Selective Expansion of Human γΔ T Cells by Monocytes Infected with Live *Mycobacterium Tuberculosis*

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Abstract

Gamma delta (γδ) T cell receptor (TCR) expressing T cells comprise 3% of human peripheral blood lymphocytes, yet their role in the immune response remains largely unknown. There is evidence both in humans and in animal models that these cells participate in the immune response to mycobacterial antigens. In mice, exposure to mycobacterial antigens leads to the expansion of γδ T cells in draining lymph nodes and lungs. In humans, γδ T cell lines with reactivity to mycobacterial antigens have been derived from synovial fluid of a rheumatoid arthritis patient, skin lesions of leprosy patients, and peripheral blood of a healthy tuberculin reactor. Very little is known, however, about the factors which induce human γδ T cells to expand. In studies comparing the human T cell response to live and heat-killed *Myobacterium tuberculosis* (MT), we have found that monocytes infected with live MT are very effective inducers of human γδ T cell expansion. After 7 d of exposure to live MT, γδ T cells were greatly increased in all healthy tuberculin reactors (PPD+) tested and frequently were the predominant T cell population. In contrast, heat-killed MT or purified protein products of MT induced a CD4+, αβTCR+ T cell response with very little increase in γδ T cells. Furthermore, a similar selective induction of γδ T cells was observed when monocytes infected with live *Salmonella* were used to stimulate T cells. Heat-killed *Salmonella,* like heat-killed MT, induced a predominantly CD4+ αβTCR+ T cell response. These findings suggest that human γδ T cells are a major reactive T cell population during the early stages of infection with living intracellular bacteria and are therefore likely to exert an important role in the initial interaction between host and parasite. (*J. Clin. Invest.* 1991. 87:729–733.) Key words: γδ T cell • monocyte • *Mycobacterium tuberculosis*

Introduction

Tuberculosis in humans is thought to result from either failure to contain mycobacteria within a primary complex of mononuclear phagocytes and recruited T cells, resulting in progressive primary disease, or from breakdown of a primary granuloma many years later, leading to reactivation-tuberculosis (1). Most individuals upon exposure to *Mycobacterium tuberculosis* (MT)[1] successfully contain the primary infection and go on to develop a vigorous delayed type hypersensitivity response (DTH) to mycobacterial antigens. Traditionally, studies of the immune response to mycobacteria have relied on dead bacteria or purified products of MT (proteins, glycolipids, polysaccharides) as reagents. Few studies have looked directly at the interaction of human T cells with mononuclear phagocytes infected with live MT. Our interest in the response of human T cells to live mycobacteria stemmed from the observation in a murine model, that protective immunity could be conferred in adoptive transfer experiments by lymphocytes from mice immunized with live mycobacteria. In contrast, only DTH was transferred by lymphocytes from animals immunized with killed organisms (2). Furthermore, recent studies have provided evidence that γδ T-cell receptor (TCR) expressing T cells participate in the immune response to mycobacteria (3, 4). γδ T cells comprise 1–5% of peripheral blood T lymphocytes and carry the CD4−, CD8− phenotype, in contrast to αβ T cell receptor (TCR) expressing T cells which make up the majority of peripheral blood T cells and are either CD4+ (helper/inducer) or CD8+ (cytotoxic/suppressor) (5). In this series of experiments, we have compared the human T cell response to live and heat-killed MT. We have found that monocytes infected with live MT were very effective inducers of human γδ T cell expansion, whereas heat-killed MT efficiently expanded CD4+, αβTCR+ T cells. γδ T cells may therefore exert an important role in the initial immune response to mononuclear phagocytes infected with living intracellular bacteria.

