negative  $CD4^+$  T cells (quadrant 3; FSC, 364). This population was not found at day

1 of a primary infection but appeared at a much later time point (in these studies, at day 10 to 12), underlining the antigen-specific recall nature of this response (Fig. 4B). The activated  $CD4^+$  cells did not express the NK-1.1 marker and therefore did not belong to the subset of unconventional T cells previously observed in the livers of mice (results not shown) (14). The operational definition "memory effector cells" was used for this population.

To discriminate active memory effector cells from the rest of the  $CD4^+$  T cells recruited to the organs as part of the inflammatory process, the phenotype of cytokine-producing  $CD4^+$  T cells was evaluated by three-color flow cytometry. Memory immune mice were infected, and liver and spleen lymphocytes were isolated 20 h later. The  $CD4^+$  T-cell population was monitored for the expression of intracellular IFN- $\gamma$  and the

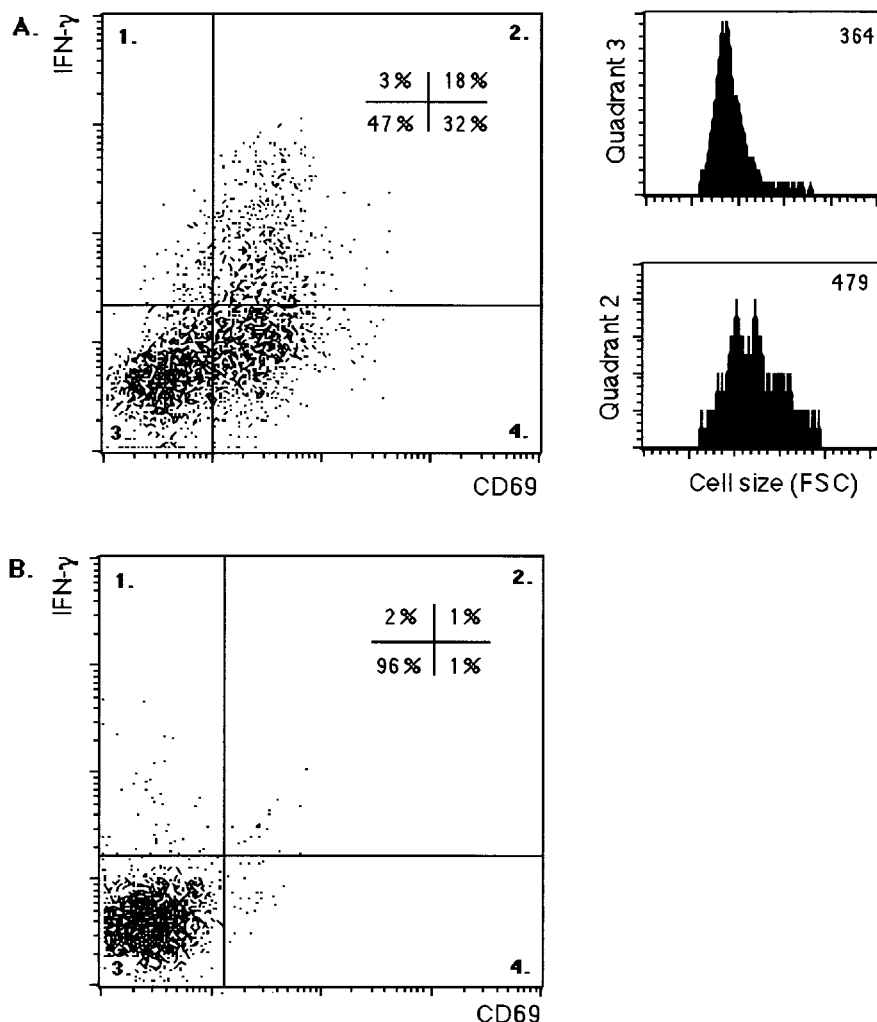


FIG. 4. Definition of CD4<sup>+</sup> memory effector T cells by three-color flow cytometric analysis of activation markers (CD69), intracellular cytokines (IFN- $\gamma$ ), and cellular size. Memory immune mice (A) and naive mice (B) were challenged with *M. tuberculosis*, and at 20 h postinfection liver lymphocytes from four mice were purified and pooled. Gated CD4<sup>+</sup> T cells from four mice were analyzed for CD69 and intracellular IFN- $\gamma$ . Cell size is shown for activated and cytokine-expressing memory effector cells (quadrant 2) versus normal nonactivated T cells (quadrant 3).

