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T lymphocyte surface expression of exhaustion markers as biomarkers of the efficacy of chemotherapy for tuberculosis

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Summary

Predictive biomarkers illustrating the effectiveness of chemotherapeutic regimens for tuberculosis still remain elusive. To date, most are predicated on assays using sputum or serum; as a result, if not predictive, treatment failure in patients may not be evident for some time. We report here the results of a simple screening study in which T cell surface markers were examined in mice infected with *Mycobacterium tuberculosis* and then treated with drugs. These studies identified certain markers, the exhaustion markers PD-1 and TIM-3, as well as the marker KLRG-1, particularly on CD8 T cells, that changed in concert with reduction of the bacterial load in the lungs. While there is no guarantee these changes would also be seen on T cells in the blood, this approach should be further investigated.

1. Introduction

Because of the involvement of various international organizations, both scientific and advocacy-based, most of the 8 million or so people who each year acquire infection with *Mycobacterium tuberculosis* can expect to receive chemotherapy for their condition. Even in individuals that respond quickly to therapy, treatment can last for a minimum of 6-9 months¹. Some of these patients, now estimated at 0.5 million per year², will turn out to have multidrug resistant tuberculosis, requiring second line drugs that are expensive, toxic, and may require up to two years of administration. While there is an impressive pipeline of new drugs in development^{1,3}, optimization and efficacy studies of these in clinical regimens in the field will obviously take years to complete.

Predictive biomarkers that could tell us if a vaccine candidate is working or whether a particular drug regimen is having the desired effect, remain elusive^{4,5}. Because of this, in the context of drug treatment, the current method to determine efficacy is low-tech; namely the detection of bacilli in sputum after two months of drug treatment. The weakness of this approach is that if the patient is actually not responding, then this provides the infection with two months of growth and concomitant pathologic damage. It has been argued⁶, for this reason, that if a biomarker existed that stringently reflected efficacy of treatment, then

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patients could be rapidly stratified into relative risk groups. This would reduce the strain on the healthcare systems, by allowing treatment duration differences depending upon whether the treatment was working or whether longer treatment options were needed.

To date, the search for biomarkers has primarily focused on serum proteins. There is an obvious reason for this, reflecting the minimal instrumentation many laboratories in developing countries have at their disposal. But this is changing, and regions of the world in which drug and vaccine trials are being conducted are becoming better equipped. Factors that are released into the blood stream that are associated with tuberculosis infection can originate from multiple sites, both infectious and lymphatic. Once there, they are diluted out into the overall plasma volume; hence, even if a particular factor is predictive, it may not be above baseline for this reason. This may be a central reason for the continuing failure to identify such markers.

In this study we did not look at serum markers associated with effective chemotherapy, but instead at markers on the surface of lung T cells themselves. While there is no guarantee such profiles would exist on lymphocytes in the blood of human patients, we propose this approach could potentially provide new and relevant clinical information if investigated. In this study we used flow cytometry to measure a variety of T cell surface markers to see if they would change as the bacterial load in mice was reduced by standard chemotherapy. Changes in certain markers were observed, both on CD4 and CD8 cells. Two molecules associated with CD8 T cell exhaustion, PD-1 and Tim-3, were particularly promising in that their expression dropped in concert with bacterial clearance. Other molecules, such as CD27 and KLRG-1 [on both CD4 and CD8 in the latter case] also seemed to reflect bacterial clearance.

2. Materials and methods

2.1. Mice

These studies were performed using specific pathogen-free female C57BL/6 mice purchased from the Jackson laboratories, Bar Harbor, ME at 6-8 weeks of age. All mice were maintained within a Biosafety level-III facility at Colorado State University for the duration of the experiments, and had free access to sterile water and standard mouse chow. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals, which were shown to be negative for 13 known mouse pathogens. All experimental procedures were approved by the Colorado State University Animal Care and Use Committee.

2.2. Bacterial Strains

M. tuberculosis strain H37Rv, originally obtained from the Trudeau Mycobacteria Collection, was grown from low passage seed lots in Proskauer-Beck liquid media containing 0.05% Tween 80 to mid-log phase, then aliquoted and frozen at -70°C until use.

