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Enhanced Macrophage Activity in Granulomatous Lesions of Immune Mice Challenged with *Mycobacterium tuberculosis*¹

Diane Ordway,² Marisa Harton, Marcela Henao-Tamayo, Rose Montoya, Ian M. Orme, and Mercedes Gonzalez-Juarrero

In this study, we evaluated the cellular influx and cytokine environment in the lungs of mice made immune by prior vaccination with *Mycobacterium bovis* bacillus Calmette-Guérin compared with control mice after infection with *Mycobacterium tuberculosis* to characterize composition of protective lesions in the lungs. Immune mice controlled the growth of the *M. tuberculosis* challenge more efficiently than control mice. In immune animals, granulomatous lesions were smaller and had a more lymphocytic core, less foamy cells, less parenchymal inflammation, and slower progression of lung pathology than in lungs of control mice. During the chronic stage of the infection, the bacterial load in the lungs of immune mice remained at a level 10 times lower than control mice, and this was associated with reduced numbers of CD4⁺ and CD8⁺ T cells, and the lower expression of protective (IL-12, TNF-α), inflammatory (TNF-α), immunoregulatory (GM-CSF), and immunosuppressive (IL-10) cytokines. The immune mice had higher numbers of CD11b⁺CD11c⁺DEC-205⁺ low alveolar macrophages, but lower numbers of CD11b⁺CD11c⁺DEC-205⁺ high dendritic cells, with the latter expressing significantly lower levels of the antiapoptotic marker TNFR-associated factor-I. Moreover, during the early stage of chronic infection, lung dendritic cells from immune mice expressed higher levels of MHC class II and CD40 molecules than similar cells from control mice. These results indicate that while a chronic disease state is the eventual outcome in both control and immune mice infected with *M. tuberculosis* by aerosol exposure, immune mice develop a protective granulomatous lesion by increasing macrophage numbers and reduced expression of protective and inflammatory cytokines. *The Journal of Immunology*, 2006, 176: 4931–4939.

The global epidemic of tuberculosis (TB)³ results in eight million new TB cases per year, with an annual projected increase of 3%. It is estimated that between 5 and 10% of immunocompetent individuals are susceptible to TB, and, of these, 85% develop pulmonary disease (1). Eradication of TB is a very difficult goal because *Mycobacterium tuberculosis* is capable of remaining in the host for long periods of time in some form of latent or chronic state. At present, the only available vaccine against TB, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), has proven unreliable to fully protect against pulmonary TB in adults (2–6). Furthermore, a thorough immunologic explanation for the variability in the efficacy of BCG is absent. Therefore, understanding the specific protective properties of BCG is vital for developing a more efficacious TB vaccine.

After pulmonary infection with *M. tuberculosis*, the infected host generates a Th1 immune response in which mycobacterial Ag-specific T lymphocytes are recruited to the lung. Both CD4⁺ and CD8⁺ T cells play a role in protection against *M. tuberculosis* (7, 8). The emergence of Th1 cells is dependent mainly on production of IL-12, TNF-α, and effector T cells producing IFN-γ (9–12). However, despite the presence of a Th1 response in some individuals, a chronic form of infection develops. Using animal models, Th1 immunity against mycobacterial infection obtained either by vaccination with BCG (13, 14) or by other vaccine strategies (15–19) or by prior exposure to virulent *M. tuberculosis* (20) does not provide >1–1.5 log reduction of the bacterial load in the lungs after a secondary exposure to virulent *M. tuberculosis*. Several vaccine strategies attempting to boost Th1 response in the lungs cause exacerbation of lung pathology (15–17, 19). Together, these studies show that some vaccine strategies, in addition to boosting Th1 responses that lead to the control of *M. tuberculosis* growth, may also increase inflammatory responses and lung immunopathology.

The development of a pulmonary granuloma is orchestrated by chemokines and cytokines produced by local cells, causing a continuous recruitment of lymphocytes, macrophages, dendritic cells (DCs), and monocytes to the local site of the infection. During the chronic granulomatous response, several inflammatory (TNF-α) and protective (IFN-γ, IL-12) cytokines promote APC priming for T cell responses in the lung (8–11, 21–25). In addition, the immunoregulatory (GM-CSF) cytokine that promotes differentiation of alveolar macrophages and DCs and the immunosuppressive (IL-10) cytokine that inhibits IL-12 function in APC (26–28) are present during the granulomatous response. The dynamic cellular immunity of the granulomatous response is designed to contain bacteria within macrophages and in some instances DCs (28, 29) within the structure of the granuloma to prevent further dissemination and disease progression. Unfortunately, as time progresses, the granuloma structure may degrade, leading to reactivation of quiescent bacilli and bacterial multiplication and active disease.

