Sequestration from Immune CD4$^+$ T Cells of Mycobacteria Growing in Human Macrophages

Preeti Pancholi,* Asra Mirza, Nina Bhardwaj, Ralph M. Steinman

CD4$^+$ helper T cells mediate resistance to tuberculosis, presumably by enhancing the antimicrobial activity of macrophages within which the *Mycobacterium tuberculosis* organism grows. A first step in resistance should be the presentation of mycobacterial antigens by macrophages to CD4$^+$ T cells. However, when the antigenic stimulus is limited to organisms growing in human monocytes, the organisms become sequestered from immune CD4$^+$ T cells. This block in presentation is selective for growing mycobacteria and not for other stimuli. Sequestration would allow replicating organisms to persist in infected individuals and may contribute to virulence.

Resistance to tuberculosis is dependent on the function of CD4$^+$ helper lymphocytes because individuals with human immunodeficiency virus (HIV) infection (*1, 2*) and mice that lack CD4$^+$ T cells (*3–5*) are highly susceptible to infection with *M. tuberculosis* and to an attenuated bovine tuberculosis strain, strain Bacille Calmette-Guerin (*Mycobacterium bovis* BCG). Immunocompetent individuals, on the other hand, develop strong CD4$^+$ T cell reactivity to many of the protein components of mycobacteria (*6, 7*). Mycobacteria are among the strongest stimuli of the CD4$^+$ T cell compartment. We find here that when BCG is grown in human monocytes, the organism becomes sequestered from immune CD4$^+$ T cells. The defect in presentation appears to be selective for antigens expressed by growing bacilli.

We used cultured human blood monocytes or macrophages to study antigen presentation by cells infected with growing mycobacteria. Monocytes were exposed to an inoculum containing live *M. bovis* BCG and then were cultured for a total of 2 or 7 days [acutely or chronically infected, respectively (*8*)]. BCG does not grow in standard tissue culture medium but does replicate in monocytes, as we monitored by staining for acid-fast bacilli and by colony counts (Fig. 1). At least 60% of the monocytes contained acid-fast bacilli. Between days 2 and 7 of culture, the number of acid-fast bacilli increased (Fig. 1, A and B), and the number of colony-forming units (CFUs) expanded two to five times (Fig. 1C).

We then determined the capacity of infected macrophages to present mycobacterial antigens to T cells from individuals infected with tuberculosis or those vaccinated with BCG (*9*). These individuals have an expanded repertoire of mycobacterial-specific CD4$^+$ T cells that proliferate when challenged with BCG and antigen-presenting cells in vitro. Acutely infected, day 2 macrophages effectively presented antigens to bulk T cells (Fig. 2A). The average amount of DNA synthesis in eight experiments was 31,907 ± 6,797 cpm for infected cells, as compared with 1,192 ± 303 cpm for uninfected cells. The response to acutely infected macrophages was comparable to that observed with uninfected cells presenting a standard purified protein derivative (PPD) preparation of mycobacterial proteins (36,093 ± 8,729 cpm). In contrast, chronically infected macrophages stimulated T cells poorly (4,299 ± 2,089 cpm, as compared with 803 ± 191 cpm for uninfected monocytes) in 11 experiments (Fig. 2B).

This defect in presentation by chronically infected macrophages was restricted to the parasitized *M. bovis* BCG. Infected day 7 macrophages presented PPD comparably (33,342 ± 10,641 cpm) to uninfected day 2 or day 7 macrophages or to infected day 2 macrophages (Fig. 2). Chronically infected macrophages could also present freshly added *M. bovis* BCG or an acetone extract of mycobacteria to bulk-primed T cells (*10*). Acutely and chronically infected macrophages could also stimulate T cells to a similar extent when presenting a monoclonal antibody (mAb) to CD3 or the superantigen staphylococcal enterotoxin A (SEA) (Table 1). In these assays, where DNA synthesis was measured relatively early (after 3 days of culture), acutely infected macrophages were already stimulating a T cell response, whereas chronically infected macrophages were less effective.