Methods

Bacterial strains and antigens. M. tuberculosis H37Ra (MT) was cultured in Middlebrook 7H9 broth for 3–4 wk and frozen at −70°C. Bacterial counts were performed by light microscopy and viability was determined by counting colony forming units (CFU) both before and after freezing. Lots with > 50% viability after freezing were used for experiments and aliquots of these lots represent the live MT. Heat-killed (dead) MT was prepared by autoclaving live MT for 20 min. Purified protein derivative (PPD) of *M. tuberculosis* was a gift from Lederle Laboratories (Pearl River, NY). Purified recombinant 64 kD

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1. *Abbreviations used in this paper:* MT, *Mycobacterium tuberculosis;* PHS, pooled human serum; PPD, purified protein derivative of *M. tuberculosis;* γδ T cell, gamma/delta T cell receptor–expressing T cell; TCR, T cell receptor.

antigen of *M. bovis* BCG was provided by Dr. Jan D. A. van Embden (Bilthoven, The Netherlands) through the UNDP/World Bank WHO Special Programme for Research and Training in Tropical Diseases (6). *Salmonella typhimurium* (wild-type TN1379) was kindly provided by Dr. Charles G. Miller (Case Western Reserve University). Live *Salmonella* was an overnight log-phase culture and heat-killed *Salmonella* was prepared by autoclaving the live organisms for 20 min.

Monocyte isolation and infection. Blood mononuclear cells from healthy tuberculin skin test reactors (PPD+) were obtained by density sedimentation over sodium diatrizoate/hypaque gradients and placed on plastic tissue culture dishes (Falcon 3003) precoated with pooled human serum (PHS) for 15 min at 37°C. After 1 h culture at 37°C in RPMI 1640 with 10% PHS, nonadherent cells were removed. After 20 min at 4°C in PBS, plastic adherent cells (>90% monocytes) were collected by scraping with a rubber policeman, washed, and counted. Enriched adherent cells (monocytes) were incubated with live or heat-killed *M. tuberculosis* H37Ra at a cell to mycobacteria ratio of 1:20 at 37°C for 1 h in RPMI supplemented with 5% PHS. Both MT preparations were sonicated for 20 s to disrupt clumps. In each experiment, live and heat-killed MT came from the same lot of live *M. tuberculosis* H37Ra. After infection, monocytes were separated from free bacteria by centrifugation and washing. For experiments with *S. typhimurium*, monocytes were incubated with live or heat-killed bacteria at a 1:2 cell to bacteria ratio. Streptomycin (100 μg/ml) was added to *Salmonella* infected monocytes 1–2 h after phagocytosis to prevent bacteria overgrowth. Phagocytosis was confirmed by light microscopy of preparations of infected cells (50–75% of monocytes contained bacteria). No differences were observed in the number of acid-fast bacilli associated with monocytes after incubation with either live or dead mycobacteria.

T cell stimulation. Nonadherent cells (10^6/well), as source of peripheral blood T cells, and treated (see above) autologous monocytes (0.5×10^6/well) were cocultured in 24-well plates (Costar, Cambridge, MA) in 2 ml of complete medium (RPMI, 10% PHS, 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin) for 7–9 d before measuring T cell phenotype. Growth indices were determined by counting viable cells per well (obtained by purification over sodium diatrizoate/Ficoll and Trypan blue exclusion) from stimulated cultures and dividing by the number of viable cells in unstimulated cultures after 7 d.

Phenotypic analysis. After 7–9 d of coculture, viable cells were harvested and analyzed by indirect immunofluorescence. Cells were stained with a saturating amount of primary monoclonal antibody for 30 min followed by FITC-conjugated goat anti-mouse IgG (Cappell Laboratories, Cochranville, PA). Monoclonal antibodies used were OKT3 (anti-CD3, IgG2a), OKT4 (anti-CD4, IgG2b), OKT8 (anti-CD8, IgG2a) (Ortho Diagnostics, Raritan, NJ), WT-31 (specific for a framework determinant on the αβTCR, IgG1; Becton-Dickinson & Co., Paramus, NJ) (7), and TCR-δ1 (specific for a framework determinant on the δ chain of the γδTCR, IgG1; kindly provided by Dr. Michael B. Brenner) (8).

