Vol. 69, No. 5

Contribution of CD8$^+$ T Cells to Gamma Interferon Production in Human Tuberculosis

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The proportions of peripheral blood mononuclear cells (PBMC), CD4$^+$ T cells, and CD8$^+$ T cells that produce gamma interferon (IFN-γ) in response to *Mycobacterium tuberculosis* were markedly reduced in tuberculosis patients, particularly in those with severe disease. Depletion of CD4$^+$ but not CD8$^+$ cells prior to stimulation of PBMC with *M. tuberculosis* abolished IFN-γ production. These results show that (i) IFN-γ production by CD8$^+$ and CD4$^+$ cells correlates with the clinical manifestations of *M. tuberculosis* infection and (ii) IFN-γ production by CD8$^+$ cells depends on CD4$^+$ cells.

Gamma interferon (IFN-γ) is believed to play a central role in immunity against tuberculosis, and IFN-γ production by peripheral blood mononuclear cells (PBMC) correlates with the clinical manifestations of tuberculosis. *Mycobacterium tuberculosis*-induced IFN-γ production by PBMC is high in healthy tuberculin reactors infected with *M. tuberculosis* but is reduced in patients with pulmonary tuberculosis (6, 11, 22, 26), particularly in those with severe disease (15). CD4$^+$ T cells are considered to be the predominant source of IFN-γ, and one study suggested that the percentage of CD4$^+$ cells producing IFN-γ was reduced in tuberculosis patients (3). However, limited information is available on the contribution of CD8$^+$ T cells to IFN-γ production in human tuberculosis (8, 24). To investigate this question, we used the enzyme-linked immunospot (ELISPOT) method to measure the frequency of IFN-γ-producing cells in PBMC, CD8$^+$ cells, and CD4$^+$ cells from tuberculin-positive and tuberculin-negative healthy donors, as well as from patients with pulmonary tuberculosis.

Study subjects. Blood was obtained from 15 healthy donors (9 tuberculin positive and 6 tuberculin negative) and 15 human immunodeficiency virus-seronegative patients with culture-proven pulmonary tuberculosis who had been treated for less than 4 weeks. Nine patients had moderately advanced tuberculosis and six had far advanced tuberculosis, based on standard chest radiographic criteria (10). The extent of interstitial or alveolar infiltrate in both lungs was expressed as a percentage of the volume of one lung. Moderately advanced tuberculosis was considered to be present if all three of the following criteria were satisfied: (i) dense alveolar infiltrate occupied less than one-third of the volume of one lung, (ii) interstitial infiltrate occupied less than the volume of one lung, and (iii) the total diameter of cavities was less than 4 cm. Far advanced tuberculosis was defined as disease that was more extensive than moderately advanced tuberculosis.

Cell culture. PBMC were obtained by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). CD4$^+$ or CD8$^+$ cells were isolated from PBMC, using positive selection with magnetic beads conjugated to anti-CD4 or anti-CD8 (Miltenyi Biotech, Auburn, Calif.) (25). In some cases, PBMC were depleted of CD4$^+$ or CD8$^+$ cells by negative selection.

PBMC were cultured in T-25 flasks at 10^6 cells/ml in RPMI 1640 with 10% heat-inactivated human serum. Cells were stimulated with 1 μg of phytohemagglutinin (PHA) per ml or 1 μg of heat-killed *M. tuberculosis* Erdman strain organisms (whole washed cells) per ml (equivalent to approximately 2 × 10^7 bacilli/ml) or were left unstimulated. In preliminary time course studies, the maximum number of spots was detected when PBMC were stimulated with heat-killed *M. tuberculosis* for 72 h or with PHA for 48 h. At these time points, supernatants were collected for measurement of IFN-γ, and cells were washed three times. One aliquot of cells was used for the ELISPOT assay, as outlined below. From two other aliquots, positively selected CD4$^+$ and CD8$^+$ cells were also used in the ELISPOT assay.

In some experiments, PBMC, CD4-depleted PBMC, and CD8-depleted PBMC were cultured as outlined above, either unstimulated or stimulated with PHA or heat-killed *M. tuberculosis*. Supernatants were collected for measurement of IFN-γ, and cells were washed three times and plated for the ELISPOT assay.

