Establishing the Role of CD8+ "Killer" T Cells in the Control Of Human Latent TB Infection

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A. Study Purpose and Rationale

Mycobacterium tuberculosis (MTB) is an enormous public health problem. According to recent data, MTB infects some one-third of the world's population and two million die from the disease annually.[1] The essential task of developing an effective vaccine for MTB will depend upon a fuller understanding of the immune mechanism involved in protection against infection. In this regard, it has long been recognized that in patients with active MTB infection there is a key role for T lymphocytes in mediating an effective immune response. The importance of CD4 T cells has now long been underscored by the observation that HIV disease markedly accelerates MTB disease progression. However, most infected individuals are able to contain the bacterium and have, not active MTB, but rather an asymptomatic, *latent tuberculosis infection* (LTBI), generally identifiable by a positive skin test. Importantly LTBI has the capacity to develop into active tuberculosis if the immune system is unable to contain it as a result of acquired immunosupression, age, or a variety of other factors still to be elaborated. Containment of LTBI may be dependent, in addition to CD4 T cells, on the existence of a robust CD8 T cell response. This hypothesis was supported initially by mice models of MTB infection. Specifically, while in mice, the control of *active* MTB infection seems to involve a prominent role for CD4 cells, the control of *latent* MTB appears to be mediated by CD8 T cells.[2] Consistent with this hypothesis, several investigators have recently identified for the first time, in humans with LTBI, antigen-specific CD8 T cells[3], and further, have demonstrated that these cells have both cytolytic abilities and a capacity to secrete interferon-γ, an essential cytokine in cellular control of MTB infection. The purpose of the experiments outline below will be to further understand control of LTBI by examining the hypothesis that in patient populations previously described to have an increased probability of progressing from LTBI to active MTB – namely the immunosupressed and the other demographic high-risk groups – that *there is an attenuated CD8 T cell response to LTBI related to this progression.* Such a finding would underscore the importance of developing a TB vaccine that induces, not only a CD4 T cell response, but also an effective CD8 T cell component that could potentially prevent reactivation disease among the billions with latent TB.

Prior to the experiments alluded to earlier, there was little prior speculation that CD8 cells would be involved in containing active MTB infection or in protective immunity. *M. tuberculosis* exists primarily intracellularly within vesicles and such intravesicular organisms are usually collected by MHC Class II molecules, and then presented to CD4 T cells. However, the importance CD8 cells was suggested by Flynn and coworkers who showed that gene knockout mice without B2-microglobulin, and thus no functional MHC Class I molecules, were highly susceptible to MTB infection.[4] The implication was that the loss of MHC I, and thus any potential for CD8 cell function, predisposed to overwhelming infection in these knockout mice. Although further investigations suggested that the immune defects that resulted from B2-microglobulin gene disruption were broader than MHC I molecules alone (extending to included CD1 and non-classical MHC molecules involved in the presentation of N-formylated peptides and glycolipids antigens via non CD8 T cell pathways), additional mice experiments were supportive of a vital role for CD8 T cells particularly. For example, work by van Pinxreren and colleagues demonstrated that the selective inactivation of CD8 cells in mice is related specifically with progression from murine LTBI to active tuberculosis. In contrast, similar progression of disease was not associated with the selective inactivation of CD4 T cell populations. The underlying mechanism of CD8 T cell action in mice does not appear to be through cytotoxic effector molecules, as experiments with granzyme and perforin knockout mice appear to indicate.[5] However, it may involve the role of the interferon gamma – a cytokine

well known to be essential in the immune response to TB – given that van Pinxeren and coworkers found that neutralization of the cyokine had similar deleterious effects as the overall loss of CD8 T cells.

