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Diabetic Mice Display a Delayed Adaptive Immune Response to *Mycobacterium tuberculosis*

Therese Vallerskog, Gregory W. Martens, and Hardy Kornfeld

Diabetes mellitus (DM) is a major risk factor for tuberculosis (TB) but the defect in protective immunity responsible for this has not been defined. We previously reported that streptozotocin-induced DM impaired TB defense in mice, resulting in higher pulmonary bacterial burden, more extensive inflammation, and higher expression of several proinflammatory cytokines known to play a protective role in TB. In the current study, we tested the hypothesis that DM leads to delayed priming of adaptive immunity in the lung-draining lymph nodes (LNs) following low dose aerosol challenge with virulent *Mycobacterium tuberculosis*. We show that *M. tuberculosis*-specific IFN-γ–producing T cells arise later in the LNs of diabetic mice than controls, with a proportionate delay in recruitment of these cells to the lung and stimulation of IFN-γ–dependent responses. Dissemination of *M. tuberculosis* from lung to LNs was also delayed in diabetic mice, although they showed no defect in dendritic cell trafficking from lung to LNs after LPS stimulation. Lung leukocyte aggregates at the initial sites of *M. tuberculosis* infection developed later in diabetic than in nondiabetic mice, possibly related to reduced levels of leukocyte chemoattractant factors including CCL2 and CCL5 at early time points postinfection. We conclude that TB increased susceptibility in DM results from a delayed innate immune response to *M. tuberculosis*-infected alveolar macrophages. This in turn causes late delivery of Ag-bearing APC to the lung draining LNs and delayed priming of the adaptive immune response that is necessary to restrict *M. tuberculosis* replication. *The Journal of Immunology*, 2010, 184: 6275–6282.

Approximately one third of the world’s population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), but this infection remains latent throughout life in ~90% of infected individuals. For those ~10% of *M. tuberculosis*-infected persons who develop active disease, the inability to mount or to maintain an immune response that enforces latent TB infection results from diverse genetic and acquired risk factors. Among the latter, HIV/AIDS has received much attention due to the dramatic increase in relative risk associated with CD4+ T cell deficiency and the observation that TB is the leading cause of death in that population (1). It has been recognized for some time that diabetes mellitus (DM) increases the risk for TB, but the magnitude of this effect has only recently been appreciated. In a review of 13 observational studies addressing the association of DM and TB disease, Jeon and Murray (2) determined that the global population-attributable risk is comparable to that of HIV/AIDS. Although the immune dysfunction associated with DM is not as severe as that caused by HIV infection, DM is a more common disorder than HIV/AIDS. The worldwide prevalence of DM in 2010 is estimated to be 285 million and is predicted to reach 439 million by 2030 (3). Of particular concern is the rapid increase in DM in developing countries with a high burden of TB (4).

Despite the public health threat posed by these convergent epidemics, the immunological basis for TB susceptibility in DM is not well understood. We previously published the first report of low-dose aerosol *M. tuberculosis* infection in mice with streptozotocin (STZ)-induced DM (5). Mice with chronic (~12 wk) but not acute (<4 wk) DM had a higher plateau bacterial burden and more lung inflammation by 8 wk postinfection (p.i.) compared with euglycemic controls. The finding that acute STZ-induced hyperglycemia and hypoinsulinemia have no major impact on TB defense suggested that impaired host defense in DM could, like many other diabetic complications, result from biochemical effects of prolonged hyperglycemia. Despite evidence of increased susceptibility, mice with chronic DM and infected with *M. tuberculosis* for >4 wk exhibited no evident deficiency in leukocyte recruitment to the lungs or in the expression of key cytokines relevant to TB defense. On the contrary, diabetic mice had significantly more pulmonary T cells, macrophages, and neutrophils than controls, whereas the relative proportions of these different populations were comparable to the controls. Similarly, the diabetic mice had more IFN-γ, IL-1β, and TNF-α protein in their lungs than control mice at 8 wk or longer after *M. tuberculosis* infection. Mice with chronic DM were capable of arresting *M. tuberculosis* replication in the lung, but at >1 log higher plateau bacterial burden than controls. Although diabetic mice had expressed more IFN-γ than controls at later time points, they had significantly lower IFN-γ expression 2 wk p.i., suggesting that TB susceptibility might result at least in part from a delay in initiating *M. tuberculosis*-specific adaptive immunity.

