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Pulmonary tuberculosis in guinea pigs is an extremely useful model for drug and vaccine testing due to the fact that its pathological disease process is similar to that present in humans. Progress in this field has been hindered because the tools necessary to undertake a complete immunological analysis of the guinea pig cellular immune response against *Mycobacterium tuberculosis* have been lacking. In this study, we combined a new flow cytometric gating strategy with immunohistochemistry to track T cells, B cells, and the MIL4 Ab, which detects both guinea pig heterophils (neutrophils) and eosinophils, to provide the first documentation of the kinetics of influx and positioning of these cell populations. The results show that the responding T cells are mostly CD4 cells and that after day 30 of the infection numbers of these cells in the lungs drops dramatically. These appear to be replaced by a steady increase in B cells and granulocytes which was associated with worsening lung pathology. These data reveal new information about the cellular phenotypes which mediate protective immunity or host immunopathogenesis during *M. tuberculosis* infection in this key animal model. *The Journal of Immunology*, 2007, 179: 2532–2541.

In the past, the primary drawback of the guinea pig model was a relative lack of specific immunological reagents with which to monitor the emerging acquired immune response in infected animals. However, this situation is gradually improving with the availability of a few Abs to T cell markers and the development of PCR-based techniques to measure key cytokines and chemokines (10, 14–19). In this study, we have taken advantage of these available Abs to monitor immunity by flow cytometry. Although this has been attempted before (10), a variety of technical difficulties have been evident, ranging from the processing of lung tissues to the initial cell-gating parameters. Traditionally, this is based on forward/side scatter (FSC/SSC)3 flow cytometric gating, the use of which has proven difficult to adequately and consistently identify the distinct fractions of guinea pig cells. Recently however, a technical solution to this has been demonstrated (18, 20) that uses gating based on SSC and a specific Ab (MIL4), which we have used here. The population of MIL4+ cells is composed of both guinea pig heterophils (neutrophils) and eosinophils. This has allowed us to track the kinetics of influx of CD4 and CD8 T cells, B cells, and MIL4+ granulocytes into the infected lung.

The disease that develops in the guinea pig following aerosol exposure to *M. tuberculosis* can be divided into acute, subacute, and chronic stages of infection based on the pattern of bacterial growth and dissemination, as well as patterns of pulmonary and extrapulmonary pathology (12, 14, 15). During acute infection, there is an initial 3-day lag in bacterial growth, followed by an ~2-wk period of rapid proliferation in the lung and draining lymph nodes. This stage is also characterized by progression of granulomatous inflammation and necrosis in the primary lesion complex of the lung and draining mediastinal lymph nodes. The subacute or bacillary phase from 2 to 4 wk is characterized by the emergence of a stationary phase of bacterial replication in the lung and lymph nodes. During this phase, the most severe inflammation within the primary lesion begins to subside but not before it has replaced a significant proportion of the normal tissue, particularly in the

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1 This work was supported by National Institutes of Health Grant AI-054697.

2 Address correspondence and reprint requests to Dr. Diane Ordway, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682. E-mail address: D.Ordway-Rodriguez@colostate.edu

3 Abbreviations used in this paper: FSC, forward scatter; SSC, side scatter.
draining lymph nodes (12, 15). Moreover, in this subacute stage, infection is established in multiple extrapulmonary sites such as the spleen and liver by hematogenous dissemination of bacilli. Concurrent with bacillemia and exponential bacterial growth in extrapulmonary sites, there is reinfection of the lung by the hematogenous route. Finally, the chronic stage is characterized by continued bacterial replication in extrapulmonary tissues but with either stationary or a gradual increase in bacterial numbers in the lung and lymph nodes. Therefore, the morbidity and mortality of guinea pigs at this stage is due to the combined effect of progressive pulmonary and extrapulmonary pathology.

The results of this study show that during the acute and subacute stages of infection the immune response involves the recruitment of more CD4 T cells, including cells expressing the activation marker CD45, with only an apparently minimal involvement by CD8 T cells. However, after the first month, at a time where the lung and lymph node pathology progressively worsens, the numbers of CD4 T cells in the lungs drops precipitously, and is replaced by B cells and MIL4<sup>+</sup> granulocytes during the chronic disease state. These data are discussed in the context of disease progression, immunopathology, and <i>M. tuberculosis</i> proliferation and dissemination.

