CONCISE COMMUNICATION

Type 2 Cytokine Gene Activation and Its Relationship to Extent of Disease in Patients with Tuberculosis

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The extent of type 2 cytokine gene expression in patients with pulmonary tuberculosis (TB) was studied by use of quantitative nested reverse-transcription polymerase chain reaction on freshly isolated peripheral blood mononuclear cells. Interleukin (IL)–4 and IL-13 mRNA expression was significantly greater in patients—median mRNA copy numbers were 1.7 and 1.1 log2 higher, respectively—than in matched tuberculin-positive control subjects. Significant correlations with radiologic extent of disease and serum IgE levels supported the biologic significance of these results. Interferon-γ mRNA copy numbers exceeded those of type 2 cytokines but were only marginally lower in patients than in control subjects. Gene expression of an IL-4 splice variant (IL-4d2) was bimodally distributed in both patient and control groups. Patients with greater IL-4d2 expression also expressed more IL-4 mRNA and had more extensive disease. Type 2 cytokines are associated with immunopathologic changes in TB patients but could be a cause or consequence of disease.

Mycobacterial infections are chiefly controlled by activation of macrophages through type 1 cytokine production by T cells, and interferon (IFN)–γ is central to this process. The nature of type 2 cytokine involvement is less clear. There have been reports suggesting that the principal type 2 cytokine, interleukin (IL)–4, is increased in patients with tuberculosis (TB) [1] when compared with that in control subjects, but this has been disputed by others [2]. In murine models of pulmonary TB infection, the appearance of IL-4 in the lung lesions (as seen by immunohistochemistry) coincides temporally and spatially with the appearance of areas of pneumonia and necrosis, leading to rapid clinical deterioration and death [3]. However, in mice with disrupted IL-4 genes, the clinical course of TB infection appears to be unchanged, with no evidence of increased resistance to the infection [4].

The confusion may be partly attributed to the diverse methodologies used and inherent difficulties in performing IL-4 assays, because very low levels of IL-4 are produced by unstimulated cells. However, stimulating the cells in culture media with various antigens and cytokines is unlikely to yield a physiologic response that reflects the in vivo cytokine milieu. The IL-4–deficient mouse model may not give definitive answers either, because IL-13 also has type 2 cytokine activity [5]. In addition, none of the previous TB studies have taken into account the presence and possible activity of an IL-4 splice variant (IL-4d2), which is detectable even in normal subjects [6] and has potential immunomodulatory functions [7].

We established a sensitive assay that allows type 2 cytokine gene expression to be quantified in fresh, unstimulated peripheral blood mononuclear cells (PBMC) from any subject, healthy or with disease [8]. We looked for differences in mRNA expression of IL-4, IL-4d2, or IL-13 between patients with TB and healthy tuberculin-positive control subjects, compared this to IFN-γ expression, and sought to relate cytokine expression levels to clinical parameters.

Materials and Methods

Patients and control subjects. Whole blood was obtained from 18 patients (age range, 18–64 years) whose first episode of pulmonary TB was confirmed by clinical findings, radiography, and sputum culture. Their ethnic origins were representative of the resident population in central London. Patients with a history of
atopy, steroid use, or other concomitant infectious or autoimmune diseases were excluded.

A blood sample was taken from the patients either before or within the first month of starting treatment. Chest radiographs (CXR) at presentation and follow-up were available for all patients. The extent of disease in each patient was assessed by 3 parameters—sputum smear for acid-fast bacilli (AFB), extent of involvement shown on CXR, and presence or absence of cavitation.

Control subjects were healthy, tuberculin-reactive, recent close contacts of patients with active pulmonary TB. Eighteen such subjects were individually matched to the patient cohort by age, sex, ethnicity, and geographic region of origin. They had no known history of bacille Calmette-Guérin (BCG) immunization or clinical evidence of a BCG scar. These control subjects were asymptomatic and had no history of chronic illnesses or atopy. Their CXR findings were normal. Their total white cell counts, differential counts, C-reactive protein levels, and erythrocyte sedimentation rates were all within normal limits. This control group was chosen because, within the limits of interpretation of the tuberculin test, such subjects are most likely to represent those recently exposed to but not with disease.