IFN-γ production. The ELISPOT assay was performed by incubating stimulated cells for 16 to 18 h in 96-well nitrocellulose-backed plates, using anti-human IFN-γ monoclonal antibodies (1-DIK and 7-B6-1; Mabtech, Nacka, Sweden), according to the manufacturer's instructions. The spots in air-dried plates were counted using a stereomicroscope. The images of dried plates were captured with a scanner (Hewlett-Packard ScanJet 6390C), saved in tagged image file format, and analyzed with an image analysis program available from the National Institutes of Health (http://rsb.info.nih.gov/nih-image/about.html). The histogram option was used to measure the density of spots in triplicate wells. The highest values from the wells with unstimulated PBMC were considered to be

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background and were deducted from values of stimulated cells on the same plate.

IFN-γ concentrations were measured by enzyme-linked immunosorbent assay, using pairs of antibodies (PharMingen, San Diego, Calif.), according to the manufacturer's instructions.

Statistical analysis. Differences between groups were compared by Student's t test or by analysis of variance, as appropriate, using the Prism software program (GraphPad Software, Inc., San Diego, Calif.). Posttests were used to determine if linear trends were present among the three groups of persons infected with *M. tuberculosis* (healthy tuberculin reactors, patients with moderately advanced tuberculosis, and patients with far advanced tuberculosis).

Frequency of IFN-γ-producing cells in *M. tuberculosis*-stimulated PBMC. Using the ELISPOT assay, the median frequency of IFN-γ-producing cells in *M. tuberculosis*-stimulated PBMC from nine healthy tuberculin reactors was found to be 16-fold higher than that in six tuberculin-negative persons ($P = 0.004$). This suggests that the assay primarily detects production of IFN-γ by previously sensitized T cells. The frequency of IFN-γ-producing cells was highest in healthy tuberculin reactors (median, $294/10^5$), intermediate in patients with moderately advanced tuberculosis (median, $126/10^5$), and lowest in patients with far advanced tuberculosis (median, $18/10^5$), and this linear trend was statistically significant (Fig. 1A) ($P < 0.05$). Two of nine patients with moderately advanced tuberculosis had a high frequency of IFN-γ-producing cells, but they were clinically indistinguishable from the other seven patients.

PHA-stimulated PBMC from all four groups had similar frequencies of IFN-γ-producing cells (Fig. 1B) ($P = 0.22$), demonstrating that the reduced frequency of *M. tuberculosis*-stimulated cytokine-producing cells from healthy tuberculin-negative persons and tuberculosis patients was antigen-specific and was not due to a generalized inability to produce IFN-γ.

We measured IFN-γ concentrations in supernatants of *M. tuberculosis*-stimulated PBMC and found that IFN-γ concentrations closely paralleled the frequency of IFN-γ-producing cells (correlation coefficient = 0.86), confirming prior studies showing that IFN-γ production varies inversely with the severity of disease due to *M. tuberculosis* (5, 15).

Frequency of IFN-γ-producing CD8$^+$ and CD4$^+$ cells in *M. tuberculosis*-stimulated PBMC. CD4$^+$ cells are considered the major source of IFN-γ produced in response to microbial pathogens. However, there is increasing evidence that CD8$^+$ T cells contribute to immunity against tuberculosis in animals (12, 16, 21) and in humans (8, 9, 17, 18). To measure the frequency of IFN-γ-producing CD8$^+$ cells, PBMC were cultured with heat-killed *M. tuberculosis* for 72 h, at which point CD8$^+$ T cells were positively selected (>95% CD8$^+$) and incubated in ELISPOT plates. The frequency of IFN-γ-producing CD8$^+$ T cells in the four groups paralleled findings for PBMC, falling with increasing disease severity ($P < 0.03$) (Fig. 2A). Because live mycobacteria activate CD8$^+$ cells more effectively than dead organisms (23), we may have underestimated the frequency of IFN-γ-producing CD8$^+$ cells.