In the current study, we sought to gain further insight to the basis of TB susceptibility in mice with chronic STZ-induced DM (STZc). We used STZ for these experiments to produce uniform hyperglycemia without the potentially confounding effects of autoimmunity in NOD mice that might interfere with the interpretation of any observed TB susceptibility and in view of the fact that hyperglycemia drives most diabetic complications. We report in this study...

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Abbreviations used in this paper: Ctrl, control; DC, dendritic cell; DM, diabetes mellitus; HbA1c, hemoglobin A1c; i.t., intratracheal; LN, lymph node; na, not available; p.i., postinfection; PPD, purified protein derivative; STZ, streptozotocin; STZc, mice with chronic STZ-induced DM; TB, tuberculosis.

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that the earliest appearance of *M. tuberculosis*-specific IFN-γ-producing T cells in the thoracic lymph nodes (LNs) and subsequently in the lung occurred later in STZc mice than control mice. Delivery of *M. tuberculosis* bacilli to the pulmonary LNs was also delayed in STZc mice but we found no evidence for impaired dendritic cell (DC) trafficking from the lung to the LNs in STZc mice challenged with intratracheal (i.t.) LPS, nor any difference in the expression of MHC class II or costimulatory molecules on migrating DCs. Examination of lung histopathology from early time points after *M. tuberculosis* infection demonstrated a delay in recruitment of myeloid cells to sites of initial macrophage infection in the lungs of STZc mice. In a similar time frame, these mice also had reduced expression of CCL2 and CCL5, chemokines that stimulate migration of macrophages and DCs. These findings pinpoint a critical lesion in protective immunity resulting from chronic hyperglycemia, wherein resident alveolar macrophages initially infected with *M. tuberculosis* after aerosol challenge fail to recruit naive myeloid cells necessary to acquire Ag and convey it to the pulmonary LN. We conclude that events preceding the priming of Ag-specific T cells are responsible for the delay in mounting what is otherwise a robust adaptive immune response against *M. tuberculosis* in diabetic mice.

Materials and Methods

**Mice**

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the Animal Medicine facility at University of Massachusetts Medical School where experiments were performed under protocols approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

**Induction of DM**

DM was established by i.p. injection of STZ (Sigma-Aldrich, St. Louis, MO) dissolved in phosphate-citrate buffer (pH 4.5). Mice were at least 8 wk old with minimum weight of 25 g when treated with STZ. Blood glucose measurements were performed with a BD Logit glucometer (Becton Dickinson, Franklin Lakes, NJ) 9 d after STZ injection and immediately prior to *M. tuberculosis* infection. Mice were considered diabetic if their blood glucose was >200 mg/dl. Mice with ketoadiposis, tested by dip stick (LW Scientific, Lawrenceville, GA), were excluded from the study. Mice were maintained with DM for 14 d before other experimental manipulations were started; a time when hemoglobin A1c (HbA1c) was elevated (Supplemental Fig. 1). HbA1c was measured in whole blood by affinity chromatography (Helena GLYCO-Tek; Helena Laboratories, Beaumont, TX).

**M. tuberculosis infection**

Aliquots of frozen *M. tuberculosis* Erdman stock in PBS plus 0.05% Tween-80 (PBS-T80) were thawed and sonicated for 5 min in a cup-horn sonifier (Branson Ultrasonics, Danbury, CT). Mice were infected with 0.5 × 10^6 colony forming units (CFU) per mouse using a Glass-Col Inhalation Exposure System (Terre Haute, IN). Two mice were sac-in collagenase IV (0.02 mg/ml) and DNase (0.5 mg/ml; both from Sigma-Aldrich). Thoracic LNs were minced and digested at 37˚C for 40 min and the digested lungs were passed through a 40-μm cell strainer. Viable cells were counted using a hemocytometer with dead cells exclusion by trypan blue (Sigma-Aldrich) staining. Lung leukocyte populations were evaluated by flow cytometry for the expression of CD3, CD4, CD8, F4/80, Gr-1, and CD19. No differences were observed between control and STZc mice in the total number of these different leukocyte types prior to *M. tuberculosis* challenge or at days 7, 14, 21, and 28 p.i. (Supplemental Fig. 2A). This is consistent with our prior observations (5). Leukocytes from lung-draining LNs were evaluated for the expression of CD11c, CD19, CD4, and CD8 at days 12, 15, 18, and 21 p.i., with no proportion differences observed between control and STZc mice (Supplemental Fig. 2B).