**Materials and Methods**

**Guinea pigs**

Female outbred Hartley guinea pigs (~500 g in weight) were purchased from Charles River Laboratories and held under barrier conditions in a biosafety level III animal laboratory. The specific pathogen-free nature of the guinea pig colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University.

**Experimental infections in guinea pigs**

Guinea pigs were challenged using a Madison chamber aerosol generation device, by delivering <i>M. tuberculosis</i> H37Rv at a low-dose aerosol of 20 bacilli. Animals were then assayed for lung, lymph node, spleen bacterial loads, histology, and cell homogenates for flow cytometric analysis on days 5, 15, 20, 30, 60, and 90 of the infection. Bacterial counts in the organs of guinea pigs (n = 4) at each time point of the study were determined by plating serial dilutions of homogenates of lungs on nutrient 7H11 agar and counting CFU after 3 wk of incubation at 37°C.

**Histological analysis in guinea pigs**

The lung lobes, spleen, and lymph nodes from each guinea pig were fixed with 4% paraformaldehyde in PBS. Sections from these tissues were stained using H&E and the Ziehl-Neelsen stain for acid-fast bacilli as previously reported (14). In guinea pigs, the concurrent progression of lung and lymph node lesions was evaluated using a histological grading system. The method for grading granulomatous lesions was based on inflammatory cell numbers and their infiltrative distribution pattern in the organs was assessed (14). Scoring of lung and lymph node lesions was based on randomly selected sections in a representative experiment from five infected guinea pigs and three noninfected guinea pigs at indicated times after infection.

**Immunohistochemistry**

Once removed from the pulmonary cavity, the cranial lobe of the lungs were embedded in OCT, frozen in liquid nitrogen, then stored at −80°C. Serial sections, 8- to 10-μm thick, from each lung were cut on a cryostat (CM 1850; Leica) using the Instrumedics tape transfer system, fixed in cold acetone, and air dried. The sections were washed, and nonspecific Ab binding was blocked with a 3% BSA-PBS solution. Thereafter, the sections were incubated overnight at 4°C with one of the following purified primary Abs listed in Table I (Sorotec). All sections were washed three times in

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**Table I. Panel of anti-guinea pig Abs used for flow cytometry and immunohistochemistry**

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Fluorescence Label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>CT7</td>
<td>FITC</td>
<td>19</td>
</tr>
<tr>
<td>CD8</td>
<td>CT6</td>
<td>FITC</td>
<td>19</td>
</tr>
<tr>
<td>Pan T cell</td>
<td>CT5</td>
<td>Allophycocyanin</td>
<td>19, 20</td>
</tr>
<tr>
<td>CD45</td>
<td>HI-1</td>
<td>RPE</td>
<td>21</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>MIL4</td>
<td>RPE</td>
<td>22</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td>MsGP9</td>
<td>FITC</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> Anti-guinea pig mAbs were purchased from Serotec.
Flow cytometric analysis of cell surface markers

Single-cell suspensions from the of the lungs and one-third portions of the whole spleens and lymph nodes were prepared as described before (17, 18). Cell suspensions from each individual guinea pig were incubated first with the CD4 (19), CD8 (19), pan T cell (19, 20), CD45 (21), MIL4 (22), and B cell (23) Abs listed in Table I at 4°C for 30 min in the dark and after washing the cells with PBS containing 0.1% sodium azide (Sigma-Aldrich). Data acquisition and analysis were done using a FACS Calibur (BD Biosciences) and CellQuest software (BD Biosciences). Compensation of the spectral overlap for each fluorochrome was done using CD4 or MIL4 or CD3 Ags from cells gated in the FSClow vs FSCmid/high and SSChigh vs MIL4neg region, respectively. Analyses were performed with an acquisition of at least T cells (100,000 total events).

Statistical analysis in guinea pigs

Data are representative of two experiments and presented using the mean values from individual guinea pigs within each group (n = 4) and ± SEM. ANOVA was completed.

Results

Bacterial growth in guinea pigs infected with M. tuberculosis

Guinea pigs exposed to ~20 bacilli of M. tuberculosis were evaluated for bacterial loads in the lung, spleen, and lymph node at indicated time points (Fig. 1). Infected guinea pigs showed an increase of ~3.5 logs over the first 20 days of infection (Fig. 1A), followed by a chronic phase of disease. A similar rise in numbers to those seen in the lungs was observed in the draining lymph nodes (mediastinal lymph node cluster) (Fig. 1B). Bacteria could be detected disseminating to the spleens of the animals by day 5, although bacterial numbers then increased at a slower rate (Fig. 1C).