We studied a group of mycobacterial-reactive, CD4$^+$ T cell clones to provide a more sensitive test of the sequestration phenomenon. CD4$^+$ T cell clones were generated from an individual with tuberculosis and an individual vaccinated with

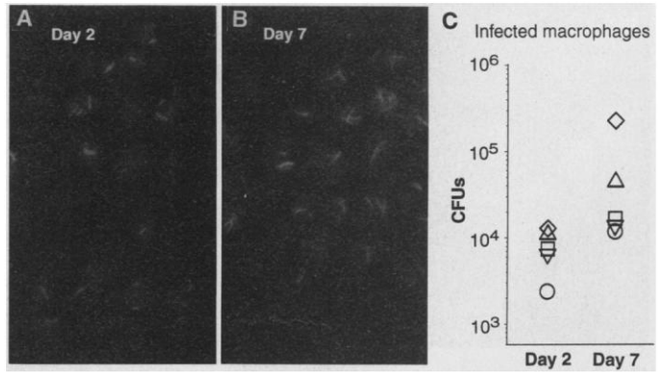

Fig. 1. Progressive growth of BCG in human monocytes. (**A**) Acutely infected day 2 monocytes. Rod shaped, brightly stained acid-fast bacilli (fluorescence auramine-rhodamine) were seen in 70 to 80% of the monocytes. The monocytes (infected and uninfected) can be identified by a faint background stain. (**B**) Chronically infected day 7 macrophages. (**C**) Viable BCG as assessed by the amount of CFUs in acutely and chronically infected monocytes. Each symbol represents a different experiment showing an expansion of CFUs between day 2 and day 7.

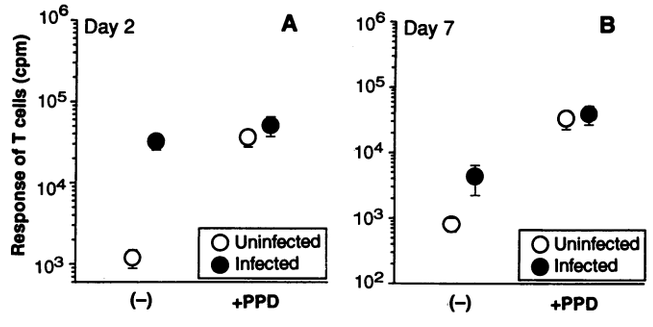

Fig. 2. T cell stimulation by acutely and chronically infected macrophages. Data shown are means ± SE of (**A**) 8 day 2 and (**B**) 11 day 7 experiments. Immune T cells (10^5 cells from five individuals vaccinated with BCG and two infected with tuberculosis) were cocultured with 2.5 × 10^4 autologous macrophages. The latter were not infected or were infected with live BCG (open and closed symbols, respectively) and either were not supplemented (–) or were supplemented with PPD at 10 μg/ml, as indicated. T cell responses were assessed by [^{3}H]thymidine uptake (1 μCi for 12 to 14 hours on the fifth or sixth day of culture). T cells from both individuals with tuberculosis and those vaccinated with BCG behaved similarly in our tests.

Laboratory of Cellular Physiology and Immunology, Rockefeller University, New York, NY 10021.

*To whom correspondence should be addressed.

BCG, as described (*11*). All of the clones were major histocompatibility complex (MHC) class II–restricted and proliferated in response to monocytes infected with *M. bovis* BCG (Fig. 3A, day 2 macrophages). Chronically infected day 7 macrophages [whether autologous or human leukocyte antigen (HLA)–DR matched] presented growing organisms poorly but exogenous PPD effectively (Fig. 3).

Table 1. T cell responses to BCG-infected macrophages. We used acutely and chronically BCG-infected macrophages to stimulate 10^5 autologous T cells from individuals with tuberculosis or those vaccinated with BCG. Infected and uninfected cells were supplemented with no stimulus, with mAb to CD3 (OKT3; 25 ng/ml), or with SEA (15 ng/ml; Toxin Tech, Sarasota, Florida). Values shown ($\times 10^3$ cpm) ± SE are the mean for DNA synthesis in the cultures of five consecutive experiments in which 1 μCi of ^{3}H-labeled thymidine was added for 12 hours at the start of the fourth day of the response.