To compare the frequencies of IFN-γ-producing CD4$^+$ and CD8$^+$ cells, the ELISPOT assay was applied to positively selected CD4$^+$ cells isolated from *M. tuberculosis*-stimulated PBMC (Fig. 2B). The results paralleled those found for CD8$^+$
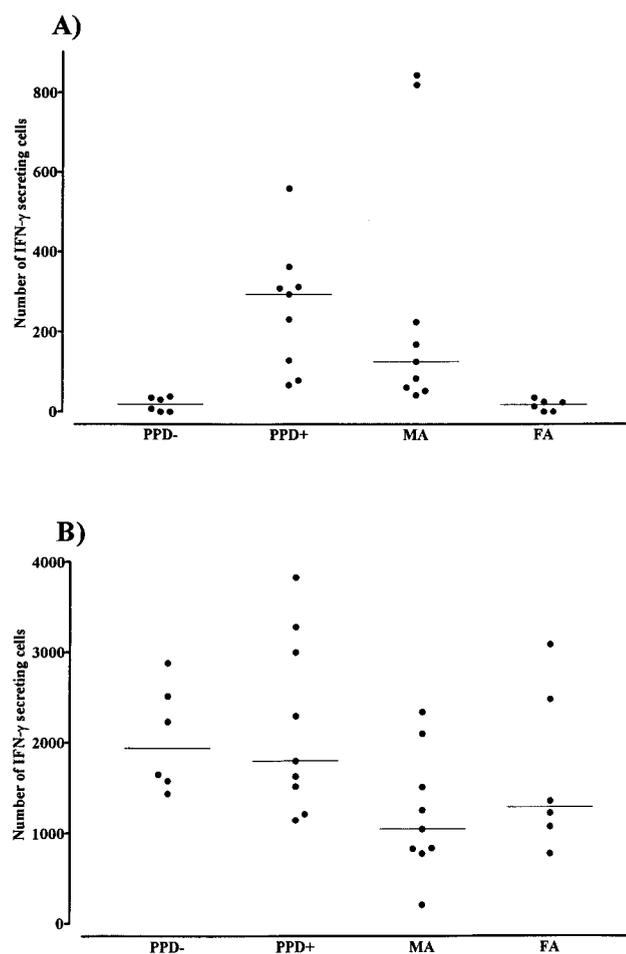

FIG. 1. Frequency of IFN-γ-producing cells in stimulated PBMC. PBMC were obtained from six healthy tuberculin-negative persons (PPD−), nine healthy tuberculin reactors (PPD+), nine patients with moderately advanced tuberculosis (MA), and six patients with far advanced tuberculosis (FA). The frequency of IFN-γ-producing cells upon stimulation with *M. tuberculosis* (A) or PHA (B) was determined by ELISPOT. Each circle represents the number of IFN-γ-producing cells per 10^5 cells for one person. The horizontal bars indicate median values.

cells. The numbers of IFN-γ-producing cells per 10^5 CD4$^+$ and CD8$^+$ T cells were similar in healthy tuberculin reactors (medians of 195 and 124, respectively) and in patients with moderately advanced tuberculosis (medians of 40 and 63, respectively).