**Ex vivo T cell restimulation**

IFN-γ secreted from lung leukocytes and thoracic LN lymphocytes was detected using an IFN-γ Ab ELISPOT pair (BD Biosciences, San Jose, CA), MultiScreen-IP ELISPOT plates (Millipore, Billerica, MA), streptavidin-alkaline phosphatase (Mabtech, Nacka Strand, Sweden), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, BCIP/NBT (Sigma-Aldrich), and *M. tuberculosis* Ag85 peptide (Ag85A243–260 sequence Ac-QDAYNGGGGNHGFDPSDG-amide; Twentyfirst Century Biochemicals, Marlboro, MA) for stimulation of T cells. The membranes of ELISPOT plates were treated with 15 μl 70% ethanol, followed by eight washes with water and then coated with detection Ab diluted 1:250 in PBS overnight at 4˚C. The plates were washed with PBS and the membrane blocked with complete cell culture medium (RPMI 1640 with 10% heat inactivated FCS, 2 mM l-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin sulfates, and 1% nonessential amino acids) for minimum 2 h at room temperature before the wells were prepared with complete medium ≥ 10 μM Ag85 peptide, followed by addition of cells in triplicate for each cell concentration and stimulation. Uncoated wells and wells without cells were used as negative controls and cells stimulated with 2 μg/ml Con A (Sigma-Aldrich), as positive controls. After 44 h incubation (37˚C, 5% CO₂), wells were washed off the membrane with PBS-T80 and biotinylated detection Ab (1:2000 in PBS-T80) was added and incubated for 2 h at room temperature. The wells were washed with PBS-T80 and PBS before incubation with streptavidin-alkaline phosphatase for 1 h at room temperature, followed by PBS wash and addition of BCIP/NBT substrate. IFN-γ spots were developed for 7–12 min before a final wash with water. The membrane was dried overnight at room temperature and the ELISPOT plates were decontaminated at 65˚C for 2 h. Analysis was performed with an automated ELISPOT reader (CTL ELISPOT reader, CTL, TECAN, OH), using CTL ImmunoSpot Academic Software version 4.0. Results were calculated as spots per 10^5 CD4+ T cells, based on the frequency of CD4+ T cells in LNs (analyzed by flow cytometry) and mean number of spots from each triplicate.

**Cytokine and NO analysis**

Lungs were homogenized in PBS-T80 and an equal volume of tissue protein extraction reagent (T-PER, Pierce, Rocky Ford, IL) was added for 30 min on ice, and the supernatant was vortexed, incubated (20 min, 4˚C), vortexed again, centrifuged (10 min, 14,000 × g), and the supernatant was filter sterilized. Lung lysates were assayed for IFN-γ by ELISA according to manufacturer’s protocol (R&D Systems, Minneapolis, MN). NO (quantified as the sum of nitrate and nitrite) was detected in lung lysates by NO Quantitation Kit (Active Motif, Carlsbad, CA) by manufacturer’s protocol. Briefly, lung lysates were added to a 96-well plate and reduced to a 96-well plate and treated with 15 μl of nitrate reductase. After 30 min incubation, Griess Reagents were added to each sample and absorbance at 540 nm was read 20 min later. Chemokines in lung lysates were screened with a Multi-Analyte ELISAArray Kit (SABiosciences, Frederick, MD) according to manufacturer’s protocol. Pooled lung lysate from each experimental group was added to the ELISAArray plate and incubated for 2 h. The plate was washed and detection Ab was added and incubated for 1 h and washed again. Bound Ab was detected by streptavidin-HRP and, after addition of the HRP substrate, absorbance was read at 570 nm using a Thermo-Multiskan Ascent ELISA reader with Ascent Software v.2.6 (Thermo Labsystems OY, Vantaa, Finland). Individual lung homogenate samples were analyzed for CXCL9 (MIG) by ELISA (R&D Systems), whereas CCL5 (RANTES), CCL2 (MCP-1), CXCL12 (SDF-1), and CCL17 (TARC) in individual samples were analyzed by a commercial multiplex sandwich-ELISA system (SearchLight Protein Array Technology, Aushon Biosystems, Billerica, MA).