2.3. Low Dose Aerosol Infection

Mice were infected via the aerosol route using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN), such that ~100 viable bacteria were deposited in the lungs of each animal⁷. The number of viable bacteria in the lungs was determined at specific time points by plating serial dilutions of individual partial organ homogenates on nutrient Middlebrook 7H11 agar, and counting bacterial colonies after ~21 days of incubation at 37°C.

2.4. Drug Treatment

Animals were treated with rifampicin (100 mg/L) and isoniazid (200 mg/L) given in the drinking water⁸. Treatment was initiated on day 30 after low dose aerosol infection and was continued for the entire duration of the experiments.

2.5. Single-Cell Suspension Preparation

Animals were sacrificed by carbon dioxide asphyxiation and were perfused through the heart with 10 ml cold PBS containing 3 U/ml heparin (Sigma-Aldrich, St. Louis, MO). The lungs were removed from the pulmonary cavity and placed in cold Dulbecco's minimal essential medium (DMEM; Cellgro, Herndon, VA). The lung tissue was disrupted using sterile razor blades and incubated for 30 min at 37°C in a final volume of 2 ml DMEM containing 0.7 mg/ml collagenase D (Roche Applied Science, Indianapolis, IN) and 30 µg/ml deoxyribonuclease (Sigma-Aldrich). Following incubation, the digested lung tissue was gently passed through a sterile 70 µm nylon screen, rinsed using 8 ml cold DMEM supplemented with 10% heat-inactivated endotoxin low fetal calf serum (Atlas Biologicals, Fort Collins, CO), 10 mM HEPES buffer (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 2% MEM-nonessential amino acids (100×; Sigma-Aldrich), 50 µM 2-mercaptoethanol (Sigma-Aldrich) and centrifuged at 200 × g. Red blood cells were lysed using Gey's solution (0.15 M NH₄Cl, 10 mM KHCO₃) and the samples were centrifuged and resuspended in DMEM plus supplements. Total cell numbers were determined by flow cytometry with liquid counting beads, as described by the manufacturer (BDPharmingen, San Jose, CA).

2.6. Flow Cytometry

For flow cytometry analysis, single-cell suspensions of lung from each mice were resuspended in PBS (Sigma-Aldrich) containing 0.1% of Sodium Azide (PBS+Na/Az). Cells were incubated in the dark for 25 min at 37°C with predetermined optimal titrations of specific antibody (directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin-chlorophyll-protein (PerCP), allophycocyanin (APC), eFluor 450, Alexa Fluor 700; or after biotin antibody incubations washed and incubated for 25 minutes more with streptavidin Qdot800 (Invitrogen), followed by two washes in PBS containing 4% sodium azide. Figures for publication were produced using Summit software (DakoCytomation, Fort Collins, CO) or FACSDiva v5.0.1 software (Becton Dickinson, NJ, USA). Viable lymphocytes were gated based upon their forward- and side-scatter characteristics and cell populations were analyzed by expression of fluorochrome-labeled markers. All analyses were performed with a minimum acquisition of 200,000 events.

Cell surface markers were analyzed with the following specific antibodies: FITC-CD44 (clone IM7), CD3e (clone 145-2C11), PE-Mtb32 H-2D^b MHC class I tetramer (Beckman Coulter Immunomics, San Diego, CA), CD62L (clone MEL-14), PerCP-CD8a (clone 53-6.7), CD4 (clone RM4-5), APC-CD3 (clone 145-2C11), CD127 (clone A7R34) FITC-KLRG-1 (clone 2F1; BD Pharmingen), APC-CXCR3 (CXCR3-173; eBiosciences), PE-CCR5 (Clone 7A4; eBiosciences). For staining PD-1, TIM-3, and CCR7, cells were stained with either anti-PD-1 (clone RMP1-14; BioLegend, San Diego, CA), anti-Tim-3 (clone RMT3-23; eBioscience), or anti-CCR7 (clone 4B12; BioLegend) followed by biotin conjugated goat anti-rat IgG (Caltag, Burlingame, CA) followed by labeling with streptavidin-APC. Cells were sequentially incubated in the dark for 20 minutes at 4°C with specific primary antibody, biotin conjugated secondary antibody, and streptavidin-APC with washes using PBS with azide between steps. Unless otherwise noted, all antibodies were purchased from BD PharMingen.