PBS and incubated with the secondary detection Ab F(ab’)_2 rabbit anti-mouse conjugated to HRP (Serotec). Finally, the reaction was developed using aminoethylcarbazole (BioGenex) as substrate. The sections were counterstained with Meyer’s hematoxylin and thereafter mounted with crystal/mount (BioGenex). Our experiments used different lung lobes of the guinea pig for immunohistochemistry and flow cytometry because we have shown the lesions are randomly scattered throughout the guinea pigs infected lung by evaluating the histopathology and magnetic resonance imaging of pulmonary lesions in guinea pigs (16).

Organ cell digestion

To prepare single-cell suspensions the lungs, lymph nodes, and spleens were perfused with 20.0 ml of a solution containing PBS and heparin (50 U/ml; Sigma-Aldrich) through the pulmonary artery and the caudal lobe aseptically removed from the pulmonary cavity, placed in medium, and dissected. The dissected lung tissue was incubated with complete DMEM containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 µg/ml; Sigma-Aldrich) for 30 min at 37°C. The digested lungs were further disrupted by gently pushing the tissue twice through a cell strainer (BD Biosciences). RBC were lysed with ACK buffer, washed, and resuspended in complete DMEM. Total cell numbers to form multifocal to coalescing inflammation that effaced large areas of pulmonary (G), lymph node (H), and splenic (I) parenchyma. B. The increase in the mean lesion scores of the lung, lymph node, and spleen was associated with the increase of bacterial loads. The histopathology (B) was characterized using a lesion scoring system (14) that showed the significant extent of lung disease compared with the other organs during chronic infection (ANOVA, *p = 0.001). A–I, Bar, 20 µm.

FIGURE 2. Histopathological appearance of infected organs. A. Representative photomicrographs from sections of formalin-fixed and paraffin-embedded guinea pig tissues from lung (A, D, and G), lymph node (B, E, and H), and spleen (C, F, and I) which were collected on days 5, 30, and 60 after M. tuberculosis challenge (A–I). A–C. Perivascular and peribronchiolar accumulations of resident epithelioid macrophages, granulocytes, and lymphocytes located in the parenchyma, close to major airways, and blood vessels. D–F. By day 30 in the lungs (D), lymph nodes (E), and spleen (F), the lesions consist of foci of mixed inflammation with areas of central necrosis (arrows) composed of nuclear and cytoplasmic debris. G–I. The lesions (arrows) progressed to form multifocal to coalescing inflammation that effaced large areas of pulmonary (G), lymph node (H), and splenic (I) parenchyma. B. The increase in the mean lesion scores of the lung, lymph node, and spleen was associated with the increase of bacterial loads. The histopathology (B) was characterized using a lesion scoring system (14) that showed the significant extent of lung disease compared with the other organs during chronic infection (ANOVA, *p = 0.001). A–I, Bar, 20 µm.

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Evaluation of infected tissue pathology

As previously described (12, 15), by day 5 of the infection the lung lesions consisted of small aggregations of resident cells close to major airways and blood vessels (Fig. 2A). As the granulomatous response increased in intensity, by day 30, lesions in all three organs consisted of foci of mixed inflammation with areas of central necrosis composed of nuclear and cytoplasmic debris (Fig. 2, AD–AF). The foci of necrosis were surrounded by predominately epithelioid macrophages and lymphocytes. At the interface with more normal parenchyma, increased numbers of lymphocytes within increased fibrous connective tissue was observed. The lesions continued to progress to form multifocal coalescing inflammation that effaced large areas of pulmonary (Fig. 2AG), lymph node (Fig. 2AH), and splenic (Fig. 2AI) parenchyma by day 60 of the infection. By this time, debris within the necrotic centers of the lesions had become mineralized (Fig. 2AG) and was surrounded by epithelioid macrophages. Fig. 2B shows an overall pathology score for each organ; it was noted that in the lungs, pathology worsened at a gradual rate over the first 30 days, but thereafter this rate was significantly accelerated compared with the other organs.

Assessment of CD4 and CD8 T cell influx using flow cytometry

Using a recently described technique (14, 20), we evaluated the influx of T cells in the lungs of guinea pigs over the course of the infection. Fig. 3 shows a typical dot-plot analysis of lung cells obtained from a representative guinea pig infected 5 days earlier with M. tuberculosis in which lymphocytes were primarily gated on SSC vs FSC (Fig. 3A) or alternatively SSC vs MIL4 (Fig. 3F). Dead cells were excluded by propidium iodide and only viable cells were gated (data not shown).

