

Naive Human T Cells Develop into Th1 Effectors after Stimulation with *Mycobacterium tuberculosis*-Infected Macrophages or Recombinant Ag85 Proteins

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Received 9 February 2000/Returned for modification 5 April 2000/Accepted 30 August 2000

Most studies of human T-cell responses in tuberculosis have focused on persons with either active disease or latent infection. Although this work has been critical in defining T-cell correlates of successful versus failed host containment, little is known about the development of *Mycobacterium*-specific T-cell responses in uninfected persons. To explore this issue, naive T cells from uninfected donors were sensitized in vitro with avirulent *Mycobacterium tuberculosis*-infected autologous macrophages. T-cell lines primed in this manner proliferated and produced cytokines after challenge with mycobacterial antigens. Of 11 such lines, 8 were high Th1 responders, 2 were low Th1 responders, and 1 was a Th2 responder. Furthermore, similar patterns and magnitudes of proliferative and cytokine responses were seen when *Mycobacterium* infection-primed lines were challenged with recombinant antigen 85 (Ag85) proteins. The addition of interleukin 12 (IL-12) during the initial sensitization increased the magnitude of Th1 responses; however, antibody to IL-12 did not eliminate Th1 responses, suggesting that additional factors contributed to the differentiation of these cells. Finally, in the presence of IL-12, recombinant Ag85B was able to prime naive T cells for Th1 responses upon challenge with *Mycobacterium*-infected macrophages or Ag85B. Therefore, under the appropriate conditions, priming with whole bacteria or a subunit antigen can stimulate *Mycobacterium*-specific Th1 effector cell development. Further definition of the antigens and conditions required to drive naive human T cells to differentiate into Th1 effectors should facilitate the development of an improved tuberculosis vaccine.

Tuberculosis remains one of the leading infectious diseases of humans, causing an estimated 6.7 million new cases and 2.4 million deaths in 1998 (27). Despite bacillus Calmette-Guérin (BCG) vaccination programs and increasing use of directly observed therapy in many developing countries, it is predicted that there will be 225 million new cases and 79 million deaths from tuberculosis between 1998 and 2030. A safe, inexpensive, and effective vaccine is of the highest priority. The development of an improved vaccine will depend on a more thorough understanding of protective host immune responses to *Mycobacterium tuberculosis* as well as the identification and characterization of any antigen(s) that induces those protective responses.

Both mouse models of infection and human patient material have provided evidence that protective immune responses to *Mycobacterium* are T-cell dependent (2, 28). It has been shown that CD4⁺ Th1 cells play an important role in the development of resistance to disease (13, 32), primarily through the production of macrophage-activating cytokines, such as gamma interferon (IFN- γ) or granulocyte-macrophage colony-stimulating factor (1, 4). In addition, CD8⁺ T cells may contribute to disease resistance either through the elaboration of cytokines or through direct cytotoxicity for *Mycobacterium*-infected cells (10, 21, 22). Most studies of human T cells in tuberculosis have focused on persons with either active disease or latent infection. These studies have been invaluable in defining the T-cell correlates of unsuccessful versus successful human immune

responses to *M. tuberculosis*. However, the roles of the different T-cell subsets and their interactions with one another to mediate protection against disease remain unclear.

More relevant to the issue of vaccine development are the types of responses that occur when T cells from tuberculosis-naive individuals are exposed to mycobacterial antigens. The antigen(s), adjuvants, and other conditions required to effectively sensitize and induce naive T cells to differentiate into protective effector cells are largely unknown but clearly warrant further investigation if a successful vaccine effort is to be undertaken.

To this end, we have focused our studies on naive human T-cell sensitization with mycobacterial antigens by developing two in vitro vaccine models. The first uses live attenuated *Mycobacterium* (HR37a)-infected macrophages (MIM) to sensitize naive human T cells. We believe that the T-cell responses observed in this system reflect what occurs in vivo on initial infection of a naive host with *M. tuberculosis*, with responses ranging from beneficial (i.e., correlating with host containment of infection) to permissive (i.e., correlating with failure to contain infection). However, since sensitization occurs in vitro, the parameters affecting T-cell priming leading to the differentiation of beneficial T cells can be manipulated and investigated. In addition, the functions of and interactions between T-cell subsets can be studied. Here, we report the use of this system to investigate certain factors affecting the development of responses to antigen 85 (Ag 85) complex proteins during infection.