Stimulus	Day 2 macrophages		Day 7 macrophages	
	Uninfected	Infected	Uninfected	Infected
None	1.0 ± 0.2	13.3 ± 5.4	0.9 ± 0.3	2.9 ± 1.1
OKT3	34.8 ± 9.5	41.6 ± 9.0	45.9 ± 10.7	55.9 ± 9.3
SEA	144.6 ± 10.5	166.4 ± 19.8	137.3 ± 8.1	151.8 ± 13.6

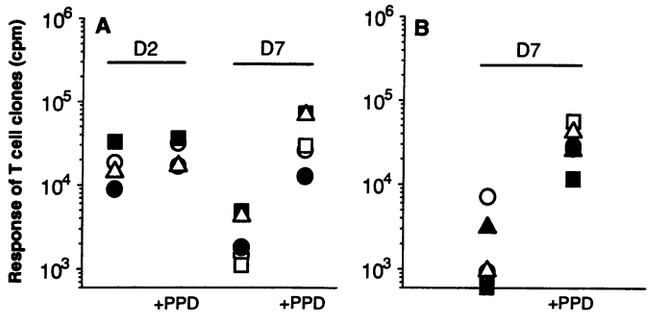

Fig. 3. Stimulation of T cell clones by acutely and chronically infected macrophages. The experiments were set up in the same way as in Fig. 2, except that the T cells were clonal populations (*11*) and were added at a concentration of 2×10^4 per well. The antigens recognized by our panel of clones were evaluated with *M. tuberculosis* Erdman fractions. Some clones respond to the culture filtrate fraction (secreted protein) and others to the cell wall (*10*). (**A**) Each symbol is the response of a different clone to an MHC-matched donor. (**B**) The symbols are different clones responding to autologous macrophages from an individual with tuberculosis. Background stimulation of individual clones in the presence of uninfected MHC-matched macrophages was in the range of 100 to 500 cpm. D2, day 2; D7, day 7.

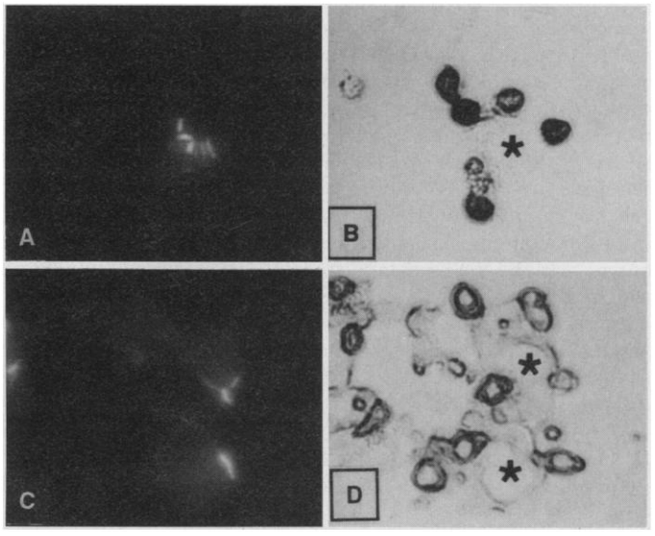

Fig. 4. Efficient presentation of PPD by macrophages chronically infected with BCG. Mycobacterial-reactive T cell clones or nonreactive clones used as a control were added for 3 days to HLA-matched monocytes on chamber slides (Lab Tek; Nunc) (*8*). Some of the monocytes had been chronically infected with BCG. We added exogenous PPD and assessed if mycobacterial-specific T cell clones could bind and respond to infected cells. We monitored T cell responsiveness by labeling the monolayers with mAb Ki-67 (Amac; Westbrook, Maine) to an S phase nuclear antigen. The antibody was visualized with an antibody to immunoglobulin conjugated to peroxidase, and then the specimens were stained with the auramine-rhodamine reagent for acid-fast bacilli. In two representative fields (from five experiments), the acid-fast bacilli are the bright or fluorescent rods (**A** and **C**). The Ki-67 mAb–stained nuclei (**B** and **D**) are the dark spots in the otherwise colorless macrophages (marked by asterisks).