Cells were analyzed by fluorescence-activated cell sorting (FACS) on a Cytofluorograph IIs (Becton-Dickinson & Co.) using the 488-nM argon line at 250 mW, Hepes/citrate saline sheath (pH 7.4) and a filter combination providing 530/22 band pass transmitting 58% of the fluorescent light at 530 nM. The cytometer was calibrated for both light scatter and immunofluorescence with immunobrite beads (Coulter Co., Cedar Ridge, CA). Cells were gated on a two-parameter plate of 90° versus forward angle scatter. The gate for viable cells was set widely to include small as well as larger lymphocytes and to avoid bias towards a particular subpopulation. The gate was kept constant for each experiment. 10,000 gated events were recorded for each cell surface marker. Fluorescence signals were amplified over a three-decade range. Markers were set visually and kept constant throughout the analysis of each experimental group. The position of the cut-off marker was determined by the distribution of cells stained with FITC-conjugated goat anti-mouse antibody alone. Populations to the left were considered negative and to the right positive for a cell-surface marker. The percentage reported for a given cell-surface marker represents the proportion of gated cells with a fluorescence signal greater than those stained with second antibody alone.

Proliferation assay. Nonadherent PBMC (10^5) were cocultured in microtiter wells in 200 μl of complete medium with 5×10^4 pretreated monocytes for 96 h. Cells were pulsed with [^{3}H]-thymidine and harvested 18 h later onto glass fiber filters. [^{3}H]-Thymidine incorporation was measured by liquid scintillation counting and expressed as the mean counts per minute of triplicate cultures.

Cytokine production. Supernatants (50 μl) from nonadherent cells cocultured with pretreated monocytes were harvested after 24 h for interleukin-2 (IL-2) and after 48 h for interferon-gamma (IFN-γ) determinations. Generally supernatants were assayed at 50% vol/vol unless cytokine activity exceeded the maximum value of the standard curves. IL-2 was measured by CTLL-2 proliferation with recombinant IL-2 (Hoffman LaRoche, Nutley, NJ) as standard (9). IFN-γ was measured by solid-phase radioimmunoassay (Centocor Inc., Malvern, PA) (10).

Results

We compared the response of human peripheral blood T cell subpopulations from healthy PPD+ persons to monocytes either infected with live MT or having ingested dead mycobacteria. T cells were stained with antibodies specific for CD3, CD4, CD8, αβTCR, and γδTCR before stimulation (baseline) and after 7–9 d of stimulation. αβTCR bearing T cells were stained with WT31 and γδTCR bearing T cells were detected with TCR-δ1. In all PPD+ individuals (5/5) tested, a marked increase in percentage of γδ T cells was noted in live MT-stimulated peripheral blood T cells compared to killed MT or PPD-stimulated T cells. A representative flow-cytometry profile is shown in Fig. 1, in which γδ T cells comprise the largest T cell population (47.6%), with a relative decrease in CD4+ (15.4%) and αβTCR+ (26.2%) T cells.

Dead MT stimulation resulted in a predominantly CD4+ αβTCR+ T cell expansion, which was similar to stimulation with PPD (data not shown); the percentage of γδTCR+ T cells

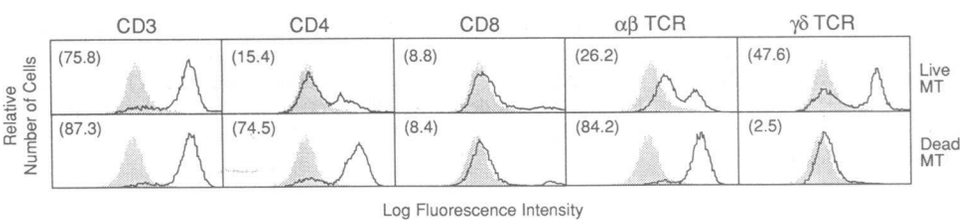

Figure 1. Phenotypic analysis by FACS of T cell subpopulations from a healthy tuberculin reactor (PPD+) after 7 d of stimulation with monocytes infected with live MT or dead MT. Log fluorescence intensity (*x*-axis as a function of relative cell number (*y*-axis) is shown for test antibodies (*solid line*) superimposed on background fluorescence (*stippled area*). Percentage of positive staining cells is shown in the upper left corner of each plot. Unstimulated cultures were 76.8% CD3+, 51.6% CD4+, 14.9% CD8+, 68.5% αβTCR+, and 0% γδTCR+. Freshly isolated peripheral blood mononuclear cells (PBMC) contained 1.6% γδ T cells.