The second in vitro model we developed represents a subunit vaccine system. Here, naive T cells were sensitized with defined mycobacterial antigens under various conditions. The effector T cells that developed were tested for antimycobacterial functions. Using this system, we found that sensitization

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with the vaccine candidate, recombinant Ag85B (rAg85B), in the presence of interleukin 12 (IL-12) effectively primes naive human T cells to become Th1 effectors against *M. tuberculosis*-infected macrophages. These two in vitro sensitization models represent novel approaches to investigating questions concerning naive human T-cell responses to mycobacterial antigens that cannot be readily addressed using murine or human in vivo systems but that may ultimately contribute to vaccine development.

MATERIALS AND METHODS

Mycobacteria. *M. tuberculosis* (H37Ra; American Type Culture Collection) was grown on Lowenstein-Jensen medium at 37°C and then transferred for further cultivation to Middlebrook 7H9 broth with ADC enrichment (Difco Laboratories, Detroit, Mich.). The culture was kept in a shaking 37°C incubator until an optical density of 0.6 (mid-log phase) was obtained (~2 weeks). The bacteria were frozen in aliquots and stored at -70°C until used. The titers in the culture were determined by colony counts of serial dilutions on Middlebrook 7H10 agar plates supplemented with OADC (Difco). Heat-killed *M. tuberculosis* (H37Ra) was obtained from Difco.

Expression, purification, and resolubilization of recombinant proteins. The genes encoding Ag85A, Ag85B, and Ag85C were cloned into the *Escherichia coli* expression vector pRSETB (Invitrogen, Carlsbad, Calif.), which was transformed into *E. coli* JM109(DE3) (Promega, Madison, Wis.). The genes for Ag85A and Ag85B were altered to replace low-use codons for the purpose of increasing the expression of rAg85 (22a). Flasks containing 50 ml of SOB broth and 200 µg of ampicillin per ml were inoculated with 300 µl of an overnight culture of transformed JM109(DE3) cells and incubated at 37°C with shaking to an absorbance at 600 nm of 0.6. Recombinant protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (Promega) at a final concentration of 1 mM and incubation for an additional 5 h.

To purify rAg85 proteins, the 5-h culture was pelleted by centrifugation, and the cells were lysed in 10 ml of 25 mM Tris-HCl (pH 7.8)-500 mM NaCl-1% Triton X-100-0.1 mg of lysozyme per ml on ice for 30 min. This lysate was sonicated (VirTis Inc., Gardiner, N.Y.), frozen in liquid nitrogen, and thawed at 37°C. Insoluble inclusion bodies were harvested by centrifugation at 7,000 rpm for 15 min and solubilized in 6 M guanidine-HCl-30 mM Tris-HCl (pH 7.8)-500 mM NaCl-10 mM dithiothreitol-2 mM EDTA. The solubilized antigen fraction was filtered through a 0.4-µm-pore-size filter (Millipore) and slowly diluted to 100 ml with 4 M urea-30 mM Tris-HCl-500 mM NaCl-6 mM reduced glutathione (Sigma Chemical Co., St. Louis, Mo.)-0.6 mM oxidized glutathione (Sigma) (pH 7.8). After 1 h of incubation at room temperature, insoluble protein was removed by filtration. The filtrate was slowly added to 1 liter of 100 mM Tris-HCl-1 M NaCl-6 mM reduced glutathione-0.6 mM oxidized glutathione (pH 7.8). This solution was concentrated to 30 ml by ultrafiltration using a stirred cell and a YM10 membrane (Millipore). After filtration through a 0.22-µm-pore-size filter, the concentrated protein was dialyzed against 100 mM Tris-HCl (pH 7.8)-1 M NaCl. Following resolubilization, polyacrylamide gel electrophoresis of rAg85B showed a single band at the expected molecular weight. The refolded Ag85 proteins were freed of endotoxin by two passages over Detoxigel affinity columns (Pierce Chemical, Rockford, Ill.), after which endotoxin contamination was less than 0.8 endotoxin units/mg of protein (*Limulus* amoebocyte assay; BioWhittaker, Walkersville, Md.).

PBMC. Human buffy coats were obtained from healthy individuals by the American Red Cross (Pacific Northwest Regional Services, Portland, Oreg.). Donated blood was serologically screened for human immunodeficiency virus, hepatitis B and C viruses, cytomegalovirus, human T-cell leukemia virus type 1, and other pathogens. Only nonreactive samples were used. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation through Ficoll-Paque (Life Technologies, Grand Island, N.Y.) and stored frozen in liquid nitrogen. Differential white blood cell counts and fluorescence-activated cell sorting analysis of common cell surface markers indicated that the samples fell within the normal ranges for lymphocytes, monocytes, and granulocytes as well as for the frequency of CD4⁺ and CD8⁺ T cells. All of the samples used were nonreactive (stimulation index [SI], <3.0) to *M. tuberculosis* antigens, as determined by testing in a primary proliferation assay with 25 µg of heat-killed *M. tuberculosis* (H37Ra) per ml. Of samples from 125 donors tested, ~77% were nonreactive by this criterion, 17% had an SI of >3.0 but <5.0, and 6% had an SI of >5.0. A subset of this last population may correspond to persons who are latently infected with *M. tuberculosis* and whose samples are possibly reactive with purified protein derivative (PPD). This suggestion is supported by U.S. data indicating that ~4% of the population has latent tuberculosis infection (7).