Though similar experiments in humans are not possible, supporting data has begun to accumulate that for humans with LTBI, CD8 T cells may also be an integral part of the immune response. It has now been demonstrated by several labs that MTB specific CD8 T cells exist in humans and, further, some ten MTB antigens have been identified as CD8 T cell targets. The functional capabilities of these CD 8 cells have been characterized, and it appears that these cells have, in vitro, interferon gamma secreting capacities, cytolytic potential and can even respond to infected macrophages.[6] It seems possibly that these MTB specific cells have activity in humans with LTBI and it is my belief as well that such a CD8 population acts to assure long-term latency in healthy hosts.

Epidemiological studies have long characterized the groups at higher relative risk for progressing from LTBI to active MTB infection. These groups include not only patients with HIV disease, but older patient populations, patients on chronic immunosupression (Relative Risk 20-74), patients with disease such as diabetes mellitus (RR 2.0-4.1), end-stage renal disease (RR 10.0-25.3), silicosis (RR 30), patients who use injection drugs as well as other groups.[7] Except in the case of large obvious defects in cellular immunity (e.g. advanced HIV disease) the exact immunological basis of accelerated progression has remained largely obscure. It is known that in non-insulin dependent diabetics there are several apparent defects in cellular immunity including a known reduced lymphocyte proliferation to mitogen as well as reduced function (chemotaxis, phagocytosis, killing) of diabetic polymorphonuclear cells.[8] Patients with ESRD and uremia appear to similarly be associated with alterations in cellular immunity and, although the details of these derangements has varied from experiment to experiment , data that suggest defects in the Th1 response (ability to contain intracellular pathogens) are convincing.[9] Based on the mouse studies presented earlier, coupled with the now demonstrated existence of CD8 T cells specific for MTB antigens in LTBI in humans, one factor in accelerated progression in these high risks groups is likely to be a defect in CD8 T cell immunity. The series of experiments that I propose will involve testing this hypothesis in both poorly controlled diabetics and patients with chronic renal disease with an assay designed to measure such TB-specific CD8 cellular activity.

B. Study Design and Statistical Analysis

Human subjects

See section below.

Study groups

This is a cross sectional study of the prevalence of TB-reactive CD8 T cells in several groups. Subjects will be recruited from consecutive new referrals to the hemodialysis center and AIM clinic at the Columbia-Presbyterian Medical Center (CPMC). PPD(-) controls will be recruited from consecutive new patients at the same clinics. PPD responses will be determined by nursing staff in these clinics and will measure at least 10mm of induration. Protocols for venipuncture of the CPMC will be observed. Subjects will fall into one of several groups: (1) PPD(+) and PPD (-) subjects with chronic renal failure newly referred for outpatient hemodialysis, (2) PPD(+) and PPD (-) subjects identified through the AIM clinic with poorly controlled Type 2 diabetes (by random finger stick glucose of >200), (3) PPD(+) and PPD (-) subjects with no risk factors also identified through the AIM clinic. The purpose of the age-matched PPD (-) recruited from each of these groups is to establish the level of *background* TB-reactive CD8 cell activity.

The number is subjects needed has been estimated assuming statistical analysis with unpaired t-testing:

$$N \text{ (in each arm)} = 1 + 16 \text{ (standard deviation / effect size)}^2$$

The effect size is the predicted difference in the outcome measure between groups, and standard deviation is derived from prior studies that estimated this cell population. Based on these studies, PPD(+) subjects with no risk factors for progression will likely have a population of approximately 0.1 TB-specific CD8 T cell/microliter +/- 0.033 cells. Though no compelling evidence upon which to base this estimate exists, we will conservatively predict poorly controlled diabetics to have approximately 0.08 cells/microliter +/- 0.033 cells and patients with ESRD to have approximately 0.05 cells/microliter +/- 0.033 cells. Assuming 80% power and testing at p = .05, this would then require **45 subjects** in the PPD (+)/diabetic arm, **45 subjects** the PPD (+)/no risk factor control arm and **8 subjects** in the PPD (+)/ESRD arm of the trial to detect a difference.

