Individuals with Pulmonary Tuberculosis Have Lower Levels of Circulating CD1d-Restricted NKT Cells

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Mycobacterium tuberculosis (MTB) is a leading cause of mortality worldwide from an infectious agent. Natural killer T (NKT) cells recognize mycobacterial antigens and contribute to anti-MTB immunity in mouse models. NKT cells were measured in subjects with pulmonary tuberculosis, MTB-exposed individuals, and healthy controls. NKT cell levels are selectively lower in peripheral blood mononuclear cells from individuals with pulmonary tuberculosis than in both MTB-exposed subjects and healthy control subjects. This apparent loss of NKT cells from the peripheral blood is sustained during the 6 months after the initiation of MTB treatment. These findings indicate that NKT cells may be an important component of antituberculosis immunity.

The tuberculosis pandemic has a major impact worldwide, with ~8 million new cases of active infection and roughly 2 million deaths each year. One-third of the world’s population is estimated to be currently infected with Mycobacterium tuberculosis (MTB); 5%–10% of individuals infected with MTB will eventually develop active disease, with higher rates among those coinfected with HIV [1].

The bacillus Calmette-Guérin (BCG) vaccine is administered shortly after birth in countries with a high prevalence of tuberculosis. It is efficacious at preventing severe forms of childhood tuberculosis, such as tuberculous meningitis and miliary tuberculosis, preventing 1 case for every 3435 and 9314 vaccinations, respectively [2]. However, the BCG vaccine has shown variable efficacy against pulmonary tuberculosis [3], highlighting the need for a better understanding of the anti-MTB immune response to facilitate new vaccine strategies.

NKT cells are an immunoregulatory subset of T cells whose frequencies and effector functions are often strongly associated with both autoimmune and infectious diseases [4, 5]. NKT cells recognize glycolipid antigens, including mycobacterial lipids [6], and contribute to an effective anti-MTB immune response in mice [7]. Also, NKT cells contribute to the formation of granulomas, a histological finding associated with MTB infection, in mice injected with deproteinized cell walls from mycobacteria [8]. To determine whether NKT cells contribute to the immune response during human MTB infection, we compared the percentages of NKT cells in the peripheral blood mononuclear cells (PBMCs) of subjects with pulmonary tuberculosis, MTB-exposed subjects, and healthy unexposed subjects.

Subjects, materials, and methods. All subjects were recruited at the Federal University of São Paulo, Brazil. Written, informed consent was obtained from all volunteers according to the guidelines of the Brazilian Ministry of Health. The study subjects are divided into 3 groups: patients with pulmonary tuberculosis (14 males and 5 females; mean age, 33 years), MTB-exposed subjects (7 males and 15 females; mean age, 40 years), and healthy control subjects (4 males and 8 females; mean age, 26 years). Patients with pulmonary tuberculosis were defined using the following criteria: (1) presentation with clinical and roentgenographic findings suggestive of MTB infection and (2) a microbiological diagnosis of tuberculosis (confirmed by the presence of acid-fast bacilli in the smear and/or an MTB-positive culture in the sputum). Eighteen of the 19 patients with pulmonary tuberculosis were given the tuberculin skin test (TST); all gave a positive response (≥10 mm induration). MTB-exposed subjects were defined using the following criteria: (1) repeated, close contact with an individual with pulmonary tuberculosis (based on above definition) and (2) lack of clinical history or chest roentgenogram suggestive of active tuberculosis. All MTB-exposed subjects presented with detectable TST responses and/or a significant number of interferon-γ–secreting cells in response to purified protein derivative (PPD) antigens in an enzyme-linked immunospot assay (data not shown).
Many of the MTB-exposed subjects may have received the BCG vaccine; therefore, responses to PPD may not indicate latent infection with MTB. Therefore, subjects are designated as MTB exposed based on their close contact exposure to subjects with pulmonary tuberculosis. Healthy control subjects were defined using the following criteria: (1) no known exposure to an individual with an active MTB infection, (2) no symptoms of MTB infection, and (3) no detectable TST reactivity at the time of sample collection. All subjects with pulmonary tuberculosis were started on a 6-month course of antituberculosis medications (rifampin, isoniazid, and pyrazinamide) on study entry.