In vitro sensitization and propagation of Mycobacterium-specific T cells from normal PBMC. Monocytes were enriched from PBMC by adherence to plastic for 45 min in 48-well tissue culture plates and then extensively washed. The cells matured into macrophages during culturing for 5 to 7 days in endotoxin-free RPMI 1640 (BioWhittaker) supplemented with gentamicin, 5 × 10⁻⁵ M 2-mercaptoethanol, L-glutamine, and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah). Mature macrophages were infected with

M. tuberculosis at a ratio of 20:1 for 4 h at 37°C. The macrophages were washed, fed with fresh medium, and recultured at 37°C for an additional 2 days. They were washed again just prior to T-cell sensitization. Histological analysis of cells adherent to LabTek slides which were stained with DiffQuik revealed that 100% of adherent cells present after 5 days of maturation and 2 days of infection were macrophages, as evidenced by morphology. No contaminating lymphocytes were detected. Slides stained with reagents to detect acid-fast bacilli demonstrated that 50 to 70% of the macrophages were infected with *M. tuberculosis*. These infected cells remained viable for up to 7 days in culture.

Autologous PBMC (10⁷) in complete medium containing 10% heat-inactivated human serum (Pel-Freez Clinical Systems, Brown Deer, Wis.) were added to *M. tuberculosis*-infected macrophages for a 48-h sensitization period. Five days after sensitization, complete medium supplemented with 10 U of recombinant IL-2 (Life Technologies) per ml was added to the cultures. Thereafter, the cells were maintained in IL-2-containing medium that was changed every 3 to 4 days. Every 12 to 16 days, the T-cell lines were boosted with autologous MIM at an approximate ratio of 10 T cells to 1 MIM. T-cell lines underwent two to four cycles of stimulation with MIM before being tested for functional activity or specificity. Infection-sensitized T cells propagated in this manner were designated Mtb T-cell lines. For some experiments, Mtb T-cell lines were sensitized in the presence of IL-12 (1 to 5 ng/ml; Pharmingen, San Diego, Calif.) or neutralizing antibody to IL-12 (100 µg/ml; Biosource International, Camarillo, Calif.) during the first exposure to MIM.

Ag85B-specific T-cell lines were generated from normal PBMC and propagated as follows. Naive PBMC were sensitized with 20 µg of rAg85B per ml in the absence (N-85B lines) or presence (T-85B lines) of 1 ng of IL-12 per ml. After 7 days, the T cells were boosted with autologous macrophages pulsed with rAg85B. Thereafter, the lines were propagated in IL-2-containing medium that was changed every 3 to 4 days and then were boosted with autologous macrophages pulsed with rAg85B every 12 to 14 days. IL-12 was given only during the initial sensitization phase.

Depletion of CD45R0-positive cells from PBMC. PBMC were depleted of CD45R0-positive cells using the MiniMac system (Miltenyi Biotec, Inc., Sunnyvale, Calif.). Briefly, PBMC were incubated with an affinity-purified mouse anti-human CD45R0 antibody (Pharmingen) and washed. Cells were then incubated with goat anti-mouse immunoglobulin G-conjugated beads and passed over the magnetic separation column. Flow cytometry indicated that the negatively enriched populations were 94 to 98% CD45RA (naive cells), with contaminating CD45R0-positive cells ranging from 0% (n = 3) to 8% (n = 1). PBMC and PBMC depleted of CD45R0-positive cells, obtained from the same donors, were used to generate Mtb T-cell lines as described above.