To verify that exogenous PPD was being presented by infected macrophages, rather than by the minority of uninfected cells in the monolayer, we used double-labeling assays in which the T cell clones could be directly shown to be responding to infected monocytes. After 3 days of macrophage–T cell coculture, the monolayers were fixed, stained with the Ki-67 mAb to antigens expressed in the DNA replication period (S phase) of the cell cycle (to identify proliferating T cells), and then stained with an immunoperoxidase stain and an acid-fast stain. Most of the acutely infected monocytes were associated with cloned T cells. However, binding of mycobacterial-reactive T cells to chronically infected macrophages was seen only in the presence of exogenous PPD. Many of these T cells were in S phase of cell cycle (Fig. 4). The T cell clones that were not mycobacterial-specific did not bind to infected or uninfected macrophage monolayers, with or without PPD (*10*). Therefore, the block in antigen presentation by chronically infected macrophages applies selectively to the BCG organisms and not to exogenous PPD. With the use of a similar approach, it was also evident that chronically infected macrophages could present superantigen and stimulate DNA synthesis in bound T cells (*10*).

Mononuclear phagocytes have conflicting roles in tuberculosis. Phagocytes are critical for containing the infection in cell collections called granulomas (*12*), and yet they are hosts that permit mycobacterial growth and most likely persistence. Mycobacteria can persist in infected individuals for years until an insult, such as steroid treatment or HIV-1 infection, results in active tuberculosis. These contrasting roles of macrophages may at least in part be mediated by differences in antigen presentation. We hypothesize that acute infection of blood-derived monocytes allows $CD4^+$ T cells to be engaged but that chronic infection can lead to sequestration of the infected cell from immune T cells, even though mycobacteria have often been used as an effective model for studying antigen presentation (*7, 13*). Our rationale for studying chronically infected macrophages was to restrict the antigenic load to mycobacteria growing within the cell, as opposed to more short-lived antigens (dead organisms, proteins, or peptides) that are likely part of the standard inocula of "live" BCG.

The mycobacterial sequestration observed differs from the phenomena of anergy and immune suppression described in mycobacterial infections (*14*). Such findings have been attributed to the compartmentalization or trapping of sensitized lymphocytes (*15*), the production of immuno-

suppressive products by both monocytes and mycobacteria (*16*), and the generation of suppressor T cells (*17*). In the system we describe, the chronically infected macrophages are not suppressive and express normal amounts of class II HLA-DR,DP,DQ (*10*) but do not present viable growing organisms. One mechanism could be that mycobacteria in the cell stop secreting proteins that act as strong antigens for T cells. This seems unlikely because our T cell clones respond to antigens in both the cell wall and culture filtrate (legend to Fig. 3). Alternatively, the presentation of antigens from the vacuoles containing growing mycobacteria could selectively be blocked. Acidification of vacuoles containing living virulent mycobacteria can be impaired (*18*), and this could block presentation on MHC class II products (*19*). Presentation defects could apply to other organisms that grow intracellularly and that alter phagosome-lysosome fusion and acidification (*20, 21*). The persistence of mycobacteria in macrophages has often been correlated with pathogenesis (*22*). Our findings point to another mechanism of persistence, the capacity of a growing organism to block the presentation of its own antigens.