**DC migration from lungs to LNs**

To induce activation and cell migration from lungs to draining LNs, 0.5 μg LPS (Sigma-Aldrich) and 1 mM CellTrace FarRed DDAO-SE (Invitrogen, Carlsbad, CA) was given in PBS in a total volume of 50 μl by i.t. instillation. Thoracic LNs were harvested after 20 h and leukocytes extracted from the tissue by enzymatic digestion as described previously. Migrating FarRed + CD11c+ cells were detected by flow cytometry using the following mAbs: CD11b-Pacific Blue (clone M1/70), CD11c-PE (clone N418), MHC class II (I-A/I-E)-Alexa 700 (clone M5/114.15.2), CD40-PE Cy5 (clone 1C10),
Histopathology

Lungs were inflated and fixed with 10% buffered formalin for 24 h and then processed for staining. Paraffin embedded tissue sections were stained with H&E for histopathology and with a 1:100 dilution of anti-purified protein derivative (PPD) polyclonal rabbit IgG (Abcam, Cambridge, MA) for detection of M. tuberculosis. Enzymatic Ag retrieval was performed prior to immunostaining and Ab was detected with polyclonal goat HRP-conjugated anti-rabbit IgG (Dako, Carpinteria, CA). Lung sections were stained with an irrelevant rabbit IgG (Abcam) for negative controls. All staining was performed by the Diabetes and Endocrinology Research Center histopathology core facility at University of Massachusetts Medical School. Stained lung sections were analyzed with a Nikon Eclipse E400 microscope (Nikon Instruments, Melville, NY) using Spot Advanced v.4.6 software (Diagnostic Instruments, Sterling Heights, MI). Percent total lung area involved with inflammation was calculated by dividing the cumulative area of inflammation by the total lung surface area examined in all sections for each lung studied as we previously described (5).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism v.5.02 (Graphpad Software, La Jolla, CA) software. Normally distributed data were analyzed with Student t test or with Welch’s unpaired t test if variances were different. One-tailed t tests were performed on samples early p.i. where difference was expected on the higher levels above zero. Nonparametric data were analyzed with the Mann-Whitney U test. Categorical data were tested with Fisher exact test. p values ≤ 0.05 were considered statistically significant.

Results

Kinetics of adaptive immunity to M. tuberculosis in LNs and lungs

To elucidate the impact of DM on the induction of M. tuberculosis-specific cell-mediated immunity, we challenged STZc mice and nondiabetic controls by low-dose aerosol infection with virulent M. tuberculosis Erdman. Leukocytes were extracted from the lungs and lung-draining LNs on successive days p.i. and restimulated ex vivo with an H-2b restricted M. tuberculosis Ag85 peptide for IFN-γ ELISPOT (Fig. 1). In control mice, IFN-γ spot-forming cells were first detected in pulmonary LNs on day 12 p.i. and in the lung by day 15 p.i. By comparison, IFN-γ spot-forming cells were not detected in LNs of STZc mice until day 15 p.i. and in the lung on day 21 p.i. Thus, the priming of naive T cells in thoracic LNs after aerosol M. tuberculosis challenge is delayed by ~3 d in diabetic mice, with a corresponding late appearance of these activated cells in the lung parenchyma. The late priming of IFN-γ T cells and their delayed recruitment to the lungs was functionally significant, as evidenced by reduced lung tissue levels of IFN-γ protein and of NO in STZc mice on day 15 p.i. (Fig. 2A), as well as lower levels of the IFN-γ-inducible chemokine CXCL9 (Fig. 2B). The late arrival of IFN-γ-producing T cells at the site of infection occurs during a period of logarithmic bacterial replication in the lung and accounts for the >10-fold higher plateau bacterial load that we previously reported in STZc mice (5).