Table I. Surface Phenotype of T Cells from PPD+ Donors after Stimulation with Live MT, Dead MT, or PPD

	CD3	CD4	CD8	αβTCR	γδTCR	Growth index
No antigen	76.0	49.8	19.3	71.8	3.0	1.0
	(55.0–87.6)	(21.1–67.6)	(14.9–24.0)	(66.5–78.9)	(0.0–4.7)	
Live *M. tuberculosis*	81.8	20.7	10.3	19.5	48.1	10.3
	(76.0–88.3)	(12.3–51.5)	(8.8–13.7)	(9.9–26.0)	(10.4–73.3)	(5.8–18.0)
Dead *M. tuberculosis*	74.6	52.5	12.4	53.8	8.1	6.2
	(40.6–87.6)	(21.3–74.5)	(6.1–23.9)	(39.8–90.9)	(3.3–19.4)	(5.0–7.5)
PPD	74.6	67.1	9.8	76.7	6.5	6.2
	(65.9–82.3)	(37.9–73.5)	(96.9–12.3)	(63.3–90.1)	(0.6–23.9)	(2.7–8.2)

Cultures of nonadherent PBMC with monocytes exposed to no mycobacteria, live MT, dead MT, or PPD (10 μg/ml) and cell surface phenotype were performed as described in Methods. The values represent the mean percentage and range (in parentheses) of each T cell surface marker for five PPD+ individuals. Baseline phenotype of the nonadherent cells was: 54.0 (46.0–60.7)% CD3+, 23.3 (11.6–35.1)% CD4+, 19.9 (15.3–24.8)% CD8+, 34.8 (21.9–41.3)% αβTCR+, and 5.0 (1.6–9.8)% γδTCR+. Growth indices were determined by counting viable cells per well from stimulated cultures and dividing by the number of viable cells in unstimulated cultures after 7 d of culture.

(2.5%) was not significantly increased above those found in freshly isolated (1.6%) or cultured, unstimulated T cells (0%).

A summary of the results from the five PPD+ individuals is shown in Table I. There was no difference in the relative number of CD8+ T cells between the stimulated and unstimulated T cell populations, suggesting that infection of monocytes with live MT does not shunt antigens preferentially into the antigen processing pathway for class I MHC restricted suppressor/cytotoxic T cells (11, 12). There appeared to be no correlation between the starting percentage of γδ T cells (1.6–9.8%) and the magnitude of the γδ T cell expansion with live MT. Dead MT induced a significant γδ T cell expansion in some PPD+ individuals (8.1%, range 3.3–19.4%), compared to unstimulated cultures (3.0%, range 0–4.7%). This finding confirms the recent report that human γδ T cells can be expanded by dead MT (13). However, the magnitude was always much smaller than the response seen with live MT (Table I). Growth indices (viable cells in stimulated cultures/viable cells in unstimulated cultures) (Table I) and parallel proliferation assays (Table II) confirmed that the increase of γδ T cells (48.1%; range, 10.4–73.3) was due to cell growth and not to selective depletion of CD4+ αβTCR+ T cells by live MT. No significant differences in IL-2 and interferon-γ (IFN-γ) were noted during the early stages of live and dead MT stimulation of T cells (Table II).