In this study, we evaluated the cellular influx and cytokine environment in mice made immune by prior vaccination with BCG to...
characterize how this might alter the composition of a protective granulomatous lesion in the lungs. These mice were able to more rapidly contain and control M. tuberculosis growth at the peak of acquired immunity compared with unvaccinated control mice. The lung granulomas present in these mice were smaller than in control mice, had bigger lymphocytic cores, but less foamy cells, less parenchymal inflammation, and slower progression of lung pathology. Because the infection quickly contained the bacteria, cytokine levels were generally lower. There were fewer DCs entering the lung lesions in the immune animals, but those present had substantially increased expression of MHC class II and CD40 molecules. These data indicate that while a chronic disease state is the eventual outcome in both control and immune mice infected with M. tuberculosis by aerosol exposure, there are qualitative and quantitative differences at several levels between the two groups of mice.

Materials and Methods

Mice

Specific pathogen-free female C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory. Mice were housed in the biosafety level 3 biohazard facility at Colorado State University, and were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiment. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Use Committee of Colorado State University.

Experimental infections

Mice were vaccinated by s.c. infection with 10⁵ CFU of BCG Pasteur. Four weeks after inoculation, BCG vaccinated and unvaccinated mice were challenged by low-dose aerosol exposure with M. tuberculosis strain Erdman using a Glas-Col (Terre Haute) aerosol generator calibrated to deliver 50–100 bacteria into the lungs. Bacterial counts in the lung and spleen (n = 5) at each time point of the study were treated and processed, as previously described (30). Briefly, bacterial loads were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar and counting CFUs described (30). Briefly, bacterial loads were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar and counting CFUs described (30). Bacterial loads were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar and counting CFUs described (30).

Histological analysis

The accessory lung lobe from each mouse was fixed with 10% Formalin in PBS. Sections from these tissues were stained using H&E.

Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded lung tissue were used to visualize expression of cytokines and acid-fast-positive bacteria. First, the paraffin was removed from the tissue sections using EZ-DeWax solution (BioGenex). Following this step, the Ag Retrieval Citra solution was used according to the manufacturer’s protocol (BioGenex) to recover Ags from Formalin fixation. After blocking the tissue endogenous peroxidase and nonspecific binding, the sections were incubated with goat polyclonal Abs recognizing murine IL-10, IL-12, GM-CSF, TNF-α, or IFN-γ (Santa Cruz Biotechnology or Serotec), followed by several washes with PBS. Then the sections were incubated with a secondary polyclonal Ab recognizing goat Abs conjugated with HRP (Santa Cruz Biotechnology), and this reaction was developed using diaminobenzidine (BioGenex) substrate. Thereafter, the sections were stained for acid-fast-positive bacteria using the TB carbolchiffin KF Kinyoun method. Finally, each section was analyzed according to the expression of a specific cytokine and its spatial relationship with mycobacteria-infected cells visualized by the acid-fast positive staining (pink color and pink arrows in pictures). Photomicrographs (Fig. 2C) were taken with a IX70 Olympus microscope with an attached ZP70 digital camera. All photomicrographs shown in these panels (Fig. 2C) correspond to sections from lung of control mice after 30 days of postchallenge with M. tuberculosis or immune mice.

The presence of apoptotic cells in lung tissue was analyzed by the TUNEL assay with the MEBSTAIN apoptosis kit (Medical & Biological Laboratories). The protocol provided by the manufacturer was modified to be used for immunohistochemistry. In brief, serial sections, 5–8 μm thick, from each lung were cut, as indicated above, and fixed for 15 min with a solution containing 4% paraformaldehyde. Thereafter, the sections were washed twice with PBS, and 50 μl of TdT buffer II was added and incubated for 10 min at room temperature. The TdT reaction was performed in the presence of biotin-dUTP, for 1 h at 37°C. Then the sections were immersed in TB solution for 15 min. After washing three times with distilled water, the sections were incubated with blocking solution for 10 min at room temperature. The blocking solution was removed, and 200 μl of streptavidin-HRP (Serotec) was incubated for 1 h at 37°C. Following three washes with PBS, the reaction was developed using 3-amino-9-ethylcarbazole (BioGenex) as substrate. The sections were counterstained and mounted, as indicated above.