Bacterial dissemination from lung to draining LNs

The induction of adaptive immunity to M. tuberculosis after aerosol challenge depends on bacterial dissemination from the initial site of infection in the lungs to the draining LNs where naive Ag-specific precursor T cells are primed (6, 7). Because we observed a delayed adaptive immune response to M. tuberculosis in diabetic mice, we reasoned that late delivery of M. tuberculosis to the lung-draining LNs might be responsible. To test this prediction, thoracic LNs were harvested from control and STZc mice every 48 h between days 5 through 11 after low-dose aerosol M. tuberculosis challenge and the entire LN homogenate from each mouse was plated for colony counting. Cultures from all mice tested at day 5 p.i. were negative for CFU. On day 7 p.i., 6 of 10 LNs in nondiabetic controls were positive for bacterial growth, whereas 3 of 9 diabetic mice had positive LN cultures at that time (Fig. 3). By day 9 p.i., the frequency of mice with detectable CFU was similar between the groups, and by day 11 p.i. 100% of the mice in both groups had positive LN cultures. In addition to having fewer positive LN cultures on day 7 p.i., the diabetic mice displayed a lower bacterial load at early time points of dissemination (Table I). On day 9 p.i., the range of CFU among positive samples was 6–196 (median 43) in the nondiabetic control mice and 1–85 (median 17) in STZc mice. These results suggested that late delivery of M. tuberculosis Ag in the form of viable bacilli contributed to the delayed priming of adaptive immunity in diabetic mice with TB.

DC migration and expression of costimulatory molecules in response to LPS

Although resident alveolar macrophages are the initial host cell population infected after inhalation of M. tuberculosis, there is strong evidence that DCs convey bacilli to the lung-draining LNs where priming of adaptive immunity takes place (8). Various reports have associated DC dysfunction with DM (9–11) leading us to speculate that the delayed priming of adaptive immunity in diabetic mice with TB might result from impaired DC trafficking to the LNs, possibly coupled with reduced expression of MHC class II and/or costimulatory molecules required to activate T cells. To evaluate
Delays in the development of innate TB immunity in diabetic mice were observed. After inhalation of Mycobacterium tuberculosis, lesions form in the lungs at sites where bacilli invade and replicate inside resident alveolar macrophages. We surveyed H&E stained lung sections from STZc and control mice over a time course of 8–28 d after low-dose aerosol M. tuberculosis infection (Fig. 5A). Lung lesions developed sooner in the control mice as compared with STZc mice and these early lesions on average were larger in the controls. Lesions were identified in some control mice by day 12 p.i. and by day 15 p.i. nearly all had visible lesions, whereas only a few of the diabetic mice had detectable lesions by 15 d p.i. (Fig. 5B). By day 28 p.i., pulmonary inflammation in the STZc mice was comparable to the nondiabetic group. This is consistent with our previous study where pulmonary TB immunopathology in STZc mice was equivalent to controls 4 wk p.i., but substantially greater in the diabetic group at 8 and 16 wk p.i. (5).

We next evaluated lung lesions in relation to foci of M. tuberculosis infection by immunohistochemical staining with anti-PPD Ab and H&E counterstaining. M. tuberculosis-infected macrophages were detectable in control and STZc mice at day 15 p.i. The infected macrophages were typically surrounded by many newly recruited myeloid cells in the control mice, but the lung sections from diabetic mice in many instances had heavily infected macrophages with few recruited leukocytes in their vicinity (Fig. 6). This finding reflects a diabetes-related defect in the innate response to initial M. tuberculosis infection of resident alveolar macrophages. Diabetic mice ultimately developed leukocyte aggregates at foci of positive anti-PPD staining that were similar in appearance to those of control mice (Supplemental Fig. 3).