3. Results

3.1. Screening CD4 and CD8 subsets for potential biomarkers of drug efficacy

In these studies mice were infected with *M.tuberculosis* H37Rv and then given chemotherapy starting on day 30. By day 120 the bacterial load in the lungs had dropped to undetectable levels. Over this period of time we isolated and screened lung T cells for a wide variety of canonical and other types of markers that are known to be expressed on T cells responding to tuberculosis infection. Many of these we excluded because they did not change [increase or decrease] concomitantly with the reduction in CFU levels in the lungs [these markers included CD44, CD69, CD11a, CD62L, CD45RB, CD27, 4-1BB, and CCR7]. In addition, in general we observed much more consistent results when we looked at CD8 T cell responses, and so for the most part these are emphasized here. A representative panel is shown in Fig.1. This Figure shows CD62L levels on CD8 cells as an example of a marker that was unchanged, and CCR7 and CD43 as examples of an intermediate pattern. In contrast, whereas CD27, PD-1, and Tim-3 were highly expressed on CD8 cells from infected but untreated mice [and remained at these high levels throughout the study; see Supplementary Fig.1], the levels of these markers steadily fell to low levels similar to those on naïve CD8 cells by the end of the period of chemotherapy. To exclude the possibility that these changes were merely non-specific and occurred on cells not contributing to the immune response we stained with a Class-I tetramer, and found a small but positive signal in all cases (Fig.2).

3.2. Expression of PD-1 and KLRG-1 as markers of drug efficacy

Because markers such as PD-1 are associated with T cell exhaustion, we also looked at other T cell activation markers. One that gave positive results was the cell surface marker KLRG-1. Where PD-1 expression was only lost on CD8+ cells after chemotherapy, KLRG-1 expression was down-regulated on both CD4 and CD8 cells. Fig.3 shows the kinetics of loss of these markers during and after chemotherapy, and a representative example of the gating strategies used for the flow cytometric analysis.

3.3. Chemokine receptor expression was not predictive

Chemokine receptors are often highly expressed on T cells in the lung, where they allow these cells to migrate and respond to the infection. Two prominent markers are CXCR3 and CCR5. In the present study however neither marker was predictive of the successful chemotherapy (Fig.4).

4. Discussion

In this simple study we began by screening a large number of cell surface molecules expressed widely on CD4 and CD8 T cell subsets in order to determine if they changed in mice receiving chemotherapy for tuberculosis and hence could be used as biomarkers for this purpose. Several of these markers are associated with activation and become highly expressed as the infection progresses, but most of the markers we looked at did not change or gave intermediate or ambivalent responses, whereas a few markers exhibited a reproducible reduction in expression as the bacterial infection was cleared by standard chemotherapy. As noted above, there is absolutely no guarantee that these changes on T cells isolated from the lungs would be reflected on T cells obtained from blood samples, but we propose that this could be investigated, especially given the current lack of validated biomarkers. Obviously, if a gold standard biomarker of effective drug treatment could be identified this would have a major impact on clinical trials of new drugs and regimens^{4, 5}.

Our most reproducible findings were found using flow cytometric analysis of the levels of expression of PD-1 and Tim-3, thought to represent functional markers of T cell exhaustion⁹⁻¹¹, and the marker KLRG-1¹². The numbers of CD4 and CD8 cells expressing the latter both dropped to very low levels after chemotherapy. This was seen in the case of PD-1 and Tim-3 expression on CD8, but not on CD4 cells. Whether our observations represent down-expression of these markers or a reduction in cellular influx of these particular T cells remains unknown.