Lung cell digestion

Single-cell suspensions were prepared, as described before (30). Briefly, the lungs were perfused with a solution containing PBS and heparin (50 U/ml; Sigma-Aldrich) through the pulmonary artery and 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 through a cell strainer (BD Biosciences). RBC were lysed with Gey’s solution, washed, and resuspended in complete DMEM. Total cell numbers per lung were determined using a hemocytometer.

Flow cytometric analysis of cell surface markers

Cell suspension from each lung was incubated with mAbs labeled with FITC, PE, PerCP, or allophycocyanin at 4°C for 30 min in the dark. After washing the cells with deficient RPMI 1640 (Invitrogen) containing 0.1% sodium azide (Sigma-Aldrich) the cells were incubated with mAbs. mAbs against CD4⁺ (clone RM4-5, rat IgG2a,k), NK-1.1 (clone PK136, ms IgG2a,k), CD8 (clone 53-6.7, rat IgG2a,k), CD3 (clone 145-2C11, Ar Ham IgG1,k), CD11c (clone HL3, hamster IgG1), CD11c (clone HL3, hamster IgG1), CD11b (Mac-1, clone M170, rat IgG2a), CD40 (clone 3-23, rat IgG2a), I-A/I-E MHC class II (clone 2C9, rat IgG2a), markers, and rat IgG2a, rat IgG2b, rat IgG1, mouse IgG2a, and hamster IgG were used in this study. These mAbs were purchased from BD Pharmingen, Serotec, or eBioscience as direct conjugates to FITC, PE, PerCP, PerCP-Cy5.5, or allophycocyanin. Data acquisition and analysis were done using a FACS Calibur BD (BD Biosciences) and CellQuest software (BD Biosciences), respectively. Compensation of the spectral overlap for each fluorochrome was done using CD4 or CD11b Ags from cells gated in the forward light scatter (FSC) vs. side light scatter (SSC) region, respectively. Analyses were performed with an acquisition of at least 100,000 or 500,000 T cell total events and a minimum of 10,000 CD11c-positive events.

Intracytoplasmic cytokine staining

Cells were first stained for cell surface markers, as indicated above, and thereafter, the same cell suspensions were prepared for intracellular staining. Staining for markers of the DEC-205 or the TNFR-associated factor (TRAF) family was done using intracellular staining of cells. Cell membranes were permeabilized according to the kit instructions (Fix/Perm kit; BD Pharmingen). Abs against DEC-205 or TRAF-1 and 2 (Santa Cruz Biotechnology) were incubated with the appropriate surface-stained cells for 30 min, and the cells were washed twice and resuspended in deficient RPMI 1640 before analysis.

Quantitative real-time PCR analysis of cytokines in lungs of control or immune mice

Lung tissue from control and immune mice was homogenized and frozen immediately in 1 ml of Ultraspec RNA reagent (Biotex Laboratories). Total RNA was extracted according to the manufacturer’s protocol. RNA samples from each group and each time point were treated with DNase (Ambion, Austin, TX). Reverse transcription was performed using the Moloney murine leukemia virus reverse transcriptase (In Vitrogen Life Technologies). cDNA was then amplified using the TaqMan Universal PCR master mix on the ABI PRISM 7700 sequence detection system (Applied Biotechnology). Samples were run in the absence of the reverse-transcriptase enzyme to confirm that signal was derived from RNA. The fold increase in signal over the 18S housekeeping gene was determined by the ABI PRISM 7700 manufacturer. The primer and probe sequences for murine IFN-γ, IL-12, 18S, GM-CSF, IL-10, and TNF-α were previously published (26, 31, 32).
Statistical analysis

Data are presented using the mean values (n = 5) utilizing replicated samples and duplicate or triplicate assays. A parametric method, the Student’s t test, was used to assess statistical significance between groups of data.

Results

Bacterial loads in the lungs and spleens of vaccinated immune mice are lower than in control mice

The bacterial loads in lungs and spleens in immune vaccinated mice and controls were compared. As previously reported 30 and 90 days after challenge with virulent M. tuberculosis, the lungs (Fig. 1A) and spleens (Fig. 1B) of immune mice had significantly reduced bacterial loads compared with control mice (13).