In vitro proliferation assays. Resting cells from Mtb or rAg85B-specific T-cell lines were cultured in quadruplicate in 96-well flat-bottom plates at 3 × 10⁴ cells/well with MIM, uninfected macrophages pulsed with heat-killed *Mycobacterium* (HKM) or rAg85, or uninfected macrophages alone. Chicken ovalbumin (OVA; Sigma) or *Leishmania* lysate was used as a control antigen. After 3 days of culturing, the cells were pulsed with 1 µCi of ³H-thymidine (6.7 Ci/mmol; NEN Research Products, Boston, Mass.) for 18 h and harvested on glass fiber filter mats. Radioactive incorporation was assessed by liquid scintillation counting. The SI was calculated as the mean counts per minute for cells cultured with antigen divided by the mean counts per minute for cells cultured without antigen. MIM cultured without T cells did not incorporate significant radioactivity (< 300 cpm).

Cytokine assays. Supernatants from proliferation assays were collected for the determination of cytokine levels. Preliminary kinetic experiments revealed that peak IL-4 production was detected in 24-h supernatants, while peak IFN-γ and IL-10 production was detected in 72-h supernatants. Cytokine levels were quantified by an enzyme-linked immunosorbent assay (ELISA) with commercial anti-cytokine antibody pairs (Pharmingen) according to the manufacturer's protocol. Human recombinant IL-4, IFN-γ, and IL-10 (Pharmingen) were used to generate standard curves. Supernatants were harvested from quadruplicate cultures and pooled and run as duplicate samples in the ELISA. Supernatants from infected or uninfected macrophages (triplicate cultures) were harvested at 24 and 48 h and tested for IL-12 using a p70-specific IL-12 ELISA (Quantikine; Pharmingen) in duplicate.

Immunofluorescence and flow cytometry. Cells from Mtb T-cell lines were tested for CD3, CD8 (Leu-2a), and CD4 (Leu-3a) (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) surface marker expression by three-color analysis using standard procedures. Data were collected using a FACScan (Becton Dickinson) and analyzed with LYSUS II software. At least 5,000 cells were analyzed per sample.

RESULTS

Mycobacterium-specific T-cell lines sensitized in vitro develop predominantly Th1 profiles. Tuberculosis-naive donors were selected by testing the proliferative responses of PBMC to heat-killed *M. tuberculosis* H37Ra. T-cell lines used in these studies were generated from nonreactive donors (SI, <3.0) and were considered naive. Figure 1 shows the proliferative re-

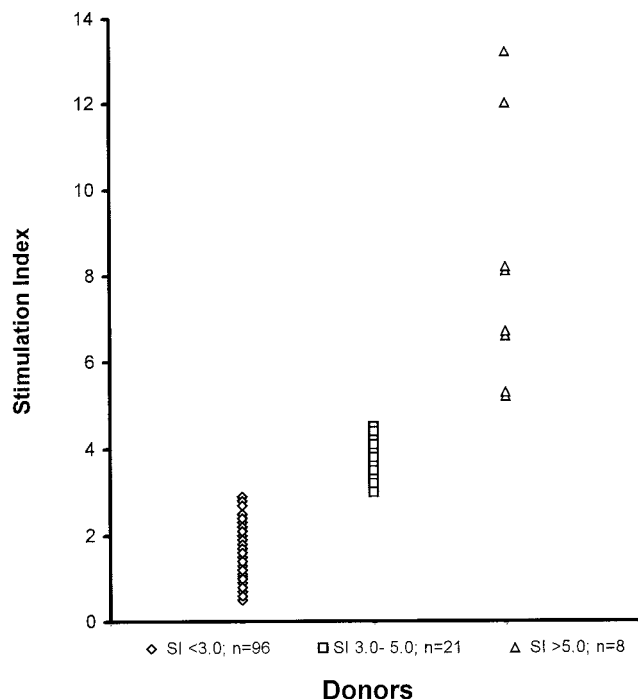


FIG. 1. Screening of donors for *M. tuberculosis* reactivity by proliferation. PBMC were tested in a standard proliferation assay using 25 µg of H37Ra per ml. The mean (standard deviation) SIs were 1.53 (0.62), 3.8 (0.45), and 8.2 (3.0), respectively, for the groups shown on the figure, from left to right.

sponses of samples from 125 donors to H37Ra. Ninety-six donors (77%) were considered tuberculosis naive by this criterion. The 17 donors whose samples were used for the following studies were chosen from this group. The average mean primary proliferation SI of these samples was 2.0. Using the methods described above, the generation of in vitro-sensitized T-cell lines was 100% efficient. Thus, the reactivity to *M. tuberculosis* antigens was an intrinsic capability of the cells and was not attributable to prior infection.