surface molecules CD45RB, CD44, and L-selectin (Fig. 5). As in the previous experiment, IFN- $\gamma$ -producing T cells constituted a significant proportion (15 to 20%) of the CD4<sup>+</sup> T cells isolated from the liver, whereas the proportion was much lower in the spleen (3 to 5%). The majority of the IFN- $\gamma$ -positive cells expressed low levels of L-selectin, but a substantial proportion (20 to 30%) expressed medium to high levels of this marker. In the spleen the cytokine-producing memory effector cells were predominantly CD44<sup>high</sup>, whereas a more heterogeneous expression of this marker was found on IFN- $\gamma$ -positive cells from the liver. Interestingly, in neither of the organs was the subset expressing IFN- $\gamma$  found among the cells with the lowest expression of CD45RB, but this subset resided predominantly in the CD45RB<sup>medium</sup> population with a fluorescence intensity of between 30 and 200.

**Adoptive transfer of immunological memory to tuberculosis by purified CD4<sup>+</sup> T-cell subsets.** A direct investigation of the CD4<sup>+</sup> T-cell subsets that mediate immunological memory to *M. tuberculosis* was done by adoptive transfer of purified T-cell subsets into nude mice followed by a virulent challenge with *M. tuberculosis*. Lymphocytes were pooled from the spleens and

blood of 12 naive and memory immune animals, and CD4<sup>+</sup> T cells were purified on columns by negative selection. CD4 cells ( $4 \times 10^6$ /mouse) from naive mice transferred only marginal levels of protection ( $0.18 \pm 0.12 \log_{10}$ ), whereas the CD4 cells from memory immune mice promoted a highly significant protection against bacterial multiplication in the spleens of donor mice ( $1.08 \pm 0.13 \log_{10}$ ;  $P < 0.0001$ ). The CD4<sup>+</sup> cells were divided by MACS into CD45RB<sup>low</sup> and CD45RB<sup>high</sup> populations, which expressed markedly different levels of the surface molecules CD44 and L-selectin (Fig. 6). These preparations were adoptively transferred into nude mice in either  $2 \times 10^5$  or  $8 \times 10^5$  cells/mouse, followed by an intravenous challenge with *M. tuberculosis*. The transfer of  $8 \times 10^5$  cells/mouse resulted in higher levels of immunity (although not statistically significant) than the transfer of  $2 \times 10^5$  cells (Fig. 7). We therefore monitored the  $\log_{10}$  resistance transferred to the liver, spleen, and lung by  $8 \times 10^5$  cells of the two subsets. In addition to cells purified from memory immune animals, we included cells from naive mice as a control for nonspecific resistance transferred by these highly purified subsets (Fig. 8). The CD45RB<sup>high</sup> subsets transferred significantly higher levels of resistance in all organs

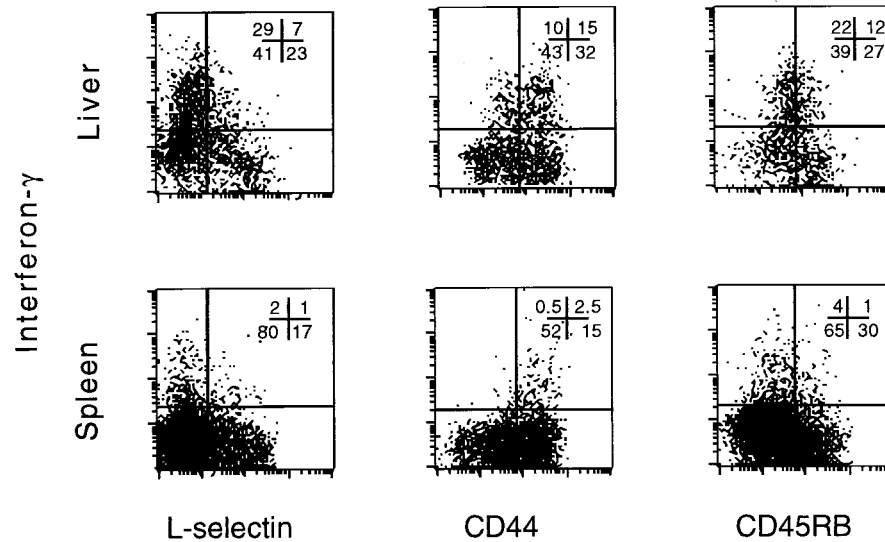


FIG. 5. Three-color flow cytometric analysis of the phenotype of cytokine-producing memory effector T cells. Pooled lymphocytes from the livers and spleens of memory immune mice ( $n = 5$ ) isolated 20 h after the infection with *M. tuberculosis* were analyzed for intracellular IFN- $\gamma$  and the expression of the surface molecules L-selectin, CD44, and CD45RB. The cutoff for positive intracellular IFN- $\gamma$  was based on anti-IFN- $\gamma$  isotype controls for each combination. The experiment was repeated with the same results.