Differences in the granulomatous response in immune mice

Immune mice were more efficient in controlling bacteria in the lungs and spleen compared with control mice. When sections from both groups of mice were compared 30 and 90 days post-M. tuberculosis challenge, the number and size of granulomas in the immune mice were smaller (Fig. 2A), as previously shown (13). These smaller granulomas consisted primarily of tight aggregates of lymphocytes (Fig. 2B).

Foamy macrophages are a prominent characteristic of the lung granuloma, but these were far less evident in the lesions forming in the immune mice 30 days (data not shown) or 90 days after challenge, and those that did arrive appeared different from those in control mice in that they had smaller cytoplasms and more condensed chromatin in the nuclei (Fig. 2B). In addition, these mice had less cell infiltration and thickening of the parenchymal walls than control mice, and the presence of bronchioalveolar cells in the bronchiolae was also less pronounced.

At the peak of acquired immunity, the presence of cytokines in the granulomatous lesion of control and immune mice was also verified using immunohistochemistry (Fig. 2C). Sections of formalin-fixed and paraffin-embedded lung tissue were used to visualize expression of cytokines and acid fast-positive bacteria. Each section was analyzed according to the expression of protective (IL-12, IFN-γ), immunosuppressive (IL-10), and immunoregulatory (GM-CSF) cytokines according to their coexpression with mycobacteria-infected cells visualized by the acid fast-positive staining.

As shown in Fig. 2C, the expression of all cytokines in the granulomatous lesions was less intense in the immune mice. Staining for IFN-γ showed mild positive staining within the granuloma, often in association with clusters of cells staining acid fast positive for mycobacteria. IL-12 showed positive staining in macrophages and foamy cells within lesions, whereas IL-10 was strongly expressed by macrophages and foamy cells in the core of the granulomas and by fewer cells at the periphery of this structure. Many cells in the granuloma coexpressed IL-10-positive staining and acid fast positive for mycobacteria in control mice, but not in immune mice. Finally, many macrophages both inside and outside the granuloma showed positive staining for GM-CSF, often with acid fast bacteria present.

Differential expression of protective cytokines (IL-12, IFN-γ), inflammatory cytokines (TNF-α), and immunoregulatory (GM-CSF) and immunosuppressive cytokines (IL-10) in immune mice

At the peak of acquired immunity, the expression of protective cytokines (IL-12, IFN-γ) (Fig. 3A), inflammatory cytokine (TNF-α) (Fig. 3B), immunosuppressive cytokine (IL-10) (Fig. 3C), and immunoregulatory (GM-CSF) (Fig. 3D) cytokine genes in the lungs of the uninfected naive, control, and immune mice was compared (Fig. 3). In all cases, the expression of cytokines in the infected tissues was higher in the control mice compared with the immune mice after challenge infection.

Decreased T cell and DC numbers in immune mice

Given the obvious differences seen by histological analysis, we conducted a comparative flow cytometric analysis of alveolar macrophages, DCs, and T cell populations from the two groups of animals following a strategy we have previously reported (33, 34). T cells from lung cell suspensions were characterized by primary gating on the viable lymphocyte population and then by a secondary gate on CD3⁺ T cells (Fig. 4A). The total number of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells was reduced in immune mice compared with controls, whereas no changes were seen in the numbers of CD3⁺ NK1.1 cells (Fig. 4B).

Macrophage and DCs were also examined, as previously described (33). The numbers of alveolar macrophages and DCs were determined by gating in the R5 region containing cells staining CD11b⁺CD11c⁺ and the R6 region containing CD11b⁺CD11c⁺/DEC-205⁺ cells (Fig. 4C). As shown in Fig. 4D, the total numbers of alveolar macrophages in lungs obtained from infected mice after 30 and 90 days of infection remained the same. Although the total numbers of alveolar macrophages in the lungs of immune mice at day 30 of the infection were reduced compared with controls, this number significantly increased at day 90, exceeding that present in control mice at 90 days. In contrast, the total number of DCs (Fig. 4E) in the lungs of immune mice at 30 and 90 days postchallenge was always lower than in the lungs obtained from controls. This had decreased even further by day 90 of the infection.
FIGURE 2. Differences in the granulomatous response in immune mice. Sections of formalin-fixed and paraffin-embedded lung tissue of control (Mtb, \textit{M. tuberculosis}) and immune (BCG-Mtb) mice on day 30 and 90 post-\textit{M. tuberculosis} challenge were used to visualize H&E staining (A and B) or expression of cytokines (IFN-\gamma, IL-12, IL-10, GM-CSF) and acid fast-positive bacteria (C). A. Shows the number and size of granulomas in immune mice were smaller 30 and 90 days post-\textit{M. tuberculosis} challenge. B. Shows immune mice have smaller granulomas, which consisted of tight aggregates of lymphocytes 90 days post-\textit{M. tuberculosis} challenge. C. Shows immunohistochemical staining of cytokine expression by immune mice and control 90 days post-\textit{M. tuberculosis} challenge analyzed according to the presence of each specific cytokine (brown color and black arrows in pictures) and its spatial relationship with mycobacteria-infected cells visualized by the acid fast-positive staining (pink color and red arrows in pictures). Pictures were taken with a IX70 Olympus microscope with an attached ZP70 digital camera. Total magnification: A = ×10; B, upper panels = ×20, lower panels = ×40; C = ×100, insets = ×10.