Long-term-infection-induced T-cell lines (Mtb T-cell lines) were prepared from 11 donors by cultivating PBMC from naive donors on autologous MIM. These lines were extensively analyzed. Table 1 demonstrates that all such Mtb lines proliferated specifically in response to in vitro challenge with MIM or HKM-pulsed autologous normal macrophages. SIs ranged from 3.0 to 34. As might be predicted, the cells proliferated more in response to live rather than dead *Mycobacterium* antigens. Analysis of cytokine production demonstrated that the majority of the T-cell lines developed Th1 profiles, producing significant levels of IFN-γ but little or no IL-4. However, the range of IFN-γ production among these Th1 cell lines varied widely, with some producing high quantities and others producing very low quantities. In most instances, IL-10 production (data not shown) paralleled that of IL-4. Only one T-cell line, Mtb.94, produced more IL-4 than IFN-γ; it was thus designated a Th2 responder. Duplicate lines generated from the same donors at a later time showed similar cytokine profiles. Not surprisingly, the levels of cytokines produced did not correlate with proliferation (Table 1) or with the percentage of CD4⁺ cells found in the lines (Table 2). For example, the percentages of CD4⁺ and CD8⁺ cells were very similar in lines Mtb.90, Mtb.94, and Mtb.101, but their cytokine profiles were quite different (Tables 1 and 2). All of the Mtb lines contained both CD4 and CD8 cells. In addition, some lines contained

TABLE 1. Proliferation and cytokine profiles of *Mycobacterium*-specific T-cell lines

T-cell line	Results obtained in the presence of:						Profile
	MIM ^a			HKM ^b			
	SI ^c	Level ^d (pg/ml) of:		SI ^c	Level ^d (pg/ml) of:		
		IFN-γ	IL-4		IFN-γ	IL-4	
Mtb.90	23	236	<5	10.6	236	<5	Th1
Mtb.94	15	19	67	7.6	<10	8	Th2
Mtb.101	14	157	20	9.2	813	<5	Th1
Mtb.102	28	331	37	8.5	54	<5	Th1
Mtb.104	10.4	193	<5	3.2	85	18	Th1
Mtb.106	33.5	215	<5	10.2	528	<5	Th1
Mtb.107	12.5	597	37	8.9	512	<1	Th1
Mtb.108	8.0	50	<5	ND	<5	12	Th1
Mtb.184	5.1	600	<5	4.0	644	<5	Th1
Mtb.186	6.1	116	<5	5.2	264	<5	Th1
Mtb.187	19.4	30	7	23.2	91	5	Th1

^a MIM ratio, 20:1.

^b HKM (15 µg/ml)-pulsed macrophages.

^c Background counts per minute for SI (mean and standard deviation), 674 ± 434. ND, not determined.

^d Supernatants were harvested from antigen-stimulated and nonstimulated cultures (T cells plus normal macrophages) and tested for cytokines. Data are reported as levels of cytokines from stimulated cultures minus unstimulated cultures. The amount of IFN-γ produced from unstimulated cultures was 1.6 ± 5.1 pg/ml (mean and standard deviation; 95% confidence interval upper limit, 5.1), and that of IL-4 was 7.7 ± 19.4 pg/ml (95% confidence interval upper limit, 20).

significant numbers of CD3⁺, CD4⁻, and CD8⁻ (double-negative) T cells (Table 2).

In order to evaluate the contribution of naive versus memory cells to the response to *M. tuberculosis*, infection-induced T-cell lines (Mtb T-cell lines) were prepared by cultivating PBMC (from naive donors; SI, <3.0) or PBMC depleted of CD45R0-positive cells (from the same donors) on autologous MIM. CD45R0-positive cells ranged from 21 to 26% of normal PBMC in the donors tested. In CD45R0-depleted PBMC, this population was reduced to 0% (n = 3) or 8% (n = 1; donor 229). Four weeks later, the cells from both types of lines were challenged in vitro with mycobacterial antigens. Table 3 demonstrates that in three of four donors, IFN-γ production from lines generated with PBMC did not differ significantly from that in lines generated with CD45R0-depleted PBMC. In one

TABLE 2. Cell surface phenotype of *Mycobacterium*-specific T-cell lines

T-cell line	% of cells that were ^a :		
	CD4 ⁺	CD8 ⁺	DN
Mtb.90	87	4	2
Mtb.94	86	4	6
Mtb.101	87	3	5
Mtb.102	71	11	9
Mtb.104	66	19	4
Mtb.106	66	19	9
Mtb.107	67	17	7
Mtb.108	75	11	7
Mtb.184	63	19	16
Mtb.186	69	14	14

^a Mtb T-cell lines were stained with antibodies to CD3-PerCP, CD4 fluorescein isothiocyanate, and CD8-phycoerythrin. DN, double negative (CD4⁻ and CD8⁻).