than the CD45RB<sup>low</sup> subsets ( $P = 0.006$ ) (Fig. 8, right panels). This was particularly pronounced in the lung, where the CD45RB<sup>low</sup> subset did not confer detectable levels of protection. The subsets purified from naive mice conferred only low levels of protection to the liver and lung, whereas a substantial level of protection was transferred by both subsets to the spleen (0.3 to 0.5 log<sub>10</sub>). In this organ there was no significant difference in the levels of resistance transferred by memory immune and naive T cells.

## DISCUSSION

The main conclusion arising from the data in the present paper is that cells that mediate protection against *M. tuberculosis* in the mouse model of memory immunity are found both in the subset expressing the classical memory phenotype (CD45RB<sup>low</sup>, CD44<sup>high</sup>, and L-selectin<sup>low</sup>) and in the subset which until recently was associated with naive cells (CD45RB<sup>high</sup>, L-selectin<sup>high</sup>, and CD44<sup>low</sup>). This finding is in agreement with recent evidence from several laboratories in-

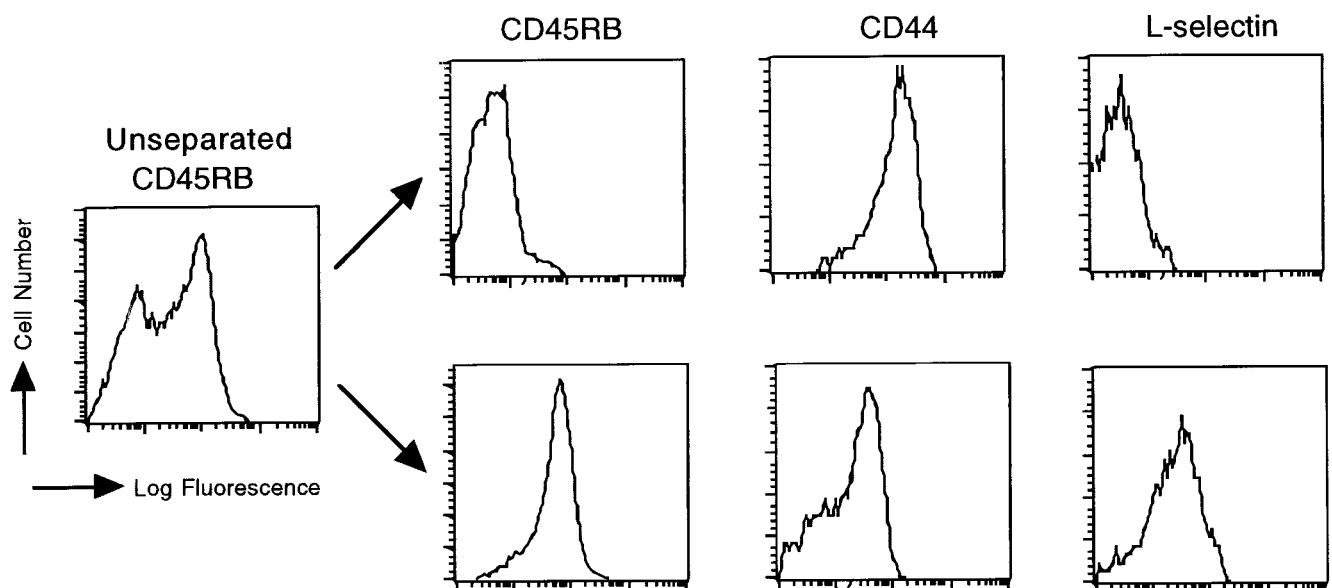


FIG. 6. Isolation of CD4<sup>+</sup> CD45RB<sup>low</sup> and CD45RB<sup>high</sup> subsets by MACS separation. CD4<sup>+</sup> T cells from pooled spleen and blood cells from memory immune mice ( $n = 12$ ) were sorted and analyzed for CD45RB, L-selectin, and CD44 expression. The subsets purified from naive mice had similar profiles.