PBMC isolation and reverse transcription–polymerase chain reaction (RT-PCR). The assay has been described in detail elsewhere [8]. In brief, PBMC were isolated from fresh whole blood by density-gradient centrifugation, and RNA was extracted from the PBMC by the phenol-chloroform method. A cRNA standard for each cytokine studied was constructed. A 10-fold dilution series of each of these synthetic cRNAs was used as an external standard for subsequent RT-PCRs. RT and 2-tube nested PCR protocols have been published elsewhere [8]. Additional PCR primers used were as follows (5′-3′): The first-round IFN-γ primers were GGCTTGTTACTGCCCAGGACCCATATGT and GATGCTCTTCGACTCCTGAAACACGACAT; the second-round primers were GCAGGTCATTGCAGTGAGCGGAT and AGGCCATCACTTGGATGAGTTCTAGT. For IL-13, the first-round primers were CCACGGTCATTGCTCTCATT and TTACAACTTGCCACCTCGA; the second-round primers were CAATCAACCAGGAAGAAG and CAGAATCCGCTCAGCATCCTC.

The PCR amplicons were detected directly from fluorescent-stained gels with an image analyzer, as described in detail elsewhere [8], and quantified by interpolation from the linear standard curves derived from amplitons of the cRNA standard series for each cytokine. The number of copies of mRNA for each cytokine was normalized against β-actin gene expression, to standardize results with respect to the initial RNA yield.

ELISA. Levels of serum IgE were determined by use of ELISA kits from ALPCO (Windham, NH), in accordance with the manufacturer’s instructions.

Statistical methods. Cytokine mRNA expression in different samples was compared by use of the nonparametric Mann-Whitney U test. Results in the figures are expressed as medians, and error bars show 25th and 75th percentiles. The Spearman rank-sum correlation was used to test the significance of correlations.

Results

Clinical parameters. Of the 18 TB patients, 14 were smear-positive for AFB, and 10 had evidence of cavitation on their CXRs. Nine patients had either minimal or moderate disease, and 9 had extensive disease, on the basis of whether ≤3 (minimal/moderate) or >3 (extensive) radiographic zones were affected by tuberculous disease.

IL-4. IL-4 mRNA was detected in all subjects, which is consistent with previous reports [6]. Exon 1 continues as exon 3 in this transcript, without frameshift (sequencing data not shown). The expression of mRNA for IL-4 was bimodal in both patient and control groups. The 2 populations within each group were designated IL-4*lo and IL-4*hi. Within the IL-4*lo population, TB patients expressed significantly more copies of mRNA for IL-4 than did control subjects (P = .04). The same is true for subjects within the IL-4*hi group (P = .002). IL-4*lo patients (n = 8) had significantly higher median levels of IL-4 mRNA expression (P = .04) than did IL-4*hi patients (n = 10). Expression of IL-13 mRNA was also higher.
Figure 2.  Interleukin (IL)-4 and IL-13 mRNA expression in patients with tuberculosis and clinical markers of disease severity. IL-4 mRNA expression was consistently higher in the group of patients with clinically more severe disease, by all 3 markers. Asterisk (*) indicates statistically significant association ($P = .04$). Median results are shown, with 25th and 75th percentiles marked as error bars. Nos. of patients in each category are described in the text. CXR, chest radiograph; AFB, acid-fast bacilli.

and that of IFN-$\gamma$ lower in the former group, but these differences were not statistically significant. The IL-4$^{\text{hi}}$ patients, as a group, were more likely to have extensive disease and visible cavitation on their CXRs at presentation than were IL-4$^{\text{lo}}$ patients, although the difference was not statistically significant.

Type 2 cytokine gene expression in TB. IL-4 and IL-13 mRNA expression was markedly greater in the PBMC of the patients with TB when compared with that in control subjects (figure 1). Median IL-4 and IL-13 mRNA log copy numbers were 1.7 and 1.1 logs higher, respectively, than control values ($P = .004$ and $P = .0009$, respectively). There was a significant positive correlation between IL-4 and IL-13 expression in both patient and control groups ($P = .00001$). The IFN-$\gamma$:IL-4 mRNA copy number ratio was markedly higher in control subjects (median, 573.21, compared with 8.877 in patients; $P = .002$).

To assess the biologic significance of the increased mRNA expression in patients, serum IgE levels were assayed as a surrogate marker of type 2 cytokine biologic activity [9]. Serum IgE levels were significantly correlated with IL-4 mRNA copy numbers in patients ($P = .032$) and in control subjects ($P = .001$), which is consistent with the known role of IL-4 in IgE isotype switching. We also found that serum IgE levels in the TB patients were significantly higher than those in control subjects, by the Mann-Whitney nonparametric test ($P = .017$).