REFERENCES AND NOTES

1. P. F. Barnes, A. B. Bloch, P. T. Davidson, D. E. Snider, Jr., *N. Engl. J. Med.* **324**, 1644 (1991).
2. C. L. Daley *et al.*, *ibid.* **326**, 231 (1992).
3. T. Pedrazzini, K. Hug, J. A. Louis, *J. Immunol.* **139**, 2032 (1987).
4. C. Leveton *et al.*, *Infect. Immun.* **57**, 390 (1989).
5. A. A. Izzo and R. J. North, *J. Exp. Med.* **176**, 581 (1992).
6. J. R. Lamb *et al.*, *Rev. Infect. Dis.* **11**, S443 (1989).
7. G. A. W. Rook, J. Steele, S. Barnass, J. Mace, J. L. Stanford, *Clin. Exp. Immunol.* **63**, 105 (1986).
8. Monocytes were isolated from Ficoll-Hypaque–selected blood mononuclear cells by adherence to plastic. The culture medium in all experiments was RPMI 1640 plus 5% heat-inactivated human serum and gentamicin (20 μg/ml). The adherent monolayer was incubated with live *M. bovis* BCG (five bacteria per cell; Trudeau Institute, Saranac Lake, NY) for 16 to 18 hours. Then we dislodged the monocytes by pipetting them, washed them free of nonbound organisms by centrifugation at 150*g* at 4°C, and cultured them an additional day (day 2, acutely infected) or 6 days (day 7, chronically infected). To stimulate DNA synthesis in T cells, we added infected macrophages (2.5×10^4) to 96-well, flat-bottomed microtest trays (Costar, Cambridge, MA) and washed the cells three times with RPMI 1640. For cytologic studies, we plated the macrophages (2×10^4 to 5×10^4) in eight-well chamber slides (Permanox Lab Tek; Nunc, Naperville, IL). Slides were air-dried before fixation in 10% formalin–phosphate-buffered saline. Mycobacteria were visualized with the fluorescent auramine-rhodamine stain (*11*).
9. We enriched T cells from individuals infected with tuberculosis or vaccinated with BCG from mononuclear cells by the formation of rosettes with neuraminidase-treated sheep erythrocytes (E rosette positive; Er^+), followed by reaction with immunoglobulin-coated dishes to deplete residual monocytes (*23*). In some cases, we enriched the $CD4^+$ subset by removing cells expressing CD8 and MHC class II molecules [B cells, monocytes, and dendritic cells (*23*)]. T cells from both individuals with tuberculosis and those vaccinated with BCG behaved similarly in our tests.
10. P. Pancholi, A. Mirza, N. Bhardwaj, R. M. Steinman, unpublished data.
11. P. Pancholi, R. M. Steinman, N. Bhardwaj, *Immunology* **76**, 217 (1992).
12. V. Kindler, A.-P. Sappino, G. E. Grau, P.-F. Piguet, P. Vassalli, *Cell* **56**, 731 (1989).
13. V. Bhardwaj and M. J. Colston, *Eur. J. Immunol.* **18**, 691 (1988).
14. P. H. Lagrange and B. Hurtrel, in Mycobacterium tuberculosis: *Interactions with the Immune System*, M. Bendinelli and H. Friedman, Eds. (Plenum, New York, 1988), pp. 171–205.
15. G. M. Bahr, G. A. W. Rook, J. L. Stanford, *Immunology* **44**, 593 (1981).
16. H. Fujiwara, K. Ohnishi, S. Kishimoto, J. J. Ellner, I. Tsuyuguchi, *ibid.* **72**, 194 (1991); J. J. Ellner and R. S. Wallis, *Rev. Infect. Dis.* **11**, S455 (1989); J. J. Ellner, *J. Immunol.* **121**, 2573 (1978).
17. R. Turcotte, *Infect. Immun.* **34**, 315 (1981); R. M. Nakamura and T. Tokunaga, in Mycobacterium tuberculosis: *Interactions with the Immune System*, M. Bendinelli and H. Friedman, Eds. (Plenum, New York, 1988), pp. 227–241.
18. A. J. Crowle, R. Dahl, E. Ross, M. H. May, *Infect. Immun.* **59**, 1823 (1991).
19. F. M. Brodsky and L. E. Guagliardi, *Annu. Rev. Immunol.* **9**, 707 (1991).
20. M. A. Horwitz and F. R. Maxfield, *J. Cell Biol.* **99**, 1936 (1984).
21. L. D. Sibley, E. Weidner, J. L. Krabenbuhl, *Nature* **315**, 416 (1985).
22. A. M. Dannenberg, Jr., in *The Mycobacteria: A Sourcebook*, G. P. Kubica and L. G. Wayne, Eds. (Dekker, New York, 1984), pp. 721–760.
23. P. Pancholi, R. M. Steinman, N. Bhardwaj, *Clin. Exp. Immunol.* **85**, 349 (1991).