Leukocyte fractions were separated using flow cytometry SSC vs FSC (Fig. 3A), showing the following cell populations lymphocytes (green) and granulocytes (orange). Representative dot plots demonstrate primary gating on SSC vs FSC and further gating on isotype controls (Fig. 3B), SSC vs CD4+ (Fig. 3C), SSC vs CD8+ (Fig. 3D), and SSC vs MIL4+ (Fig. 3E).

The new gating strategy shows representative dot plots of primary gating on SSC vs MIL4 (Fig. 3F), allows clear separation of lymphocytes (green), MIL4lowSSChigh (orange), MIL4lowSSClow monocytes (red), MIL4negSSChigh granulocytes (blue). Further separation of R1 lymphocytes shows isotype controls (G), MIL4neg vs CD4+ (H), MIL4neg vs CD8+ (I) and further separation of the granulocytes shows SSC vs MIL4+ (J).

**FIGURE 3.** Flow cytometric gating techniques. Dead cells were excluded by propidium iodide and viable cell were gated. Leukocyte fractions from organ of infected guinea pigs were separated using flow cytometry SSC vs FSC (A); this plot demonstrates lymphocytes (green) and granulocytes (orange) and further separation of the lymphocytes shows isotype controls (B), SSC vs CD4+ (C), SSC vs CD8+ (D), and further separation of the granulocytes shows SSC vs MIL4+ (E). Gated CD4+ cells which are autofluorescent and located outside the SSC vs FSC lymphocytes (green, arrows) gate (A) and this is also occurs in the gated positive SSC vs MIL4+ cells which are false positives being located outside the SSC vs FSC (A) granulocytes (orange, arrows) gate. The new SSC vs MIL4 (F) gating shows lymphocytes (green), MIL4lowSSChigh (orange), MIL4lowSSClow monocytes (red), MIL4negSSChigh granulocytes (blue). Further separation of R1 lymphocytes shows isotype controls (G), MIL4neg vs CD4+ (H), MIL4neg vs CD8+ (I) and further separation of the granulocytes shows SSC vs MIL4+ (J).

Thereafter however, these levels dropped again close to levels seen in age-matched uninfected controls. A much smaller rise was observed in terms of CD8 cell influx (Fig. 4B), which was not sustained after day 15 of the infection. Upon initial aerosol infection of the guinea pig, our results support an increase of CD4+ and CD8+ T cells over the course of infection.

By day 15 of the infection, we observed a very substantial rise in CD4 cell levels in the lungs which peaked on day 30 (Fig. 4A).

These data provided an overall assessment of T cell levels but not their positioning in lesions. To address this, we followed up by performing immunohistochemistry on the infected lung tissues.
After infection, M. tuberculosis was assayed by flow cytometry for percentages (%) of CD4+ and CD8+ T cells. A and B, The total percentage of MIL4++CD4+ and MIL4++CD8+ T cells, respectively, in the lungs of uninfected (naive) guinea pigs (□) and M. tuberculosis-infected guinea pigs (●). C and D, The percentages of CD4+ and CD8+ T cells, respectively, in the lymph nodes of uninfected guinea pigs (□) and guinea pigs infected with M. tuberculosis (●) at the indicated times after infection. E and F, The percentages of MIL4++CD4+ and MIL4++CD8+ T cells, respectively, in the spleens of uninfected guinea pigs (□) and guinea pigs infected with M. tuberculosis (●) at the indicated times after infection. G and H, The total percentage of MIL4++CD4+ and MIL4++CD8+ T cells, respectively, in the blood of uninfected (naive) guinea pigs (□) and M. tuberculosis-infected guinea pigs (●). Results are expressed as the mean percentage of MIL4++CD4+ and MIL4++CD8+ T cells (±SEM, n = 4). (ANOVA, *, p ≤ 0.005) compared with naive guinea pigs.

Fig. 5 shows the results of this study. The increasing numbers of CD4 cells over the first 30 days was consistent with the flow cytometry data. By day 30, there were large aggregations of CD4 cells forming a distinct layer surrounding the necrotic center of the lesion (Fig. 5, C and D), but by day 60 the staining distribution of these cells had become more diffuse (Fig. 5, E and F). In contrast, far fewer CD8 cells had accumulated by day 30 and were distributed along the periphery of the lesions (Fig. 5, I and J). By day 60, very few could be detected, and appeared to be mostly beyond the actual boundaries of the granulomas (Fig. 5, K and L).