TABLE 3. IFN- γ production by Mtb T-cell lines generated in the presence or absence of CD45R0-positive cells^a

T-cell lines	IFN- γ produced (pg/ml) with the following challenge antigen:		
	MIM	H37Ra	rAg85B
Mtb.231	76	122	111
Mtb.231 R0 depleted	42	260	66
Mtb.232	135	747	55
Mtb.232 R0 depleted	140	477	126
Mtb.229	37	98	67
Mtb.229 R0 depleted	260	226	99
Mtb.234 ^b	<10	<10	<10
Mtb.234 R0 depleted ^b	<10	<10	<10

^a Mtb T-cell lines were generated with normal PBMC or with PBMC depleted of CD45R0-positive cells (R0 depleted) and propagated as described in the text. Four weeks later, the cells were challenged in vitro with mycobacterial antigens, and supernatants were tested for IFN- γ production.

^b These T-cell lines were specific for mycobacterial antigens, as determined by proliferation (SI, 3 to 13).

donor, IFN- γ levels were somewhat higher in lines generated from CD45R0-depleted PBMC. Of interest, the sample from donor 234 exhibited no IFN- γ or IL-4 (data not shown) production but was *Mycobacterium* specific, as evidenced by proliferation (SIs ranged from 3 to 13). Thus, the contribution of CD45R0-positive cells to the responses seen were minimal.

Mtb T-cell lines respond to challenge with Ag85 complex proteins. We analyzed cytokine production from Mtb T-cell lines that were stimulated in vitro with normal autologous macrophages pulsed with rAg85A, rAg85B, and rAg85C. All Mtb T-cell lines responded to challenge with Ag85 complex proteins by production of IFN- γ or IL-4 (Table 4). Cells from Mtb lines challenged with OVA or *Leishmania* lysate did not proliferate (SI, <2) or produce IFN- γ (<20 pg/ml). Mtb lines which produced high concentrations of IFN- γ in response to MIM also produced high quantities of IFN- γ in response to rAg85 proteins. Mtb.94, the one Th2 cell line generated, produced only IL-4 in response to rAg85A. Responses to Ag85 proteins were of a very high magnitude, ranging from 50 to

TABLE 4. Cytokine profiles of Ag85-elicited *Mycobacterium*-specific T-cell lines^a

T-cell line	Level (pg/ml) of the indicated cytokine produced with:							
	rAg85A		rAg85B		rAg85C		MIM	
	IFN- γ	IL-4	IFN- γ	IL-4	IFN- γ	IL-4	IFN- γ	IL-4
Mtb.186	11	<5	27	6	831	13	101	<5
Mtb.144	169	<5	73	<5	ND	ND	129	<5
Mtb.149	1,178	<1	746	<5	ND	ND	420	<5
Mtb.173	300	<5	270	<1	400	<1	500	<5
Mtb.183	250	<5	300	<5	300	<5	501	<5
Mtb.184	41	<5	20	<5	129	<5	319	<5
Mtb.191	34	<5	82	<5	30	<5	65	11
Mtb.193	<10	<5	22	<5	141	<5	82	<5
Mtb.94	<10	55	ND	ND	ND	ND	19	50

^a Resting T cells from *Mycobacterium*-specific T-cell lines were cultured with MIM or with autologous macrophages pulsed with 15 μ g of rAg85A, rAg85B, rAg85C, or no antigen. Supernatants were harvested from triplicate cultures at 24 h (IL-4) and 72 h (IFN- γ) and tested by an ELISA. Results are expressed as levels of cytokines from stimulated cultures minus unstimulated cultures. ND, not determined.

TABLE 5. IL-12 potentiates *Mycobacterium*-specific IFN- γ production

T-cell line	IFN- γ production ^a (pg/ml) with:						IL-12 production ^b (pg/ml) with:	
	MIM		rAg85B		MIM	MIM + PBMC ^b		
	- IL-12	+ IL-12	- IL-12	+ IL-12				
Mtb.187	<5	174	<5	ND	ND	7	<5	
Mtb.191	65	184	63	ND	ND	<5	10	
Mtb.184	34	599	50	97	206	30	17	
Mtb.193	82	397	141	22	77	7	14	
Mtb.186	129	1,510	ND	12	394	<5	<5	

^a Mtb T-cell lines were generated by stimulating normal PBMC with MIM in the absence (-) or presence (+) of IL-12 (1 ng/ml) or neutralizing antibody to IL-12 (100 μ g). They were challenged in vitro with MIM or rAg85B-pulsed normal macrophages for 3 days. Supernatants were harvested at 72 h, and IFN- γ production was determined by an ELISA. ND, not determined.