Pulmonary chemokines

The slow development of leukocyte aggregates around M. tuberculosis-infected macrophages in diabetic mice suggested that innate signals required for the evolution of such lesions might be deficient. We performed a multiplex ELISAArray to survey levels of 12 chemokines in pooled samples of lung homogenate from STZc and control mice (4 mice per group) on days 12, 15, and 18 p.i. (Fig. 7A, Supplemental Fig. 4). By this method, control mice exhibited higher levels of CXCL9/MIG than STZc mice, consistent with previous ELISA result (Fig. 2B). The ELISAArray also indicated possible differences in the levels of several chemokines including CCL2/MCP-1, CCL5/RANTES, CXCL10/IP-10, CXCL12/SDF-1, and CCL17/TARC that were all higher in control than in STZc mice. We next analyzed the concentrations of individual chemokines (CCL2, CCL5, CCL17, and CXCL12) in unpooled lung samples from day 15 p.i. using a multiplex ELISA system. By this method, levels of CCL2 and CCL5 were higher in control mice than STZc mice (Fig. 7B). This finding may be relevant to delayed recruitment of leukocytes to the lung-draining LNs.

Table I. Dissemination of M. tuberculosis to the lung-draining LNs

<table>
<thead>
<tr>
<th>Time (d p.i.)</th>
<th>Control</th>
<th>STZc</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 d p.i.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% LNs positive for CFU</td>
<td>60 89 100</td>
<td>33 89 100</td>
</tr>
<tr>
<td>Mean CFU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 43 1180</td>
<td></td>
</tr>
<tr>
<td>STZc</td>
<td>0 0 17 1520</td>
<td></td>
</tr>
<tr>
<td>Range CFU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1–6 196* 230–384</td>
<td></td>
</tr>
<tr>
<td>STZc</td>
<td>2–4 1–85 281–2150</td>
<td></td>
</tr>
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<td>n</td>
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</tr>
<tr>
<td>Control</td>
<td>11 10 9 9</td>
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<tr>
<td>STZc</td>
<td>9 9 9 5</td>
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Nondiabetic (Ctrl) mice and mice with ≥3 mo STZc were infected with ~100 CFU M. tuberculosis Erdman by aerosol and then lung-associated LNs were harvested for plating and colony counting on the indicated days p.i.

\( p < 0.05 \) (one-tailed test).

na, not available.
myeloid cells surrounding *M. tuberculosis*-infected alveolar macrophages, were seen earlier in control mice than in STZc mice. Reduced expression of certain chemokines was also noted at time points preceding the widespread expression of adaptive immunity in the lung. Delivery of *M. tuberculosis* to the lung-draining LNs occurred later in STZc than control mice, with a corresponding delay in the detection of T cells primed to produce IFN-γ in response to *M. tuberculosis* Ag in the LNs initially and subsequently in the lung. The physiological consequence of reduced pulmonary IFN-γ was supported by the finding of reduced IFN-γ–inducible factors including NO (the product of inducible NO synthase) and CXCL9 in the diabetic mice. These changes may not be the only defense functions impaired by chronic hyperglycemia but they could explain several features of TB disease in diabetic mice, including their capacity to induce stationary persistence of *M. tuberculosis* but at a much higher bacterial load than controls, with concomitantly increased chronic inflammation at later time points.

Human TB is primarily a disease of the lung, which is the initial site of infection in nearly all cases. Evading (and/or suppressing) immunity allows the bacillus to establish a foothold in the host before the expression of IFN-γ and other defense factors forces a shift in bacterial gene expression and function resulting in stationary persistence (12). The lung appears to be uniquely vulnerable to TB. High-dose i.v. *M. tuberculosis* challenge in mice delivers logs more bacteria to the liver and spleen than the lung, but after several weeks bacterial burden in the lung exceeds that in other organs and pulmonary inflammation becomes the main feature disease (13). Low-dose aerosol infection of mice is followed by an ~3 wk period of logarithmic *M. tuberculosis* growth in the lung that is constrained only after a Th1-biased adaptive immune response is expressed (14, 15).