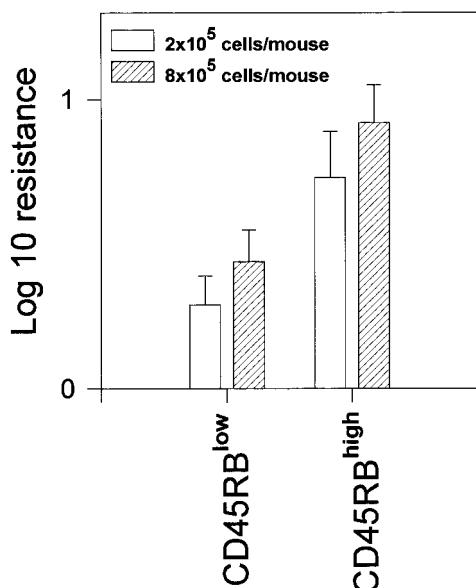


FIG. 7. Adoptive protection by  $2 \times 10^5$  and  $8 \times 10^5$  purified CD45RB<sup>low</sup> and CD45RB<sup>high</sup> cells. The purified subsets were transferred into nude mice which were challenged with *M. tuberculosis*, and bacteria in the spleen were enumerated 14 days later. Data are given as log<sub>10</sub> resistance (for the calculation, see Materials and Methods).

dicating that memory cells can revert to a quiescent state in which they are indistinguishable from naive cells (recently reviewed in reference 8). The concept was initially based on adoptive transfer experiments with a rat model (7) but was later supported by data from long-term human cell lines which were found to reexpress the high-molecular-mass isoform of CD45R (31). More recently, antigen-experienced cells have been marked with bromodeoxyuridine and demonstrated to return to the naive phenotype over time (16, 39). However, the concept of memory reversion is still open for debate, as demonstrated by a recent study by Young et al. in which responses to various recall and neo-antigens was studied (41). This study confirmed the classical observation that recall responses are contained within the CD45RO subset (26,28), but it additionally provided evidence that the predominant response to neo-antigens was in the CD45RA population. The poor responses to recall antigens in the CD45RA population in that study were used by the authors as an argument against reversion of the memory phenotype (41).

In this ongoing debate, the data provided by our study are strongly in favor of CD45R isoform switching, and our data do not support either CD44 or L-selectin as stable memory markers for CD4<sup>+</sup> T cells, although molecules like CD44 has been suggested elsewhere for CD8 memory cells (20).

It is an open question whether the maintenance of memory cells in a activated or primed state requires continuous contact with antigen or occurs simply through bystander contact with IFN- $\alpha$  and - $\beta$  released from activated cells during infection and inflammation (38). It has also been hypothesized that once primed, T cells may be readily reactivated by low-affinity cross-reacting antigens and that such intermittent stimulation may maintain the memory population in a primed state (9). In contrast to this hypothesis, Bunce and Bell have recently demonstrated a full reversion of the memory population in animals devoid of antigen, whereas only small amounts of persisting antigen prevent reversion (12). Taking this observation into account, our findings suggest that during the natural infection,

small depots of antigens remain after the termination of the primary immune response and maintain a significant proportion of the T cells in the primed state. This prolonged expression of the primed memory state may be an advantage for host immunity, as recently suggested by reports from Zinkernagel and colleagues (6, 19). In these studies only CD8<sup>+</sup> T cells persistently activated with antigen efficiently protected against challenge in peripheral organs like the lung, whereas memory expressed in the spleen seemed to be independent of continuous antigen exposure. Although these studies did not address the phenotype of the memory cells, they suggest that stores of antigen which maintain a significant proportion of the memory population in the primed state may be necessary for the optimal expression of memory in peripheral organs. Such a scenario would be in agreement with the preferential migration through peripheral tissues by CD45R<sup>low</sup> cells, which was originally described by Mackay et al. based on studies with sheep (24, 25) and interpreted as evidence for different recirculation pathways by naive and memory T cells (23).