^b Mature macrophages (from donors 187, 191, 184, 193, and 186) were infected with MIM (ratio, 20:1) or mock infected (data not shown). After 2 days, autologous naive PBMC were cocultured with MIM. Supernatants were harvested at 48 h and tested for IL-12 p70 by an ELISA.

150% the cytokine production seen in response to MIM. Mtb lines in which the three recombinant proteins were tested individually showed no greater response to one Ag85 protein than to the others.

Regulation of Mtb T-cell line Th1 responses by IL-12. In order to determine if IL-12 affected the cytokine profile of *Mycobacterium*-specific T cells in this model, new Mtb T-cell lines were generated in the presence or absence of IL-12 or neutralizing antibody to IL-12. Mtb lines sensitized in the presence of IL-12 produced 3- to 30-fold more IFN- γ upon subsequent challenge with MIM or rAg85B-pulsed macrophages (Table 5) than Mtb lines sensitized in the absence of IL-12. However, the addition of antibody to IL-12 to cultures at the initiation of the Mtb lines did not reduce or eliminate IFN- γ production below the levels seen in Mtb lines initiated in the absence of IL-12. Therefore, the elimination of endogenous IL-12 during sensitization did not inhibit the development of *Mycobacterium*-specific Th1 cells. This conclusion is further supported by the fact that endogenous IL-12 production from MIM in both the presence and the absence of PBMC did not correlate with IFN- γ production by Mtb lines generated in the absence of exogenous IL-12 (Table 5).

Immunization with rAg85B can prime naive T cells for *Mycobacterium*-specific responses. In order to test the immunogenicity of rAg85B in a human vaccine system, naive T cells from six naive donors were sensitized with rAg85B in the presence or absence of IL-12 (1 ng/ml). These cells were propagated as continuous Ag85B-specific T-cell lines by periodic boosting with rAg85B-pulsed normal macrophages. IL-12 was given only at the time of initial sensitization. Ag85B-specific T-cell lines were tested for specificity by proliferation and cytokine production after 4 to 6 weeks in cultures. All T-cell lines proliferated in response to MIM and HKM- or rAg85B-pulsed normal macrophages (Table 6). Ag85B-specific T-cell lines generated in the absence of IL-12 proliferated to mycobacterial antigens, demonstrating specificity, but produced little or no IFN- γ . In contrast, five of the six Ag85B-specific T-cell lines sensitized in the presence of IL-12 produced high quantities of IFN- γ ; little or no IL-4 production was detected (data not shown). Thus, in the presence of IL-12, rAg85B can prime human Th1 cells which recognize the natural ligand present on MIM. We found similar results when using rAg85A as the

TABLE 6. Immunization with rAg85B induces *Mycobacterium*-specific human T-cell responses^a

T-cell line	Results obtained in the presence of:					
	MIM		HKM		rAg85B	
	SI	IFN- γ level	SI	IFN- γ level	SI	IFN- γ level
N-85B.149	5.0	<5	2.0	71	2.0	<5
T-85B.149	3.2	40	2.0	223	2.1	92
N-85B.159	1.0	62	ND	ND	3.8	240
T-85B.159	2.0	190	ND	ND	4.6	641
N-85B.173	ND	43	ND	61	ND	<5
T-85B.173	ND	864	ND	3,106	ND	336
N-85B.186	10.0	<5	10.7	<5	4.5	12
T-85B.186	6.8	<5	10.4	31	15	107
N-85B.187	10.7	5	3.6	<5	6.6	<5
T-85B.187	3.8	594	1.5	260	2.7	734
N-85B.191	4.2	<5	2.3	<5	2.4	<5
T-85B.191	3.3	50	2.3	36	2.3	48

^a Resting cells from rAg85B-specific T-cell lines were stimulated in vitro with MIM (20:1) or HKM (15 μ g/ml)- or rAg85B (10 μ g/ml)-pulsed normal macrophages for 3 days. Supernatants were harvested and tested for the IFN- γ concentration (picograms per milliliter) by an ELISA. Proliferation was assessed by measuring ³H-thymidine incorporation. ND, not determined. T-cell lines designated with "N" were generated by immunization of normal PBMC with rAg85B. T-cell lines designated with "T" were generated by immunization of normal PBMC with rAg85B plus IL-12 (1 ng/ml).

sensitizing antigen in this system (data not shown). Surface marker analysis of the Ag85B-expressing T-cell lines demonstrated that they contained 80 to 85% CD4⁺ cells and 10 to 15% CD8⁺ cells. Of interest, no significant surface phenotype differences between lines generated in the absence (N-85B) or the presence (T-85B) of IL-12 were detected.