IFN-γ production per cell. To evaluate IFN-γ production per cell, we used an integrated image analysis system (4) to quantify the density of each spot in the ELISPOT plate. A spot density of zero was assigned to two patients with far advanced tuberculosis, for whom no spots were present. The median spot density of CD8$^+$ cells was highest in healthy tuberculin reactors, intermediate in patients with moderately advanced tuberculosis, and lowest in patients with far advanced tuberculosis (test for linear trend, $P = 0.003$) (Fig. 3A). Similar findings were observed for CD4$^+$ cells ($P < 0.0001$, Fig. 3B). Comparing the values in Fig. 3A with those in Fig. 3B, the spot densities of CD4$^+$ and CD8$^+$ cells were found to be similar in all
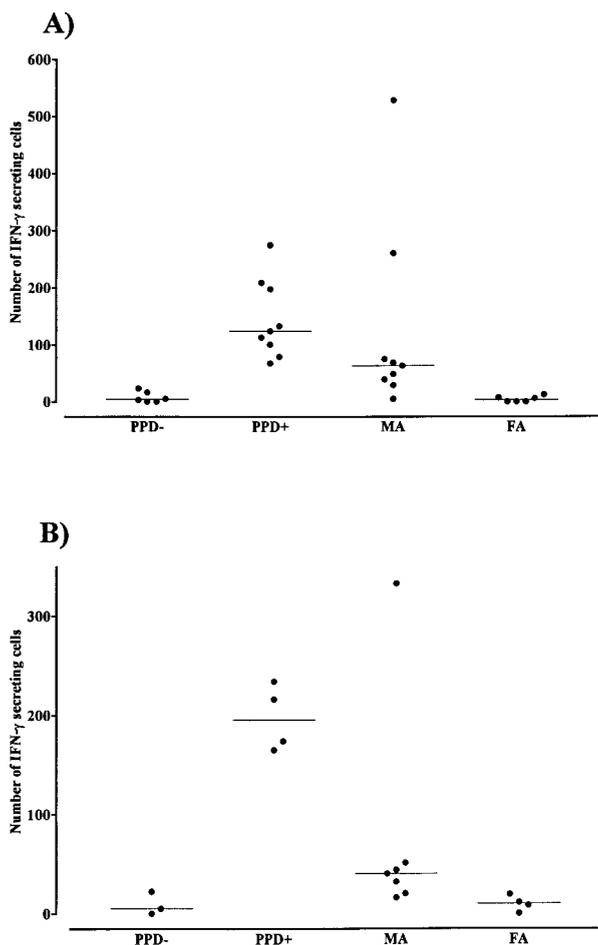
FIG. 2. Frequency of IFN-γ-producing CD8⁺ cells (A) and CD4⁺ cells (B) in *M. tuberculosis*-stimulated PBMC. PBMC were obtained from six healthy tuberculin-negative persons (PPD−), nine healthy tuberculin reactors (PPD+), nine patients with moderately advanced tuberculosis (MA), and six patients with far advanced tuberculosis (FA). The frequency of IFN-γ-producing cells after stimulation with *M. tuberculosis* was determined by ELISPOT. Each circle represents the number of IFN-γ-producing cells per 10^5 CD8⁺ or CD4⁺ cells for one person. The horizontal bars indicate median values.

FIG. 3. IFN-γ production per CD8⁺ cell (A) and CD4⁺ cell (B). IFN-γ production per cell was estimated by measuring the density of spots on the ELISPOT plates for healthy tuberculin reactors and tuberculosis patients shown in Fig. 2, using an integrated image analysis system. The y axis shows spot density in arbitrary units. Each circle represents the mean spot density for one person. The horizontal bars indicate median values.

three groups, indicating that the amounts of IFN-γ produced per cell were similar for CD4⁺ and CD8⁺ cells.

Effects of depleting CD8⁺ or CD4⁺ T cells on IFN-γ production. Our data above demonstrate that both CD8⁺ and CD4⁺ T cells contribute to IFN-γ production by *M. tuberculosis*-stimulated PBMC. To determine if CD8⁺ or CD4⁺ cells were required for the reciprocal subpopulation to produce IFN-γ, we depleted PBMC from three healthy tuberculin reactors of either CD8⁺ or CD4⁺ cells prior to culture with *M. tuberculosis* and measured the frequency of IFN-γ-producing cells after 72 h. CD4⁺ cells comprised 48% ± 7% (mean ± standard error) of PBMC, 71% ± 7% of CD8-depleted PBMC, and 0.2% ± 0.1% of CD4-depleted PBMC. CD8⁺ cells comprised 29% ± 2% of PBMC, 0.8% ± 0.6% of CD8-depleted PBMC, and 58% ± 6% of CD4-depleted PBMC.