To evaluate the specificity of this γδ T cell expansion, we tested the ability of monocytes infected with *Salmonella typhimurium*, another intracellular bacterial pathogen, to induce γδ T cell proliferation. Using the same experimental method, expansion of γδ T cells (29.2%) was reproducibly found in response to live *Salmonella*, in one of five persons tested. A representative experiment is shown in Fig. 2. As seen with MT, live bacteria were again able to induce γδ T cells. These experiments suggest that the γδ T cell expansion by live bacteria is specific for live MT in some individuals but that this expansion is not unique to mycobacteria. The interaction of monocytes and live intracellular bacteria apparently results in signals which preferentially induce γδ T cells to proliferate. Prior priming with antigens may be one explanation for the greater consistency of the γδ T cell expansion to *M. tuberculosis* as compared to the response to *Salmonella*. We tested three healthy PPD-negative individuals. Live MT induced a γδ T cell response in two persons (7.1 and 49.0%). Both of them, despite negative tuberculin skin tests, had a proliferative response to mycobacterial antigens suggesting some degree of sensitization to mycobacterial or cross-reactive bacterial antigens (14).

It has been proposed that γδ T cells may be uniquely responsive to heat-shock proteins of both pro- and eukaryotic origin (15–19). To address the possibility that the live MT-induced γδ T cell response was due to the enhanced expression of heat-shock proteins, two approaches were used. First, monocytes were stimulated for 3 d with 1,000 U/ml of IFN-γ before adding a responding T cell population (in the presence or absence of mycobacterial antigens) (19). No γδ T cell induction was seen (Table III). Second, T cells from a PPD+ person, known to be responsive to the 65-kD heat-shock protein of *M. bovis*-BCG (6), were stimulated with monocytes pulsed with purified recombinant 65 kD. Again no γδ T cell proliferation was observed. Furthermore, as noted above, stressing monocytes with live *Salmonella* did not induce a γδ T cell response in four out of five persons tested. These results suggest that the γδ T cell expansion by live bacteria may not result from the induction of heat-shock proteins. Alternatively, infection of monocytes with live bacteria results in the induction of unique

Table II. Proliferative and Cytokine Response of T Cells to Monocytes Infected with Either Live or Dead Mycobacterium tuberculosis

	Proliferation		IL-2		IFN-γ	
	Live MT	Dead MT	Live MT	Dead MT	Live MT	Dead MT
	cpm		*U/ml*			
subject 1	62820	44728	0.7	0.5	444	140
subject 2	80690	92296	4.0	4.5	192	140
subject 3	82926	87189	<0.01	<0.01	192	136

Nonadherent PBMC (10^5) from three PPD+ individuals were cultured in 96-well plates in 200 μl of complete medium with 5×10^4 monocytes pretreated with live MT, dead MT, or no mycobacteria. Supernatants (50 μl) were harvested after 24 h for IL-2 and after 48 h for IFN-γ determination. Cultures of nonadherent cells and monocytes without bacteria had 1,790, 532, and 2,889 cpm, respectively, and produced no detectable IL-2 and IFN-γ.

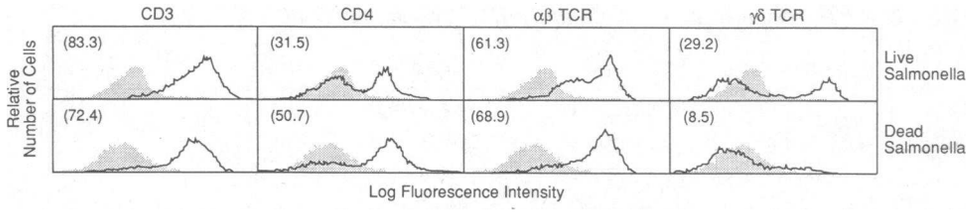

Figure 2. Phenotypic analysis by FACS of T cell subpopulations after 7 d of stimulation with monocytes infected with either live or dead *Salmonella typhimurium.* Log fluorescence intensity (*x*-axis) as a function of relative cell number (*y*-axis) of test antibodies (*solid line*) superimposed on background fluorescence (*stippled area*) is shown. The percentage of positive cells is shown in the upper left corner of each plot. Unstimulated cultures contained 4.3% γδ T cells. In this PPD+ individual, the γδTCR+ T cells in live MT-stimulated cultures were 25.7%.