Both the Programmed death-1 (PD-1) and T cell immunoglobulin mucin 3 (Tim-3) molecules are expressed by T-cells and are regarded as negative regulators of the immune system^{11, 13}. High expression of these markers have been identified as markers of exhausted T cells in chronic disease states, including infections, autoimmunity, and cancers^{14, 15}. PD-1 has recently been investigated specifically in the context of *M.tuberculosis* infection, using PD-1/-gene disrupted mice¹⁶. In these animals there was uncontrolled bacterial proliferation and severe pathology, and evidence of a complete disruption of the host inflammatory response. When taken in comparison with studies looking at chronic viral infections¹⁷, these observations suggest that PD-1 plays an early homeostatic role in controlling inflammation in tuberculosis, but as the infection is contained and becomes chronic then promotes mechanisms leading to T cell exhaustion. These results also suggest PD-1 has a function in innate immunity, and this has been further revealed by recent studies showing that PD-1 can limit the function of dendritic cells and macrophages as well as T cell independent B cell responses¹³. The T cell marker T cell immunoglobulin mucin- (TIM)-3 seems to fall into a similar category¹⁸. It was first identified as a molecule specifically expressed on IFN-gamma-secreting CD4 TH1 T cells and CD8 T cells. Like PD-1, TIM-3 seems to act as a negative regulator of T cell functions by triggering cell death upon interaction with its ligand, galectin-9. Engagement of these two molecules results in signaling that inhibits T-cell proliferation, cytokine production, and cytolytic function¹⁹.

KLRG-1, originally identified as killer cell lectin-like receptor G1, is similar to the exhaustion markers in a sense, in that it has been identified as a marker for terminal differentiation and replicative senescence, particularly within the CD8 T cell subset. In mice clearing acute viral infections, KLRG-1-negative CD8 cells were shown to be capable of a greater proliferative burst and had enhanced cytokine production¹². As with viral infections, this suggests that removal of the chronic or progressive *M.tuberculosis* infection allows either the replacement or reversion of exhausted T cells as the infection is cleared by effective drug treatment.

Given their involvement in immunity to tuberculosis, specifically migration of T cells to sites of infection, we also thought it worthwhile to look at the expression of the chemokine receptors CXCR3 and CCR5, which are often found on activated T cells in models of tuberculosis²⁰. In mice chronically infected with tuberculosis²¹ there were high numbers of CXCR3+ cells in the lungs, and this receptor has been implicated in early formation of the granuloma²². CCR5 is also increased in tuberculosis²³; it does not appear to play any central role in protection, but does influence cell recruitment and degree of cellular influx^{24, 25}. In our studies here however, we saw no consistent or sustained effect of drug treatment on the expression of CXCR3 or CCR5 on lung T cells.

To date, studies on biomarkers have focused on serum markers, for obvious practical reasons. An easy approach is simply to take serum samples from patients undergoing treatment and to determine if any biomarkers line up with lung X-ray data. In such a study⁶ various correlations were found between certain serum protein markers [C-reactive protein, granzyme B, LAG-3] and the degree of cavitary disease. Similarly, another study²⁶ observed some associations between various markers [soluble IL-2R α , granzyme B, soluble

TNF receptors, nitrotyrosine, IFN γ] and a slower response to treatment. A further study²⁷ observed different profiles in patients with different forms of disease; pleural TB patients had lots of inflammatory markers in their blood [pro-inflammatory cytokines and chemokines], whereas patients without effusions had higher levels of IL-12p40 and soluble CD40L, indicating expression of protective TH1 immunity.