NKT cell populations were defined using combinations of the following antibodies: phycoerythrin (PE)–labeled anti-Vα24, fluorescein isothiocyanate–labeled anti-Vβ11 (both from Beckman Coulter), and Per-CP–labeled anti-CD3 (BD Biosciences). Purified CD1d:1g recombinant fusion protein (DimerX; BD Biosciences) loaded with α-GalCer (Kirin Brewery) and purified invariant NKT antibody (clone 6B11; BD Biosciences) followed with PE-labeled anti–human IgG1 (Caltag Laboratories). Flow cytometry was performed on a FACSCalibur (BD Biosciences); data were analyzed using FlowJo software (version 8.1.1; Tree Star). “Frequency minus one” stained control samples were used to determine gate selection for all antibody panels [9]. Therefore, nonspecific events were excluded from the Vα24+Vβ11+ gate and were not included in the analysis of NKT percentages. The Mann-Whitney U test was used to compare NKT percentages between the different groups.

**Results.** We first determined whether the use of Vα24 and Vβ11 antibodies was a valid approach to define NKT cells in our subjects. Human invariant NKT cells express a canonical T cell receptor incorporating Vα24IgαQ with a limited Vβ repertoire, the vast majority using Vβ11 [10]. Therefore, NKT cells are often defined using anti-Vα24 and anti-Vβ11 antibodies. However, a fraction of the Vα24+Vβ11+ T cells may be variant T cells that naturally chose those gene segments during rearrangement; therefore, the percentage of 6B11+Vβ11+ cells and Vα24+Vβ11+ cells were compared directly among individual subjects. The 6B11 antibody recognizes the CDR3 region of the Vα24IgαQ chain and binds exclusively to invariant NKT cells. The numbers of events within the Vα24+Vβ11+ and the 6B11+Vβ11+ gates from individual subjects were very similar, confirming the invariant NKT specificity of the Vα24+Vβ11+ gate (figure 1). NKT cell populations are also defined using antigen-loaded CD1d tetramers or dimers. The number of dimer–α-GalCer+Vβ11+ events was similar to those detected by the other 2 gating strategies; this indicates that NKT cells are accurately defined as Vα24+Vβ11+ cells in our cohort (figure 1).

The percentages of Vα24+Vβ11+ cells were compared between patients with pulmonary tuberculosis, MTB-exposed subjects, and healthy control subjects. A significantly lower percentage of NKT cells was detected in the pulmonary tuberculosis group than in both the MTB-exposed ($P = .0013$) and healthy control ($P = .0005$) groups (figure 2A–2C). The Vα24+Vβ11+ and Vα24+Vβ11+ T cell subsets were also compared, and there was no significant decrease of either of these T cell populations in the pulmonary tuberculosis group compared with the other 2 groups. This indicates that the lower levels of NKT cells present in the PBMCs of infected subjects was not due to nonspecific T cell loss (figure 2A–2C). Interestingly, there were significantly lower levels of Vα24+Vβ11+ T cells in the MTB-exposed subjects than in the other 2 groups (figure 2B).

NKT cells are capable of down-regulating their T cell receptor on activation with α-GalCer in mouse models [11, 12]. Individuals with pulmonary tuberculosis may contain circulating NKT cells that have lost surface expression of the T cell receptor because of activation with MTB glycolipids. Therefore, to ensure that this phenomenon did not occur, intracellular cytokine staining with anti-Vα24 and anti-Vβ11 antibodies was performed on a subset of the pulmonary tuberculosis samples. No increase in NKT cell frequencies was detected, indicating that NKT cells were truly lower in frequency in the PBMCs of these subjects (data not shown).