HSP of either host or bacterial origin which are recognized preferentially by γδ T cells and for which there is variable induction or responsiveness among individuals.

Discussion

T cells are thought to play a critical role in the protective immune response to *M. tuberculosis.* Most studies have focused on the role of CD4+ αβ TCR+ helper T cells in regulating the immune response which follows infection with mycobacteria, with the mononuclear phagocytes as the major effector cell in controlling mycobacteria growth. Although reactivity by γδ T cells to mycobacterial antigens can be demonstrated, their role in mycobacterial immunity remains unclear. It is known that they are capable of secreting cytokines (IL-2, IFN-γ, lymphotoxin) and can function as cytotoxic cells in a major histocompatability complex (MHC) unrestricted manner (20, 21). Cytotoxicity for infected target cells may be one way in which γδ T cells contribute to the immune response to intracellular bacterial pathogens. Very few antigens recognized by γδ T cells have been characterized and the molecules used for antigen presentation to them are still unknown (16–19). Although significant reactivity to the 65-kD mycobacterial heat-shock protein has been demonstrated in murine studies (22), human γδ T cells have not demonstrated the same degree of responsiveness (13). In addition, very little is known about the factors which lead to the induction of human γδ T cells.

Our findings indicate that monocytes infected with live bacteria provide a strong signal for the activation and expansion of human γδ T cells, in contrast to monocytes which have phagocytosed heat-killed organisms or have been exposed to purified protein antigens from these organisms. The importance of this observation is that after infection, in vivo, intracellular bacteria such as MT reside within mononuclear phagocytes, and the T cell response which follows infection is directed toward these living bacteria and their products. The expansion of γδ T cells by live MT and *Salmonella* suggests, therefore, that γδ T cells have an important role in the early immune response to live intracellular bacteria. The mechanism by which live intracellular bacteria induce γδ T cells remains unclear. Possibilities include the expression of unique bacterial antigens or super-antigens of γδ T cells by cells infected with live bacteria, and the secretion of unique growth factors (cytokines) for γδ T cells by infected cells. Additional possibilities include the expression of host antigens (such as HSP) as the result of intracellular infection, which are preferentially recognized by γδ T cells and the enhanced expression of antigen-presenting molecules for γδ T cells by infected macrophages. Further studies will address these questions and help define the antigenic specificities and functions of γδ T cells in the protective immune response against intracellular pathogens such as mycobacteria and *Salmonella.* The observation that live mycobacteria expand γδ T cells provides a direct approach to their definitive characterization.

Table 3. Surface Phenotype of T Cells, 7 d after Stimulation with 65 kD Antigen of M. bovis BCG and IFN-γ Pretreated Monocytes

	CD3	CD4	CD8	γδTCR
Live *M. tuberculosis*	68.8	37.0	15.4	17.0
65 kD	74.4	53.6	16.3	0.7
IFN-γ	68.1	58.9	15.1	0.6
IFN-γ + PPD	70.5	49.4	18.1	0.0

Fresh nonadherent PBMC from a PPD+ individual were incubated either with monocytes infected with live MT, monocytes pulsed with 10 μg/ml of purified recombinant 65-kD antigen of *M. bovis* BCG, or monocytes pretreated with IFN-γ (1,000 U/ml) for 3 d. The IFN-γ-treated monocytes were isolated from the same donor 3 d earlier and washed three times before use in the assay. One group of IFN-γ-treated monocytes was pulsed with PPD (10 μg/ml) upon addition to the fresh nonadherent cells. After 7 d, T cells were harvested, stained, and analyzed by FACS. Unstimulated T cells after 7 d of culture were 79.8% CD3+, 57.9% CD4+, 17.4% CD8+, and 0% γδTCR+.

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