The slow kinetics of adaptive immunity in TB differs from the response to many other respiratory infections. Chackerian et al. (6) reported slower *M. tuberculosis* dissemination from lung to thoracic LNs in TB-susceptible C3H mice compared with the more resistant strain C57BL/6, and that dissemination preceded priming of adaptive immunity. Studies using fluorescent *M. bovis* BCG or fluorescent *M. tuberculosis* H37Rv (8, 16), or using adoptive transfer of fluorescently-labeled DCs and macrophages pulsed in vitro with irradiated *M. tuberculosis* H37Rv (17), established that DCs preferentially carry bacilli to the LNs. Work from several groups confirms that priming to *M. tuberculosis* after aerosol challenge is primed in the lung-draining LNs (7, 17–19), although priming can occur at other sites in mice lacking LNs (20) or lacking CCR7 (21). Our data presented in this paper suggest that DM exacerbates the already slow pace of generating immunity to *M. tuberculosis*. This effect of DM could shed light on key factors regulating the kinetics of TB immunity in the euglycemic host.

The sluggish development of myeloid aggregates around *M. tuberculosis* infection because these two chemokines promote migration of DCs and monocyte/macrophages.

**Discussion**

We previously reported that mice with chronic, but not acute, hyperglycemia had increased TB susceptibility evidenced by higher pulmonary bacterial burden and more widespread immunopathology (5). A robust Th1-biased cell mediated immune response developed after 4 wk p.i. with many key factors for successful TB defense expressed in abundance. Experiments in the current study tested the hypothesis that TB susceptibility in STZc mice is due to delayed initiation of adaptive immunity. The first lung lesions detectable by light microscopy after aerosol challenge, aggregates of foci of *M. tuberculosis* infection because these two chemokines promote migration of DCs and monocyte/macrophages.

**FIGURE 4.** DC migration from lung to LNs in response to LPS. Pulmonary DCs were simultaneously labeled with FarRed and activated with LPS by i.t. instillation. After 20 h, the lung-draining LNs were removed and leukocytes were analyzed by flow cytometry. A, Gating strategy to identify newly emigrated DC as FarRed+ CD11c+ cells. B, Proportion of FarRed+ CD11c+ cells in LNs of control and STZc mice 20 h after LPS stimulation. C, Expression of CD40, CD80, CD86, and MHC class II on a gated population of FarRed+ CD11c+ cells.
expression of CCL2, the lungs of STZc mice had lower levels of the macrophage and DC chemoattractant CCL5. Jang et al. (25) reported that *M. tuberculosis* induces expression of CCL5 mRNA and protein by macrophages, whereas Salam et al. (26) showed that CCL5 contributes to host protective DC activation against *M. tuberculosis* in vivo. We posit that in STZc mice, alveolar macrophages infected aerogenically with *M. tuberculosis* have a relative defect in generating signals, including CCL2 and CCL5, that recruit naive macrophages and DCs to the site of infection. DCs are thereby delayed in acquiring *M. tuberculosis* and consequently late in delivering Ag to the LNs despite intact capacity for LN homing. Although late delivery of *M. tuberculosis* from the lung to the LNs might also be explained by an effect of DM to inhibit DC trafficking to the LNs, we found no difference in the frequency of migrating DCs of STZc or control mice stimulated with LPS. The response of DC to *M. tuberculosis* and LPS surely differs in many ways but Khader et al. (18) reported that tracheal instillation of LPS or

**FIGURE 5.** Pulmonary lesions at early time points after *M. tuberculosis* infection. Ctrl and STZc mice were challenged with ~100 CFU *M. tuberculosis* Erdman by aerosol and the lungs were subsequently harvested, inflated, and fixed with formalin and then processed for H&E staining. **A**, Leukocyte aggregates (arrows) were easily identified in nondiabetic Ctrl mice but not in mice with chronic DM (STZc) by microscopy at ×20 magnification. **B**, Total cross-sectional lung area from all tissues sections examined (3–6 mice per group at different time points) and the combined areas of inflammation within these sections were measured by video microscopy. Percent total area involved with inflammation (top panel) was calculated as (total area of inflammation/total lung area surveyed) × 100. The mean values for nondiabetic Ctrl mice (filled circles) and for STZc mice (open circles) diabetes are indicated by horizontal lines. The number lesions of any size visible at ×20 magnification on H&E stained lung sections was counted (bottom panel). Horizontal lines indicate mean values for control and STZc groups. *p < 0.05.