24. We thank P. Brennan for *M. tuberculosis* Erdman fractions and V. Schauf, W. Rom, the staff of the Rockefeller University Hospital, and J. Adams. Supported by grants AI24775 (to R.M.S.) and AR39552 (to N.B.) from the NIH, the New York Arthritis Chapter, a Heiser Fellowship (to P.P.), the Kaskel Fund, the Llewellyn Burchell Charitable Trust, and Clinical Research Center grant MOI-RR00102.

23 December 1992; accepted 26 February 1993

CD19 of B Cells as a Surrogate Kinase Insert Region to Bind Phosphatidylinositol 3-Kinase

David A. Tuveson, Robert H. Carter, Stephen P. Soltoff, Douglas T. Fearon

Antigen receptors on B and T lymphocytes transduce signals by activating nonreceptor protein tyrosine kinases (PTKs). A family of receptor PTKs contains kinase insert regions with the sequence tyrosine-X-X-methionine (where X is any amino acid) that when phosphorylated mediate the binding and activation of phosphatidylinositol 3-kinase (PI 3-kinase). The CD19 membrane protein of B cells enhances activation through membrane immunoglobulin M (mIgM) and was found to contain a functional analog of the kinase insert region. Ligation of mIgM induced phosphorylation of CD19 and association with PI 3-kinase. Thus, CD19 serves as a surrogate kinase insert region for mIgM by providing the means for PI 3-kinase activation by nonreceptor PTKs.

The response of B lymphocytes to antigens is mediated by receptors that induce the proliferation and differentiation of these cells. The paradigm of signal transduction through antigen receptors is that of certain receptor PTKs (*1*), such as the platelet-derived growth factor (PDGF) receptor, that have two regions essential for inducing cell growth: the PTK domains that mediate intra- and intermolecular phosphorylation of tyrosines and the kinase insert regions in which autophosphorylated tyrosines that have the motif Tyr-X-X-Met (where X is any amino acid) mediate the binding and activation of PI 3-kinase (*2, 3*), an enzyme that phosphorylates phosphatidylinositol (PI) in the position of the inositol ring. One of the subunits of PI 3-kinase, p85, contains two Src homology-2 domains specific for phosphotyrosines in the Tyr-X-X-Met motif (*3, 4*). Mutation of the tyrosines in the two Tyr-X-X-Met motifs in the kinase insert region of the PDGF receptor eliminates the binding and activation of PI 3-kinase and the growth-inducing activity of the receptor (*3*).

Antigen receptors lack these functional cytoplasmic domains of the receptor PTKs but are coupled indirectly to nonreceptor PTKs (*5*) through association with other membrane proteins that share conserved cytoplasmic motifs (*6*). Nonreceptor PTKs have been suggested to interact with PI 3-kinase (*7, 8*), but members of the Src family lack phosphotyrosines with a Tyr-X-X-Met motif, and the interaction may involve other proteins (*9*).

CD19 is a B cell–specific membrane protein that is 95 kD (*10*) and that when coligated with mIgM reduces the number of antigen receptors that must be ligated to activate B cells by two orders of magnitude (*11*) and that also associates with comple-

D. A. Tuveson, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
R. H. Carter, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
S. P. Soltoff, Division of Signal Transduction, Department of Medicine, Beth Israel Hospital, Harvard Medical School, Boston, MA 02115.
D. T. Fearon, Department of Medicine and Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.