It would be very useful to be able to have validated biomarkers that not only tell us if a drug regimen is working or not, but also if after apparently successful chemotherapy the patient is beginning to relapse. One such marker we have noted in the guinea pig model of tuberculosis is the [putative selectin] CT-4, a marker that increases in expression on CD4 T cells as animals start to show evidence of bacterial re-growth²⁸⁻³⁰. Indeed, we would note in closing that given the absolute plethora of CD markers now available, further research and screening of such markers has the obvious potential to reveal cellular biomarkers that could replace current serum-based approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Lienhardt C, Vernon A, Raviglione MC. New drugs and new regimens for the treatment of tuberculosis: review of the drug development pipeline and implications for national programmes. *Curr Opin Pulm Med*. 2010; 16:186–193. [PubMed: 20216421]
2. World Health Organization. Anti-Tuberculosis drug resistance in the world, Report #4. 2008.
3. Ginsberg AM, Spigelman M. Challenges in tuberculosis drug research and development. *Nat Med*. 2007; 13:290–294. [PubMed: 17342142]
4. Wallis RS, Pai M, Menzies D, Doherty TM, Walzl G, Perkins MD, Zumla A. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. *Lancet*. 2010; 375:1920–1937. [PubMed: 20488517]
5. Walzl G, Ronacher K, Djoba Siawaya JF, Dockrell HM. Biomarkers for TB treatment response: challenges and future strategies. *J Infection*. 2008; 57:103–109.
6. Djoba Siawaya JF, Bapela NB, Ronacher K, Veenstra H, Kidd M, Gie R, et al. Immune parameters as markers of tuberculosis extent of disease and early prediction of anti-tuberculosis chemotherapy response. *J Infection*. 2008; 56:340–347.
7. Kelly BP, Furney SK, Jessen MT, Orme IM. Low-dose aerosol infection model for testing drugs for efficacy against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 1996; 40:2809–2812. [PubMed: 9124846]
8. Orme IM. Characteristics and specificity of acquired immunologic memory to *Mycobacterium tuberculosis* infection. *J Immunol*. 1988; 140:3589–3593. [PubMed: 3129497]
9. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol*. 2009; 83:9122–9130. [PubMed: 19587053]
10. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, et al. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. *Eur J Immunol*. 2009; 39:2492–2501. [PubMed: 19676072]

11. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *PNAS*. 2010; 107:14733–14738. [PubMed: 20679213]
12. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med*. 2008; 205:625–640. [PubMed: 18316415]
13. Brown KE, Freeman GJ, Wherry EJ, Sharpe AH. Role of PD-1 in regulating acute infections. *Curr Opin Immunol*. 2010; 22:397–401. [PubMed: 20427170]
14. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev*. 2010; 236:219–242. [PubMed: 20636820]
15. Wang S, Chen L. Immunobiology of Cancer Therapies Targeting CD137 and B7-H1/PD-1 Cosignal Pathways. *Curr Top Microb Immunol*. 2010 E-published.
16. Lazar-Molnar E, Chen B, Sweeney KA, Wang EJ, Liu W, Lin J, et al. Programmed death-1 (PD-1)-deficient mice are extraordinarily sensitive to tuberculosis. *PNAS*. 2010; 107:13402–13407. [PubMed: 20624978]
17. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity*. 2007; 27:670–684. [PubMed: 17950003]
18. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*. 2005; 6:1245–1252. [PubMed: 16286920]
19. Zhu C, Anderson AC, Kuchroo VK. TIM-3 and Its Regulatory Role in Immune Responses. *Curr Top Microbiol Immunol*. 2010 E-published.
20. Lin PL, Pawar S, Myers A, Pegu A, Fuhrman C, Reinhart TA, et al. Early events in *Mycobacterium tuberculosis* infection in cynomolgus macaques. *Infect Immun*. 2006; 74:3790–3803. [PubMed: 16790751]
21. Chakravarty SD, Xu J, Lu B, Gerard C, Flynn J, Chan J. The chemokine receptor CXCR3 attenuates the control of chronic *Mycobacterium tuberculosis* infection in BALB/c mice. *J Immunol*. 2007; 178:1723–1735. [PubMed: 17237422]
22. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, et al. Early granuloma formation after aerosol *Mycobacterium tuberculosis* infection is regulated by neutrophils via CXCR3-signaling chemokines. *Eur J Immunol*. 2003; 33:2676–2686. [PubMed: 14515251]
23. Pokkali S, Das SD, L R. Expression of CXC and CC type of chemokines and its receptors in tuberculous and non-tuberculous effusions. *Cytokine*. 2008; 41:307–314. [PubMed: 18226915]
24. Vesosky B, Rottinghaus EK, Stromberg P, Turner J, Beamer G. CCL5 participates in early protection against *Mycobacterium tuberculosis*. *J Leukocyte Biol*. 2010; 87:1153–1165. [PubMed: 20371596]
25. Algood HM, Lin PL, Flynn JL. Tumor necrosis factor and chemokine interactions in the formation and maintenance of granulomas in tuberculosis. *Clin Infect Dis*. 2005; 41 3:S189–193. [PubMed: 15983898]
26. Brahmabhatt S, Black GF, Carroll NM, Beyers N, Salker F, Kidd M, et al. Immune markers measured before treatment predict outcome of intensive phase tuberculosis therapy. *Clin Exp Immunol*. 2006; 146:243–252. [PubMed: 17034576]
27. Djoba Siawaya JF, Chegou NN, van den Heuvel MM, Diacon AH, Beyers N, van Helden P, et al. Differential cytokine/chemokines and KL-6 profiles in patients with different forms of tuberculosis. *Cytokine*. 2009; 47:132–136. [PubMed: 19570688]
28. Ordway D, Henao-Tamayo M, Shanley C, Smith EE, Palanisamy G, Wang B, et al. Influence of *Mycobacterium bovis* BCG vaccination on cellular immune response of guinea pigs challenged with *Mycobacterium tuberculosis*. *Clin Vaccine Immunol*. 2008; 15:1248–1258. [PubMed: 18508930]
29. Ordway D, Palanisamy G, Henao-Tamayo M, Smith EE, Shanley C, Orme IM, et al. The cellular immune response to *Mycobacterium tuberculosis* infection in the guinea pig. *J Immunol*. 2007; 179:2532–2541. [PubMed: 17675515]