Twelve subjects with pulmonary tuberculosis were studied longitudinally to determine whether NKT cells increased in the PBMCs in the first 6 months after the initiation of treatment, all with successful clinical responses. Nine of the 12 subjects consistently exhibited NKT percentages lower than 0.1% of PBMCs for all time points measured (figure 2D). Three subjects demonstrated higher NKT cell levels: 2 subjects exhibited a rise in NKT percentages after the initiation of therapy, and 1 subject...
Figure 2. Lower levels of NKT cells in the peripheral blood mononuclear cells (PBMCs) of patients with active tuberculosis (TB) than in those of both Mycobacterium tuberculosis (MTB)–exposed subjects and healthy control subjects. The percentages of NKT cells (Vα24+Vβ11+ [A]) and non-NKT cells (Vα24–Vβ11– [B] and Vα24–Vβ11+ [C]) in the PBMCs of patients with active pulmonary TB, MTB-exposed subjects, and healthy control subjects were measured using 4-color flow cytometry. Each circle represents 1 subject; the solid bars represent the median values from all subjects within each group. The Mann-Whitney U test was used to determine statistical significance between the subject groups.

Discussion. We have shown that the percentages of circulating NKT cells are selectively and significantly lower in individuals with active pulmonary tuberculosis than those in uninfected individuals. Also, the levels of NKT cells in peripheral blood did not increase in the majority of infected subjects during the first 6 months after the initiation of antituberculosis drug treatment. These data suggest that NKT cells are integral components of the immune response to MTB.

There are several explanations for this apparent loss of NKT cells from the circulation of patients with pulmonary tuberculosis. The circulating NKT cells may have undergone apoptosis as a result of chronic stimulation by glycolipid MTB antigens. However, this possibility is incongruent with our finding that the NKT levels did not rise during the 6 months of treatment in most patients, as we would have predicted that the decline in mycobacterial burden would allow NKT cells to recover to homeostatic levels. Measurement of NKT cell frequencies in individuals both before and after the onset of active MTB infection would help determine whether the infection does in fact induce NKT loss from the blood.

NKT cells may have relocated from the blood to granulomas at the site of infection and, from there, may exert effector functions that hold MTB in its latent form. Circulating NKT levels are low in 2 diseases characterized by granulomas of the lungs: tuberculosis (figure 2A–2C) and sarcoidosis [13]. NKT cells have been observed in granulomatous lesions of individuals with sarcoidosis [14] and leprosy [15], and Vα14– NKT cell–deficient mice have higher numbers of granulomas after infection with Mycobacterium bovis BCG [16]. Future longitudinal studies of both PBMCs and granulomatous lesions from individuals with pulmonary tuberculosis will help determine whether NKT cells home to the lungs from the circulation to contribute to granuloma formation.
As the circulating NKT levels before the onset in symptoms of the subjects with tuberculosis are not known, it is also possible that individuals with fewer NKT cells are more susceptible to MTB infection. It would be of interest to assess individuals with latent MTB infection longitudinally to determine whether the loss of NKT cells, from the circulation and/or the lung, is associated with the reemergence of active MTB disease. Also, males and individuals with systemic lupus erythematosus are likely to have low or abnormal levels of NKT cells [17, 18], respectively, and both groups also appear to be more susceptible to MTB infection and disease [19, 20]. It would be interesting to note whether the MTB-exposed subjects with the lowest NKT cell frequencies are more likely to present with active disease.

Analysis of NKT cell frequencies and ex vivo effector functions in both the circulation and the site of infection in large cohorts of subjects with pulmonary tuberculosis will help elucidate the exact role of this unique T cell population in anti-tuberculosis immune responses.

Acknowledgments

We thank Wassim Ballan, Amedeo Cappione, and Keith Garrison for their critical reading of the manuscript, Helena Tomiymana and Tania Silva for help with laboratory work, and Jason Barbour for help with the statistical analysis. We also thank the Kirin Brewery for the α-GaCer.

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