Expression of the CD45 marker on CD4+ and CD8+ cells in infected tissues

The up-regulation of CD45 (leukocyte common Ag) molecules on T cells indicates signal transduction of Ag receptor signaling during immune responses and hence is an indication of T cell activation (24). We took advantage of an available mAb to evaluate the percentage of cells expressing CD45 on CD4 and CD8 T cells over the course of the infection.

Fig. 6 shows the expression of CD45 on T cells within the lung was expressed to a lesser extent in comparison to the other organs and blood assayed. As shown in Fig. 6A, CD45+ was up-regulated on CD4+ and CD8+ cells from the lungs of guinea pigs after 30 days of infection and began waning by day 60. Percentages of CD4+ and CD8+ cells residing in the lymph nodes showed a strong expression of CD45 by day 15, peaking by day 30, and declined during chronic infection (Fig. 7B). Increases in CD45 expression on CD4+ and CD8+ T cells was evident by day 20 in the spleen (Fig. 7C) and blood (Fig. 7D) which thereafter these molecules were down-regulated during chronic disease.

The influx of B cells into the infected tissues

B cells are a known component of the granulomatous response to tuberculosis in the mouse (25). To determine whether these cells entered lesions in the guinea pig, we attempted to detect these cells by flow cytometry and by immunohistochemistry. As shown in Fig. 7A, increases in MIL4 B cell levels in the lung were negligible in the lungs until around day 60, at which time they increased substantially. Similar rises were seen in the other organs occurring somewhat earlier (Fig. 7, B–D). Immunochemical analysis (Fig. 7E) provided the additional information that these cells were distributed on the periphery of the necrotic center of the lung granulomas.

The influx of granulocytes into the infected tissues

Granulocytes are a known component of the guinea pig granuloma, and we have suggested previously that degranulation of these cells is the initial cause of the characteristic necrosis occurring very early in the disease process (12). The MIL4 Ab binds heterophils and eosinophils. As shown in Fig. 8A, their kinetics of appearance in the lungs was fairly similar to B cells, accumulating in increased numbers from day 60 onward. This was also apparent in the lymph...
nodes and blood (Fig. 8, B and D), whereas the spleen data indicated a very early rise in these cells (Fig. 8C). Immunohistochemical analysis (Fig. 8E) showed that these cells in the lungs were positioned in and around the central necrotic core. We also documented during chronic infection large aggregates of these cells located with acid-fast-staining bacilli within macrophages (Fig. 8F) located in the pulmonary airways associated with secondary lesions.

**Discussion**

In this study, we used a recently described technique (20) to allow us to perform a comprehensive analysis by flow cytometry of the influx of lymphocytes and granulocytes into the lungs and other organs in guinea pigs infected with *M. tuberculosis*. The primary findings of this study revealed a substantial influx of CD4 T cells, many expressing the activation marker CD45, but few CD8 cells, into the lung tissues over the first 30 days of the infection. Although this time represents the start of a phase of chronic disease in this animal model that can last for another 80–100 days or so, soon after day 30, we observed a dramatic loss in the CD4 response. At this time, lesions instead became more and more occupied by B cells and MIL4+ cells which detect both guinea pig heterophils (neutrophils) and eosinophils. This is important new information that requires a re-evaluation of current concepts regarding the pathogenesis of tuberculosis in this important animal model.

The drop in CD4 numbers appears to represent a true effect and not a dilution by other cell types entering the granulomas. We suspected this in a previous study (12) where we observed that the lymphocyte “mantle,” an early characteristic of the guinea pig granuloma, exhibited an obvious reduction in the density of staining after day 30. This is confirmed in the present study; in our immunohistochemical analysis, CD4 cells took up position surrounding the developing core of necrosis, but by day 60, this layer was smaller and staining for CD4 more diffuse. This has been in the mouse model as well; although the granuloma in the mouse does not initially show any necrosis, the large aggregates of lymphocytes seen in lesions slowly disappear and are replaced by fibrosis (26).

Few CD8 cells were detected by flow cytometry, and immunohistochemistry showed that they tended to be on the periphery of the cell layers in the granuloma, rather than within the main inflammatory cell zone.