FIGURE 3. Immune mice express less TNF-\alpha, GM-CSF, IFN-\gamma, and IL-12 genes in the lungs during \textit{M. tuberculosis} infection. Lung tissue from naive (■), control (□), or immune mice (◆) 30 days postchallenge with \textit{M. tuberculosis} was homogenized and frozen immediately in 1 ml of Ultraspec RNA reagent (Biotex Laboratories). Total RNA was extracted according to the manufacturer’s protocol. The cytokine expression is determined using real-time RT-PCR. The expression of IFN-\gamma and IL-12 (A), TNF-2 (B), IC-10 (C), and GM-CSF (D) genes in the lungs of control mice was higher than in immune mice.
FIGURE 4. Early decrease in T cells and DEC-205⁺ cells in the lungs of immune mice. Lung cells obtained from control and immune mice were assayed by flow cytometry at days 30 and 90 postchallenge. 

A, Shows a representative dot plot of lung cells from a representative mouse primarily gated on FSClow vs SSClow and secondarily gated on CD3⁺ cells. 

B, Shows the total number of cells expressing CD4⁺, CD8⁺, or NK1.1⁺ surface markers in the lungs of control (hatched bars) and immune mice (□) (n = 5). 

C, Shows a representative dot plot of lung cells obtained from a representative mouse primarily gated on FSCmid/high and SSCmid/high and secondarily gated according to the expression of CD11c vs CD11b markers. Two representative CD11b/CD11c dot plots of lung cells obtained from representative mice in the control (Mtb, M. tuberculosis) (left) or immune (BCG-Mtb) (right) groups are shown as well. 

D, Shows the total number of alveolar macrophages in R5 from control mice (hatched bars) and immune mice (□) (n = 5). 

E, The total number of DEC-205-positive cells (DEC 205⁺) in R6 from control mice (hatched bars) and immune mice (□) (n = 5).
Increased expression of MHC II and CD40 molecules in immune mice during the chronic stage of the infection

We have demonstrated previously that during the chronic stage of pulmonary infection with *M. tuberculosis*, lung DCs do not up-regulate MHC class II expression and only have significant up-regulation of CD40 molecules during the early stages of this process (33, 34). Importantly, we found in this study, however, that this was not the case in immune animals. Fig. 5 shows changes in MHC class II and CD40 molecule expression on alveolar macrophages (R = 5; CD11b⁺/CD11c⁺) and DCs (R = 6; CD11b⁺/CD11c⁺/DEC-205) during the early (day 30) and later stage (day 90) of chronic infection with *M. tuberculosis*. The expression of MHC class II molecules (Fig. 5A) on alveolar macrophages gated within region R5 and DEC-205-positive cells gated in R6 obtained from lungs of immune mice was significantly higher than that seen on similar cells obtained from the lungs of control mice. These levels were very high on day 30 and had reduced somewhat at day 90, but still remained higher than levels seen in control mice.

During the peak of acquired immunity, the expression of CD40 molecules (Fig. 5B) on alveolar macrophages (R5) and DEC-205-positive cells (R6) obtained from lungs of immune mice was also significantly higher than on similar cells obtained from control mice. However, 90 days post-*M. tuberculosis* challenge, the expression of CD40 molecules on alveolar macrophages (R5) remained increased in the immune mice compared with control mice. The level of CD40 molecule expression by DEC-205-positive cells (R6) in control and immune mice did not differ.