Depletion of CD8⁺ cells modestly reduced the frequency of IFN-γ-producing cells (Fig. 4A), presumably because of the removal of CD8⁺ cells and an increased number of cells that
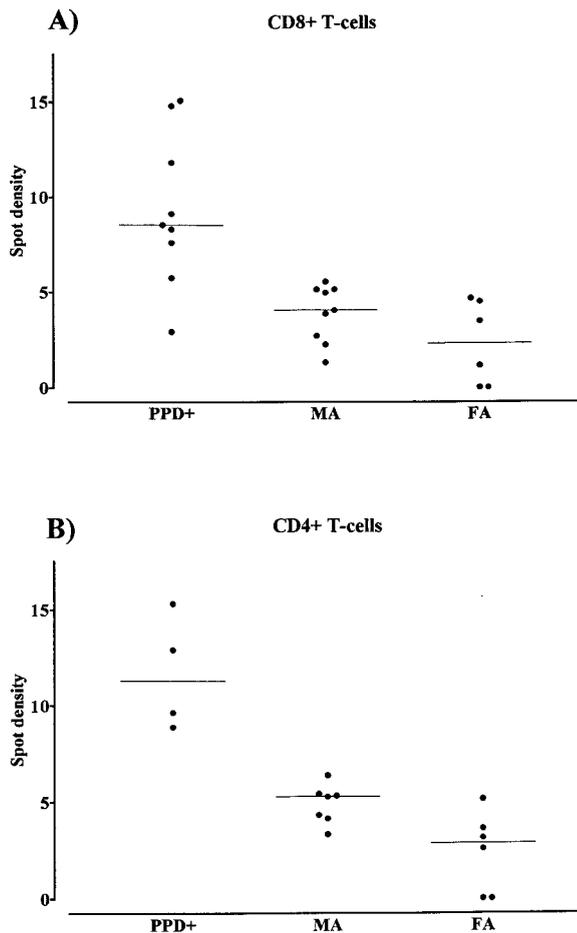
do not produce IFN-γ, such as B cells. In contrast, depletion of CD4⁺ cells completely eliminated IFN-γ-producing cells. These results were confirmed by measurement of IFN-γ levels in supernatants (Fig. 4B). Depletion of CD4⁺ cells may have abolished IFN-γ production by eliminating CD4⁺ monocytes and reducing the number of antigen-presenting cells. However, the percentages of CD4⁺ cells were similar in CD8-depleted and CD4-depleted PBMC (4.4 and 3.4%, respectively). These findings demonstrate that CD4⁺ cells are required for CD8⁺ cells to produce IFN-γ in response to *M. tuberculosis*.

Conclusions. In this study, we found that the proportions of PBMC and CD4⁺ and CD8⁺ cells that produce IFN-γ in response to *M. tuberculosis* were reduced in patients with tuberculosis, particularly in those with far advanced disease. IFN-γ production per CD4⁺ or CD8⁺ cell was also reduced in tuberculosis patients. These results indicate that IFN-γ production by CD8⁺ and CD4⁺ cells correlates with the clinical manifestations of *M. tuberculosis* infection in humans. The proportions of cells producing IFN-γ and the amounts of IFN-γ produced per cell were similar for CD8⁺ and CD4⁺

A)

B)
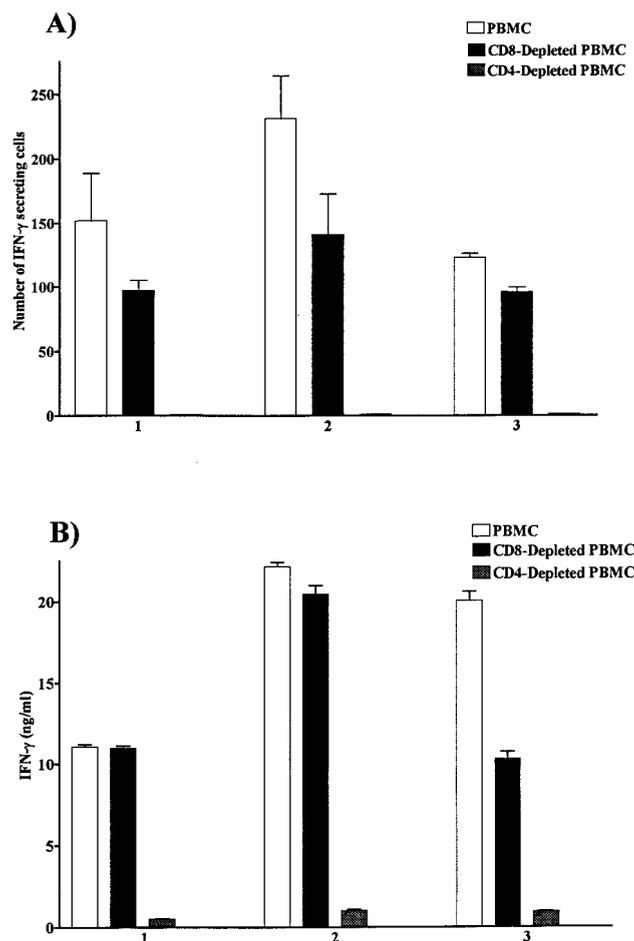

FIG. 4. Effect of depletion of CD8$^+$ or CD4$^+$ cells on IFN-γ production. PBMC, CD8-depleted PBMC, and CD4-depleted PBMC from three healthy tuberculin reactors were cultured with *M. tuberculosis* for 72 h. The frequency of IFN-γ-producing cells per 10^5 cells was determined by ELISPOT (A), and IFN-γ concentrations in supernatants were measured by enzyme-linked immunosorbent assay (B). Mean values and standard errors are shown.