Of further note was the observation that despite the obvious dissemination of the infection to the spleen, no major increase in either CD4 or CD8 T cell numbers was observed. This was
unexpected, and this raises the question as to whether whatever mechanism shut down the T cell response in the lungs was also operative in the spleen. In fact, it was proposed by Smith (28) and are based on his classical studies that some form of immunosuppressive event induced the breakdown of the chronic disease state and resulted in fatal reactivation disease in this model. This still may be so, particularly in clinical situations, but if a suppressive event occurred in our study it obviously was induced very early on.

B cells are a known component of the mouse granuloma (26), and we show here a similar event in the guinea pig model. In addition, we demonstrated that B cells surged in the blood and lymph nodes, respectively, in the spleens of infected (naive) guinea pigs (open symbols) and guinea pigs infected with M. tuberculosis (closed symbols) at the indicated times after infection. Results are expressed as the mean percentage CD45+ cells (±SEM, n = 4). (ANOVA, α, p ≤ 0.005) compared with naive guinea pigs.

The morphologic differences in the guinea pig model allows a classification of the course of the disease as acute, subacute, and chronic infection (12, 14, 15). The development of pulmonary and extrapulmonary pathology reflect the different stages of the disease process. In the acute and subacute stages of the infection, the increasing primary lesion pathology scores were very similar in both the lung and draining lymph nodes, coincident with the climbing bacterial load in each organ. The establishment of the extrapulmonary infection, or bacillemic phase, was reflected by the first appearance of lesions in the spleen which we show here appeared to continue to grow progressively during the chronic infection with little or no T cell response. As the chronic stage disease developed, reinfestation of the lung probably occurred by the hematogenous route, and was reflected by an increase in lung lesion scores which represent the progressive development of secondary lesions (12, 14, 28, 30). Given the data gained above, it seems likely that the predominant lymphocyte population entering these secondary lesions were B cells.

The loss of the early CD4 T cell population will require further investigation. The decline was rapid and may have resulted from cell necrosis or perhaps apoptosis. We would favor the former hypothesis, for the simple reason that our earlier studies (12) found very few apoptotic bodies in these tissues. An alternative explanation is that the lung digest procedure became increasingly inefficient at liberating T cells from the diseased tissue. However, we do not prefer this explanation due to our lung digest procedure of surgical dissection of granulomas followed by tissue teasing and passage through a fine mesh screen. Another possibility is that the T cells entering the lungs are either short-lived or not replenished due to progressive destruction of peripheral lymphoid tissues in the chronic infection. The trigger for this event remains completely unknown, but may start to become apparent as we develop more Abs to guinea pig cell markers that can be used for flow cytometry. One possibility we should consider, based on our recent observations in the mouse in which we observed a rapid fall-off in Th1 CD4 T cell activity in the lungs of mice infected with strain HN878, is the emergence of regulatory T cells (29). Regardless of the reason, the loss of the CD4 response raises the question of whether effective new vaccines function by preventing this T cell loss. If so, this could represent a new surrogate marker for vaccine efficacy in this gold standard testing model.

FIGURE 6. Expression of CD45 on CD4+ and CD8+ T cells during M. tuberculosis infection. Lung, lymph node, spleen, and blood cells obtained from naive guinea pigs and guinea pigs challenged with M. tuberculosis were assayed by flow cytometry for CD4+ and CD8+ T cell expression of CD45 on days 5, 15, 20, 30, 60, and 90 of the infection. A and B. The total percentage of CD4+CD45+ and CD8+CD45+ T cells, respectively, in the lungs (A) and lymph nodes (B) of uninfected (naive) guinea pigs (open symbols) and M. tuberculosis-infected guinea pigs (closed symbols). C and D. The percentages of CD4+CD45+ and CD8+CD45+ T cells, respectively, in the spleens of uninfected (naive) guinea pigs (open symbols) and guinea pigs infected with M. tuberculosis (closed symbols) at the indicated times after infection. Results are expressed as the mean percentage CD45+ cells (±SEM, n = 4). (ANOVA, α, p ≤ 0.005) compared with naive guinea pigs.
adjacent macrophages containing large numbers of acid-fast bacilli. It is unknown as yet whether the loss of T cells in the lungs and the subsequent increase in B cells and MIL4⁺ granulocytes are related or just coincidental. The Th1/Th2 paradigm probably holds in the guinea pig model, based upon studies relating protective immunity and vaccine effects to established Th1-type cytokines and chemokines (32). B cells are known to produce proinflammatory cytokines, and express chemokine receptors such as CXCR5 and CCR7 that may promote their migration to inflammatory sites (33), although in the mouse model at least there is no evidence these cells interfere with protective immunity (34). Granulocytes are also strongly associated with Th2 immunity, and play a major role in the immunopathogenesis of asthma and allergic rhinitis (35). Eosinophils in particular can mediate Ab-dependent cellular cytotoxicity, produce IL-4, and hence strongly influence cytokine production, chemokine receptor expression, and cellular influx into the respiratory tract (36). In vitro at least, Th2 cytokines can interfere with macrophage activation (37), decrease TLR2 signaling (38), and reduce production of NO synthase (inducible NO synthase) (38). Therefore, even if a potentially emerging Th2 environment is not the cause of the CD4 cell decline, it may still directly interfere with any subsequent expression of protective immunity.