30. Ordway DJ, Shanley CA, Caraway ML, Orme EA, Bucy DS, Hascall-Dove L, et al. Evaluation of standard chemotherapy in the guinea pig model of tuberculosis. *Antimicrob Agents Chemother.* 2010; 54:1820–1833. [PubMed: 20160055]

Figure 1.

Flow cytometric analysis of cell surface markers expressed on CD8 T cells on day-120 of the infection. Hatched [red] lines – cells from naïve mice. Solid [green] lines – cells from infected mice. Open [blue] lines – cells from animals receiving drug treatment. These are also indicated by arrows in the case of PD-1 [drug treatment reduced expression back to naïve animal levels] and CCR7 [intermediate, ambivalent pattern] as representative examples [N: naïve mice; IN; infected mice; IN/Tx; infected and drug treated mice]. Experiments were repeated twice and the histograms are from one representative sample among 5 samples recovered in each data point, from the organs of 4-5 mice.

Figure 2.

Flow cytometric analysis showing total CD8 T cells [green] and CD8 T cells staining with an antigen-specific Mtb32 H-2D^b MHC class I tetramer [blue]. Experiments were repeated twice and the histograms are from one representative sample among 5 samples recovered in each data point, from the organs of 4-5 mice.

Figure 3.

Changes in numbers of CD4 and CD8 cells that stained positive for PD-1 or KLRG-1. [A] Numbers of CD4 cells and [C] numbers of CD8 cells recovered from the lungs of mice infected with *M. tuberculosis* over the course of drug treatment [open squares] versus age-matched uninfected controls [also drug treated; black squares] [Mean numbers of cells $\pm$ SEM, n=5]. Representative gating strategies, doublet discrimination and exclusion of dead cells, for KLRG-1 [B] and PD-1 [D] are also shown. In each case the data point represents cells recovered from the organs of 4-5 mice.

Figure 4. Changes in numbers of CD4 and CD8 cells that stained positive for CXCR3 and CCR5. Numbers of CD4 cells [left] and CD8 cells [right] recovered from the lungs of mice infected with *M.tuberculosis* and drug treated [open squares], or age-matched uninfected controls [also drug treated, black squares]. Mean numbers of cells $\pm$ SEM, n=5. In each case the data point represents cells recovered from the organs of 4-5 mice.