cells, suggesting that they both contribute significantly to IFN-γ production. However, depletion of CD4$^+$ but not CD8$^+$ cells prior to stimulation of PBMC with *M. tuberculosis* completely abolished IFN-γ production, demonstrating that CD4$^+$ cells are required for CD8$^+$ cells to produce IFN-γ.

Many investigators have found that *M. tuberculosis*-induced IFN-γ production by T cells is reduced in tuberculosis patients compared to healthy tuberculin reactors (6, 11, 22, 26), and one study demonstrated that the percentage of *M. tuberculosis*-stimulated CD4$^+$IFN-γ$^+$ cells is decreased in tuberculosis patients (3). Our present report confirms and extends these observations. First, we found that *M. tuberculosis*-induced IFN-γ production paralleled the percentages of CD4$^+$ and CD8$^+$ cells that produce IFN-γ. In addition, we found reduced production of IFN-γ per CD4$^+$ and CD8$^+$ cell in tuberculosis patients, compared to healthy tuberculin reactors. Our findings differ from those of Surcel and colleagues, who reported that the frequency of IFN-γ-producing cells in *M. tuberculosis*-stimulated PBMC of tuberculosis patients was higher than that in

healthy tuberculin reactors (19). Two factors may explain this difference. First, Surcel et al. studied a heterogeneous group of patients with pulmonary and extrapulmonary tuberculosis, whereas we evaluated a more homogeneous population of patients with pulmonary disease. Second, our ELISPOT assay may be more sensitive, as the mean frequency of IFN-γ-producing cells was approximately 10-fold higher in the present study than in the report of Surcel et al.

Although CD4$^+$ T cells clearly play a central role in immunity to *M. tuberculosis*, a growing body of evidence indicates that CD8$^+$ cells also contribute to immune defenses against this pathogen. In murine models, elimination of CD8$^+$ T cells by gene deletion greatly increases susceptibility to tuberculosis (16). CD8$^+$ cells can combat mycobacterial infection by lysing infected cells (13) or by producing IFN-γ (12, 21) or molecules with antimicrobial activity (17, 18). In humans, *M. tuberculosis*-specific CD8$^+$ cytolytic T cells have been isolated from bronchoalveolar lavage fluid, suggesting that they contribute to immune responses in the lung (20). In addition, CD8$^+$ cells that produce IFN-γ and exhibit cytolytic activity are present in the blood of healthy tuberculin reactors and of persons vaccinated with bacillus Calmette-Guérin (8, 9, 14, 24). However, the relative contributions of CD4$^+$ and CD8$^+$ T cells to IFN-γ production in humans have not been previously evaluated. Our present results show that a similar proportion of CD4$^+$ and CD8$^+$ cells produce IFN-γ in response to *M. tuberculosis* and that CD4$^+$ and CD8$^+$ cells produce comparable amounts of IFN-γ per cell, suggesting that CD8$^+$ cells contribute significantly to IFN-γ production in persons infected with *M. tuberculosis*.

Although CD8$^+$ and CD4$^+$ cells were similar in their capacities to produce IFN-γ, depletion of CD4$^+$ cells from PBMC prior to stimulation with *M. tuberculosis* completely abolished IFN-γ production by CD8$^+$ and other cells, whereas depletion of CD8$^+$ cells did not have this effect. This suggests that CD4$^+$ cells are essential for CD8$^+$ cells to produce IFN-γ in response to *M. tuberculosis*. In many experimental systems, CD4$^+$ T cells are required for induction of CD8$^+$ T-cell responses and maintenance of long-term CD8$^+$ memory T cells. This effect may be mediated through CD40-dependent or CD40-independent mechanisms, including interleukin-2 production by CD4$^+$ cells (1, 2, 7). Studies are currently under way in our laboratory to evaluate this interaction between CD4$^+$ and CD8$^+$ T cells.

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Editor: S. H. E. Kaufmann