Correlation with clinical parameters. Expression of IL-4 mRNA correlated with clinical measures of disease severity (figure 2). Radiologically advanced disease and cavitation on CXR, as well as sputum smear positivity for AFB on clinical presentation, were all associated with greater IL-4 mRNA expression. The association of disease extent shown on radiography with IL-4 mRNA expression was statistically significant ($P = .04$). Interestingly, there was also a lower IFN-$\gamma$:IL-4 mRNA copy number ratio in patients with greater extent of disease shown on their CXRs ($P < .05$).

Discussion

IL-4 detection. Previous studies using conventional assays have reported IL-4 to be almost undetectable in PBMC from TB patients and normal subjects [10]; the levels of detectable IL-4 are so low that the data cannot be adequately interpreted. The nested RT-PCR assay that we used has a 5-log$_{10}$ working range [8], and we believe its sensitivity allows us to study type 2 cytokine expression more accurately, since IL-4 mRNA expression can be quantified even in unstimulated cells from normal subjects.
Association of type 2 cytokines with disease. There have been few studies examining quantitatively the extent of involvement of type 2 cytokines in TB. We chose control subjects who would represent an “exposed but non-diseased” healthy cohort and compared their state of cytokine activation with that of the TB patients. Our results show that increased type 2 cytokine activity is associated with the presence of disease and that IL-4, in particular, further relates to radiologic extent of disease. Although these observations were made at the mRNA level, the correlation of IL-4 mRNA expression with serum IgE levels supports the probability that the mRNA levels relate to biologically active quantities of cytokine. It has recently been shown that IgE is undetectable in mice with simultaneous disruption of IL-4 and IL-13 genes [11], thus underlining the crucial role of these type 2 cytokines in IgE synthesis.

In our study, type 2 cytokine gene activation relates to unfavorable disease outcomes, but we do not yet know whether this association is a cause or consequence of disease. We do not dispute previous evidence that a relative depression of type 1 cytokine expression may be an important feature of immunopathology in TB patients; indeed, our data supports this contention. However, we observe that the relative excess of type 2 cytokine mRNA in patients (compared with that in control subjects) is far greater than their relative lack of IFN-γ (figure 1). This suggests that these two effects are only partially a result of their mutually antagonistic actions; type 2 cytokines may be associated with immunopathology through a different mechanism(s). It is also possible that the increased presence of type 2 cytokines may only be a consequence of a dysregulated immune response to infection, in which an imbalance of pro- and anti-inflammatory mediators favors mycobacterial growth. This is consistent with our observation that the IFN-γ:IL-4 mRNA copy number ratios were significantly lower in patients than in control subjects, and, among the patients, were lower in the group with greater radiologic extent of disease.

Immunopathologic mechanisms. One possible mechanism of the association of type 2 cytokines with disease is through the effect on tumor necrosis factor (TNF-α)-mediated cytotoxicity. T helper cells may mediate local tissue inflammation, which is IL-4 dependent [12]. In TB-infected mice, susceptibility to toxic effects of TNF-α injected into the footpads temporally coincides with the emergence of type 2 cytokines in the lungs [13]. Lawrence et al. [14], studying Trichinella spiralis infection in mice, also found that IL-4 may regulate enteropathy caused by TNF. We do not yet know if a parallel situation exists in TB patients; this is the subject of current investigations.

IL-4d2. The significance of the bimodal expression of IL-4d2 in both patient and control populations is unknown and has not been previously reported. There are many good reasons to measure IL-4d2 expression in human disease: in vitro functional studies suggest that IL-4d2 may be an antagonist of IL-4-induced T cell proliferation, IgE synthesis, and CD23 expression [7]. Our preliminary work showed a non-significant trend toward a greater extent of disease in the IL-4d2 patient population, which also supports an immunomodulatory role for IL-4d2 but requires further investigation.

Conclusion. We have shown that, although the mRNA copy number for IL-4 remains ~10 times lower than that of IFN-γ in TB patients, there is, nevertheless, a striking (1.7 log) increase in IL-4 gene expression relative to that in control subjects. The elevation of type 2 cytokine gene expression in TB patients may be a secondary effect of the inflammatory process. It could also contribute to complex immune interactions, which result in immunopathologic consequences in the host. How such responses influence the immunopathology of TB requires clarification, since it is relevant to immunotherapeutic approaches in TB management.

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References

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