Reduced expression of TRAF-1 in immune mice

TRAF are known to block apoptotic signals by activating signaling pathways that promote cellular survival and longevity (35–37), and our previous studies showed colocalization of DEC-205 and TRAF-1 by cells in the foamy cell layers in the granulomas of chronically infected mice (34). To observe whether this changed in any way in immune animals, we looked at the ability of control and immune mice to express TRAF-1 on DEC-205 cells during the early and late stages of the ensuing chronic infection. We found that DEC-205-positive cells in the R5 region (alveolar macrophages) and in the R6 region (DCs) expressed less TRAF-1 markers in the immune mice (Fig. 6, A and B). As before, TRAF-1 was expressed in foamy cells in the granuloma that stained positive for DEC-205, but there was very low expression of TRAF-1 in the immune mice (Fig. 6C). Because TRAF-1 protects cells from apoptosis, we looked for apoptotic cells by TUNEL analysis and found these to be few in number and located outside the granuloma in control mice (Fig. 6D). Immune cells showed an increased frequency of such cells, again in tissues exterior to the granuloma itself.

Discussion

The results of this study show that there are several quantitative and qualitative differences between the granulomatous response of control and immune mice to challenge in the lungs with *M. tuberculosis*. In addition to the classically demonstrated (38–40) drop in the bacterial load, we show in this study that memory immune mice show obvious differences in lung histopathology, in the nature of the cellular composition of the ensuing granuloma, in the expression in granulomatous tissues of key cytokines, and in the influx of macrophages. Of these, the most prominent are the macrophages, which we show in this figure differ considerably in immune mice, both in terms of their expression of molecules involved in Ag presentation as well as in their expression of antiapoptotic markers.

BCG-vaccinated immune mice differ from control animals by the possession of an activated population of cells that can be found in lung lymphatic organs and that are capable of rapidly secreting IFN-γ (13, 41). This results in the capacity to mediate an accelerated expression of protective immunity, resulting in control and containment of the infection at a lower level (usually a 10-fold reduction) than in control mice, as demonstrated several times (38–40) and confirmed above. Despite this event, however, the vaccinated mouse fails to eliminate the infection, and so this lower bacterial load still remains and enters a chronic form of disease, as noted by several laboratories (1, 40, 42). It has been suggested that as a result of this event, there is no major quantitative and qualitative difference between control and memory animals other than the reduction in the bacterial load (20).

We examined this issue further in the study reported in this work. Gross lesions in immune mice were smaller as anticipated (13), and were dominated by lymphocytes. Foamy cells were present in these animals, but they had obvious morphological differences compared with cells seen previously in control mice (34), an observation that we cannot explain at this point. We showed previously (34) that these cells curiously express the DC marker DEC-205, which may indicate that they are of DC origin rather than alveolar macrophages as generally thought.

Because the infection was contained more efficiently and effectively in the immune mice, there was a reduced need to produce key cytokines in the granulomatous lesions, as demonstrated above. As a result, the expression of the protective Th1 cytokines IL-12 and IFN-γ was reduced compared with control mice. A similar reduction was seen for TNF-α, although this cytokine and to some extent IFN-γ were strongly coexpressed in tissues, often adjacent to bacilli detected by acid fast staining.

**FIGURE 5.** Immune mice had early up-regulation of expression of both MHC class II and CD40 molecules on alveolar macrophages and DC. The mean fluorescence channel (MFC) of MHC class II (A) and CD40 (B) molecule expression on cells in the R5 region and DEC-205-positive cells in R6 from samples obtained from control (□) and memory immune mice (□) at 30 and 90 days postchallenge is shown. The results correspond to the average ± SE (n = 5) of the MFC from each group of mice at 30 and 90 days postchallenge.
As expected, flow cytometric analysis showed fewer CD4\(^+\) and CD8\(^+\) T cells within the lungs of immune mice. As the infection progressed through the chronic stage, there was a progressive increase in the number of alveolar macrophages (CD11b\(^+/\)CD11c\(^{high}/\)DEC-205\(^{low}\)) and lower numbers of DCs expressing CD11b\(^+/\)CD11c\(^{high}/\)DEC-205\(^{high}\) compared with the control mice. A further important difference in the DC subset was the relative expression of the class II MHC and the CD40 molecules during the chronic stage of the infection. In complete contrast to our earlier demonstration (34) that DC function in terms of the capacity for Ag presentation is clearly shut down during the chronic disease stage, in the immune mice this activity was obviously highly enhanced. Even though this had dropped considerably by day 90 of the infection, this still remained higher than in controls. DCs also harbor intact live bacteria, but the degree to which these bacilli can be killed by the cell is very low compared with alveolar macrophages (28, 43). Thus, an increased number of alveolar macrophages that have a higher capacity to present Ag results in an increased ability of immune mice to eliminate the bacteria more efficiently.