C. Study Procedure

Phlebotomy

The subjects will undergo only brief phlebotomy of the hand or lower arm during which approximately 20cc of blood will be obtained.

Overview of procedure

Autologous CD8 lymphocytes derived from peripheral blood mononuclear cells (PBMC) with magnetic beads will be used as effector cells in a INF-gamma ELISPOT assay, with monocyte-derived dendritic cells (DC) infected with Mtb as antigen presenting cells. ELISPOT plates will be developed after 18 hours of incubation and the spots will be counted. The experiments will include at least two separate effector frequency determinations for each donor. TB-specific CD8 cell frequencies will be obtained by linear regression analysis from lines derived from multiple experiments with each subject. Values generated by the assay (e.g. 1 TB-reactive CD8 cell/4000 CD8 cells) will be converted to concentrations (e.g. 0.1 TB-reactive CD8 cell/microliter) by obtaining total donor CD8 counts by standard lab based protocol to correct for differences in these counts between subjects.

Procedure details

Dendritic cells (DC) will be obtained in the manner outlined in previous publications. This will involve isolation by centrifugation over Ficoll-Hypaque (Sigma, St Louis, MO) solution, growth in a medium supplemented by GM-CSF and IL-4 (Immunex, Seatle, WA) for 18 hours and 5-7 days of growth followed by harvesting with cell-dissociation media (Sigma).[10] CD8 T cells will be separated from PBMC via both positive and negative selection using respectively anti-CD4 Ab coated magnetic beads and then anti-CD8 Ab coated magnetic beads (Miltenyi Biotec, Auburn, CA) per the manufacturer's instructions. Flow cytometry will be used to assure that the cells are a pure population of CD8+ T cells. The ELISPOT assay (Millipore, Bedford, MA), which will use irradiated autologous MTB-infected DC as antigen presenting cells (2 x 10 4) and known quantities of autologous purified CD8+ cells as effector cells. The plates will be coated with mouse anti-INF gamma mAb (Mabtech, Nacka, Sweden) and an ELISA reaction carried out. Spots will be quantified using a Zeiss Axiolplan 2 microscope and KS EPISPOT software (Carl Zeiss Vision, Hallbergmoos, Germany).

D. Study Drugs

Not applicable.

E. Medical Device

Not applicable

F. Study Questionnaires

Actual questionnaire is still under development. Topics to be addressed will include height, weight, history of positive tuberculin skin test, history of latent TB eradication therapy, country of birth, history of BCG vaccine, comorbid diseases, prescription drugs, current or recent acute illness, history of TB exposure, use of alcohol or illicit substances, history of lung disease.

G. Study Subjects

The subjects will be US-born women, aged 45-65, with no acute illness, not currently on corticosteroids or other immune system modulating agent. Unfortunately subjects born in tropical climates have an increased risk of exposure to non-tubercular mycobacteria and harboring potentially cross-reacting CD8 T cells. Similarly a history of BCG vaccination will also be a disqualification. Subjects can have no other risk factor for progression of latent TB. The subjects who are PPD(+) will have an induration of at least 10mm as determined by nursing staff at AIM or in the hemodialysis center/renal clinic.

H. Recruitment of Subjects

As described previously.

I. Confidentiality of Study Data

All results will be confidential and participants will be referred to only by number.

J. Potential Conflict of Interest

Not applicable.

K. Location of the Study

The study will recruit from the renal clinic at CPMC, employee health department of CPMC, and the pulmonary clinic at CPMC.

L. Potential Risks

Mild discomfort at the site of the phlebotomy, minimal risk of bleeding and superficial infection.

M. Potential Benefits

While no potential benefit to the individual participant exists, there is a societal value achievable through a better understanding of human correlates of immunity in the development of a rational TB vaccine.

M. Alternative Therapies

Not applicable.

N. Compensation

None.

O. Cost to Subjects

None.

P. Minors

None.

Q. Radiation/Radioactivity

None.

R. References

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