The accumulation of lymphocyte subsets during the recall of a protective memory immune response to tuberculosis has been the subject of two earlier studies (2, 15). In those studies, changes in the relative sizes of different subsets in the lymphoid organs were monitored, and increased numbers of highly activated CD45RB<sup>low</sup>, CD44<sup>high</sup> CD4<sup>+</sup> T cells were found. Compared to those studies, the monitoring of lymphocyte traffic in perfused nonlymphoid organs, as in the present study, provides a much more sensitive monitoring of T cells attracted to the site of tuberculosis infection. However, T cells, and in partic-

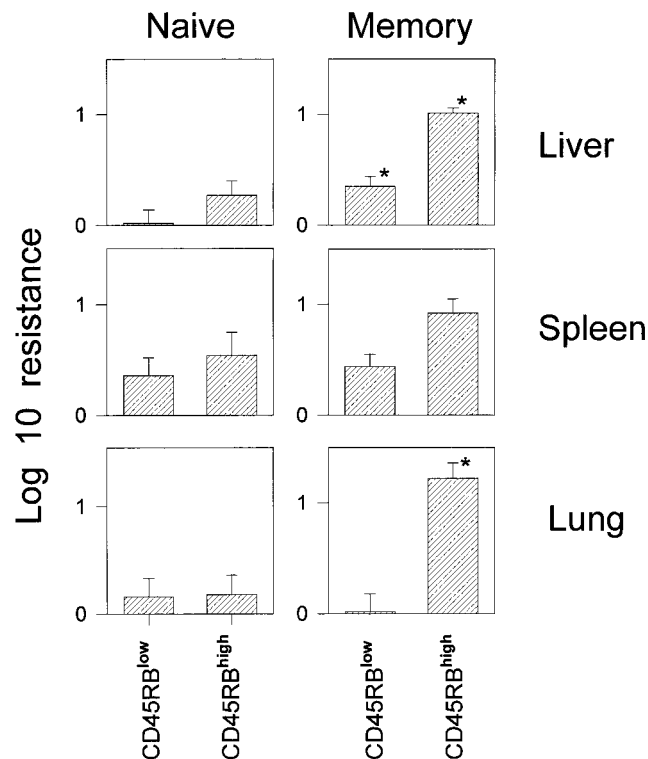


FIG. 8. Adoptive protection of *M. tuberculosis*-infected liver, spleen, and lung by purified T-cell subsets. Purified CD4<sup>+</sup> T-cell subsets ( $8 \times 10^5$  cells/mouse) from naive and memory immune mice were transferred into nude mice, and bacteria were enumerated 14 days after challenge with *M. tuberculosis*. Data are given as log<sub>10</sub> resistance. Significant differences between protection transferred by cells from naive and memory immune mice are indicated by asterisks.

ular activated T cells, migrate to inflammatory sites in a nonantigen-specific manner, attracted by chemokines such as RANTES which are released by a variety of stimulated cell types (37). Adding to this, T cells adhere to inflamed endothelium, promoted by integrins such as LFA-1 and VLA-4 (36) and, as recently demonstrated, mediated by the activated form of CD44, which binds to hyaluronate and starts the rolling and extravasation (13). It is therefore necessary to distinguish antigen-activated effector cells from the nonspecific part of the cellular exudate. In the present study this was done by simultaneous monitoring of activation markers and intracellular cytokines, which allowed the identification and phenotypic characterization of specific memory T cells actively involved in the recognition of *M. tuberculosis*.

After antigen stimulation, T cells change their phenotype. Thus, as recently discussed by Westermann and Pabst (40), biopsies taken after challenge can reveal a preferential accumulation of memory cells even if cells with a naive phenotype have initially entered. To minimize this methodological bias, we have monitored the cells recruited to the site of infection shortly after the reinfection (<20 h) and have identified antigen-activated memory effector cells with a heterogeneous expression of L-selectin and CD44 and intermediate levels of CD45RB. In contrast to the case at this early time point, CD4<sup>+</sup> T cells isolated from the liver from day 5 on express the classical memory phenotype, with low levels of CD45RB and L-selectin and high levels of CD44, and possibly are cells expanded at the site of infection. Even if this indicates that phenotypic switches occur relatively late after antigen exposure, it is not possible to formally exclude that some changes may occur even before 20 h. In this regard, some reports indicate that changes, particularly between the various isoforms of the CD45R molecule, can occur very rapidly (33, 34). This may be particularly relevant for L-selectin, where a down-regulation immediately after extravasation may explain the observation that most of the cytokine-producing cells were found among L-selectin<sup>low</sup> cells, whereas the purified CD45RB<sup>high</sup>, L-selectin<sup>high</sup> cells adoptively transferred high levels of resistance. Importantly, however, both the direct monitoring of memory effectors in the organs and the adoptive transfer experiments identified cells mediating long-lived immunity to tuberculosis in the memory subset which had reverted to a naive phenotype.

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