The second major difference was that these DCs in the immune mice had a much lower expression of the antiapoptotic TRAF-1 marker compared with the high levels seen in control mice. These class of molecules is antiapoptotic (37), but this did not seem to dramatically affect the integrity of the lung tissues in that we could not detect many apoptotic cells, and those we did find were not in the granuloma. Although apoptosis has been implicated both as a protective and cross-priming mechanism, in our hands it is a minor event within the granuloma (34, 44).

It is clear from flow cytometric analysis (33, 34) that cells with the characteristics of DCs and macrophages are an integral component of the lung granulomatous process. In addition, foamy cells, which figure prominently in the more advanced stage of the granuloma during the chronic disease stage, stain brightly for DEC-205, indicating that they are either of DC origin themselves or are CD11c-positive alveolar macrophages that begin to express

**FIGURE 6.** The lung granulomas from immune mice had decreased expression of the antiapoptotic TRAF-1 marker. By flow cytometric and immunohistochemistry analysis, we compared the antiapoptotic marker, TRAF-1, in CD11b\(^+/\)CD11c\(^{high}/\)DEC-205\(^{low}\) cells obtained from the lungs of control or immune mice. A, Shows representative dot plots of lung cells from control (Mtb, M. tuberculosis) and immune (BCG-Mtb) mice primarily gated on TRAF-1 marker expression on cells in the DEC-205\(^{+}\) cells gated as R5 and R6 regions. B, Represents the percentage of TRAF-1-expressing cells in the R5 region and R6 region cells in R6 from samples collected at 30- and 90-day control (○) and immune (□) mice. Results are represented from the average (± SE) (n = 5) percentage of TRAF-1-positive cells. C, Immunohistochemical staining for TRAF-1 in lung sections from control (Mtb) and immune (BCG-Mtb) mice. Control mice showed increased expression of TRAF-1 on foamy cells in the lung lesions. Immune mice showed few cells stained for TRAF-1. D, Demonstrates TUNEL analysis showing cells staining positive for apoptosis were few in number and were located outside the granuloma in control mice (G) (left panel). Immune mice showed increased frequency of cells encountered staining positive for apoptosis (right panel) in the area exterior to the granuloma. Total magnification: C and D, ×20; D, insets, ×100.
this molecule for some unknown reason. In the immune mice, however, foamy cells could still be detected in lesions, but appeared to have a different morphology.

The biggest difference, however, was in the degree of expression of MHC class II and CD40 molecules by DCs in the R6 gate, which was far higher than in lesions in control mice. Thus, although the lesions in the former group were smaller, they still contained DCs that were capable of high levels of Ag presentation. One possible explanation is that these lesions are highly lymphocytic and presumably primarily consist of effector memory T cells secreting IL-2 and IFN-γ. This latter cytotoxic is a potent inducer of DC activation (45, 46). The control mice lesions during the chronic stage are much larger, but analysis of the numbers of IFN-γ-secreting CD4+ and CD8+ cells clearly shows a considerable drop during the chronic phase (30), which would be consistent with the observed low levels of MHC class II and CD40 molecules. The cytokine IL-10 may also contribute to this process (27, 28, 47, 48); as shown above, this cytokine was strongly expressed in the control mice, but was minimally present in the immune animals.

Activated DCs in lung lesions would be expected to accelerate the emergence of the memory immune response, and continued expression of class II and CD40 molecules would presumably expand reactive T cell clones even further. Indeed, directed vaccination targeting DCs via DEC-205 has been shown recently to be an effective immunization strategy (26, 49, 50). Previous studies demonstrating infection of DC with BCG induce up-regulation of these Ags (46). In addition, BCG-infected DCs used as a vaccine strategy provided higher protection against virulent challenge with Mycobacterium tuberculosis (51, 52). Finally, our data indicate that vaccination strategies should consider the ratios of T cells to alveolar macrophage and DCs for optimal induction of protective immunity.

Prime-boosting strategies, currently popular in the TB vaccine field, should also benefit from these processes.

Disclosures

The authors have no financial conflict of interest.

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