**FIGURE 6.** Anti-PPD immunohistochemistry of lung sections from diabetic (STZc) and Ctrl mice with TB. Mice were infected with ~100 CFU *M. tuberculosis* Erdman by aerosol and then lungs were isolated 15 d later for immunohistochemistry with H&E counterstain. *M. tuberculosis*-infected macrophages are distinguished by brown staining (original magnification ×400).

**FIGURE 7.** Pulmonary chemokines in diabetic and Ctrl mice with TB. Mice were infected with *M. tuberculosis* Erdman and lung lysates were prepared 15 d p.i. for analysis by ELISArray of samples pooled from 4 mice per group (A), or by ELISA of samples from individual mice (B). *p < 0.05.
irradiated *M. tuberculosis* increased DC trafficking to the draining LNs at a comparable level. These results, and our finding that expression of class II MHC, CD40, CD80, and CD86 on migrating DCs are not influenced by DM, points to a delay in DCs acquiring *M. tuberculosis* from infected macrophages as a key factor regulating the kinetics of adaptive immunity in DM.

Adverse effects of DM on TB defense in rodent models have been confirmed by other laboratories. Saiki et al. (27) and Yamashiro et al. (28) used high-dose i.v. infection of STZ-treated mice and found evidence of increased TB susceptibility (increased mortality or increased bacterial burden, respectively). The latter study reported trends for lower IFN-γ levels in lung, liver, and spleen on day 14 p.i. Sugawara and colleagues published two studies on diabetic rats aerogenically infected with *M. tuberculosis* Kurono. One study compared GK/Jcl rats that spontaneously develop type 2 DM to Wistar rats (29), whereas the second compared the type 1 DM model KDP rats to nondiabetic LETL controls (30). Hyperglycemia in the type 2 DM study was tracked only by glycosuria and DM was present for a short duration prior to infection. GK/Jcl rats had a higher plateau lung burden of *M. tuberculosis* by 5 wk p.i. compared with nondiabetic controls. IFN-γ mRNA in lung was lower in GK/jcl diabetic than Wistar control rats 3 wk p.i. but subsequently higher in the diabetic than control group at 7 and 12 wk. Increased lung CFU was also recorded in diabetic KDP rats compared with LETL controls (7 wk p.i.). Pulmonary IFN-γ mRNA levels measured only at 7 wk p.i. were higher in the KDP rats. The picture emerging from these combined studies fits with our model where DM increases TB susceptibility by causing a delay in the priming and expression of adaptive immunity, followed by exaggerated leukocyte infiltration and inflammatory cytokine expression in the setting of a higher bacterial burden at later stages of TB disease.

It is now appreciated that DM is an acquired risk factor for human TB with a global impact comparable to that of HIV/AIDS, making it imperative to learn more about the mechanistic basis of susceptibility. From our studies with diabetic mice, we can make several predictions: human TB risk will correlate with poor glycemic control; diabetic patients may have higher rates of postprimary TB disease; diabetic patients with TB disease will have higher bacterial load and higher levels of inflammatory cytokines, including IFN-γ, TNF-α, and IL-1β; as a consequence of higher bacterial burden, treatment failure, and other adverse outcomes of TB disease will be more frequent in patients with DM. Although studies addressing these points are so far limited, DM has been linked to greater expression of inflammatory cytokines after PPD restimulation of whole blood from TB patients, and cytokine expression within diabetic TB patients was increased in those with elevated levels of HbA1c (31). In other studies, DM was associated with delayed clearance of bacilli from sputum during treatment (32, 33). We plan to use data from the mouse model as a basis to design clinical studies in human populations with DM and TB. Our data in mice, and limited data from diabetic humans with TB, suggest that like the vascular, renal, and neuropathic complications of DM, the immune deficiency resulting in TB susceptibility is a consequence of prolonged hyperglycemia and its downstream effects of superoxide overproduction leading to abnormal protein glycation and other adverse effects (34). Future experiments with our mouse TB/DM models will be directed to more fully characterizing the immunological mechanisms of susceptibility and identifying the biochemical pathways involved.

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Disclosures

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References