An obvious further parameter that is centrally important is the pathologic process itself. It is possible that T cells in the lung, and almost certainly in the draining lymph nodes, were replaced by the progressive granulomatous inflammation and necrosis, a key feature of the subacute and chronic disease stages. In this and previous studies (12, 14, 15), we have shown that as part of the primary lesion complex, granulomatous and necrotizing lymphadenitis progresses more rapidly than the expansion of the primary lesions of the lung. The draining lymph nodes, as well as being the first extrapulmonary lymphoid tissues to encounter the infecting organism and the primary site of T cell priming (39–41), are also the first site of rapidly progressive destructive pathology. Spread of bacteria due to lymphatic drainage from the lung may then lead to bacillosis in the subacute phase of the infection. In addition, pulmonary lymphatics in the guinea pig can themselves be regarded as being part of the primary lesion complex, and can be the site of

FIGURE 7. B cell influx into the lungs, lymph nodes, spleen, and blood of guinea pigs during M. tuberculosis infection. Lung, lymph node, spleen, and blood cells obtained from naive guinea pigs and guinea pigs challenged with M. tuberculosis were assayed by flow cytometry for B cells on days 5, 15, 20, 30, 60, and 90 after the infection. A and B, The total percentage of MIL4 neg B cells, respectively, in the lungs and lymph nodes of uninfected (naive) guinea pigs (□) and M. tuberculosis-infected guinea pigs (●). C and D, The percentages of MIL4 neg B cells, respectively, in the spleens and blood of uninfected guinea pigs (□) and guinea pigs infected with M. tuberculosis (●) at the indicated times after infection. Results are expressed as the mean percentage of MIL4 neg B cells (±SEM, n = 4). (ANOVA, * p ≤ 0.005) compared with naive guinea pigs. E, Immunohistochemical staining of B cells in lung tissue sections from guinea pigs after 30 and 60 days of infection. Left upper and lower panels = bar, 100 μm; right upper and lower panels = bar, 10 μm.
granulomatous and necrotizing lymphatic vasculitis (15). Capture of dendritic cells containing bacilli as a result of this lymphangitis, in concert with the rapid involvement and destruction of the draining lymph nodes, may be the reason T cells are depleted in the lungs during the chronic infection.

Current concepts of the pathogenesis in the guinea pig is based on early studies (28, 30, 42). This model supports during acute infection with \( M.\) \( \text{tuberculosis} \) the initial presence of bacilli results in the absence of tissue damage. However, as the infection progresses, excessive cellular mediated immunity classified as, “delayed type hypersensitivity” is generated in association with a strong cytolytic T cell activity which leads to necrotic core degeneration of the center of the granulomatous lesions and eventual death. In addition, this model supports that the central necrosis initially restricts bacterial growth. Then, as the infection progressed into chronic disease, Smith (30) theorized that reactivation of the lung granulomas and eventual lung consolidation and death was due to some immunosuppressive event. Contrary to current dogma (42), we have suggested that the development of the primary lesion necrosis is a very early event in the disease process and may reflect the degranulation of MIL4\(^+\) granulocytes observed in these lesions (12); as shown above, numbers of these cells were already rising by day 5 of the infection. Moreover, our belief that the chronic stage of the disease was controlled by the ongoing T cell response, as shown in the mouse model (43), is clearly incorrect. Based on the data presented above, a new appraisal of the pathogenesis of tuberculosis in this important animal is required.

Disclosures
The authors have no financial conflict of interest.

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