DISCUSSION

The development of resistance to disease caused by *M. tuberculosis* is still poorly understood and probably involves multiple innate as well as acquired immune components. T-cell responses, in particular, have been shown to be critical. Specifically, these include the CD4⁺ Th1 subset, which secretes IFN- γ , as well as CD8⁺ cells, which produce cytokines and directly lyse infected cells. Roles for other T-cell subsets, including CD1-restricted CD3⁺ cells (12, 33) and γ δ T cells (5, 16), are also being investigated.

We were interested in studying vaccine-induced human T-cell responses to *M. tuberculosis*. To this end, we developed an in vitro system whereby naive human T cells were sensitized and propagated with an attenuated live vaccine (Mtb T-cell lines) or a subunit vaccine (Ag85B lines). These models may be useful for investigating the antigens and other conditions required to induce the differentiation of naive T cells into beneficial effector cells against *M. tuberculosis*. The advantages of this in vitro system are (i) the sensitization parameters can be controlled and easily manipulated and (ii) early events and cell interactions can be delineated. Neither of these objectives can be easily accomplished using human cells sensitized in vivo.

Mtb T-cell lines mounted largely beneficial responses, as expected. Mtb lines proliferated and produced cytokines in response to challenge with live or dead bacteria. Of 11 Mtb lines generated from different donors, 10 developed a Th1

profile of cytokine production, although 2 of these lines produced very low levels of IFN- γ (<50 pg/ml). Only one line (Mtb.94) showed an overt Th2 cytokine profile. In general, cytokine levels produced by Mtb lines were low, but this result is not surprising, as these cells were primed in vitro for a limited period of time.

The type of immune responses seen with this system appeared to be mounted largely by the CD45RA or naive cell population. Depletion of CD45RO-positive (memory) cells from the initial PBMC population did not affect the type or magnitude of responses which developed. Therefore, the contribution of cross-reacting memory cells, which may have originally been primed by related bacterial antigens, appeared minimal.

Although the number of naive donors tested was small, the profiles seen in our Mtb T-cell lines in vitro appeared to closely fit the T-cell profiles that accompany natural infection in humans. Normally, about 90% of individuals newly infected with *M. tuberculosis* develop protective immune responses, whereas less than 10% develop primary progressive disease. While we have no direct knowledge of how these donors would respond to in vivo infection, we speculate that the moderate to high Th1 responders might be at low risk for the development of active disease, while the Th2 responder (Mtb.94) might be at high risk. The two low Th1 responders (Mtb.108 and Mtb.187) are more difficult to classify, but their clinical status might be more dependent on the presence of other protective responses (cytotoxic T cells). Thus, the majority of responses seen in this model are beneficial ones and may be analogous to those seen in PPD-positive (PPD⁺) asymptomatic individuals.

Since sensitization in vitro with avirulent *M. tuberculosis* induces T-cell responses which parallel at least some of those seen during vivo sensitization, it may represent an alternative to the use of patient material for certain studies, particularly those investigating early T-cell responses to new infection. We have used a similar in vitro model to study early human T-cell responses to *Leishmania* infection (31). Like infection with *Mycobacterium*, infection with *Leishmania* usually results in resistance to disease. Using an in vitro model of human *Leishmania* infection, we found that only 50% of individuals developed Th1 responses to *Leishmania*. However, >75% of individuals mounted cytotoxic responses which lysed *Leishmania*-infected macrophages. This result suggested that cytotoxic effector activity, in addition to Th1 responses, contributed to resistance to human disease caused by *Leishmania*. Further investigation is needed to determine if the CD8⁺ T cells present within Mtb T-cell lines are cytotoxic. Using a similar in vitro system, involving short-term coculturing of *M. tuberculosis*-infected macrophages with autologous PBMC from tuberculosis patients, PPD⁺ asymptomatic individuals, or naive donors, other investigators demonstrated cytokine differences between the groups. In the naive PBMC cocultures, high-level IFN- γ production in association with low-level IL-2 production suggested a role for natural killer cell-derived cytokines in the development of a successful *M. tuberculosis*-specific immune response (20).

When Mtb T-cell lines were challenged with rAg85 proteins, the quantities of IFN- γ produced in response to the Ag85 proteins correlated with the quantities produced in response to MIM or HKM. However, in some instances, more IFN- γ was elicited by rAg85 than by MIM, demonstrating the potency of Ag85 proteins. Not surprisingly, IL-4 was produced by the one Th2 Mtb T-cell line in response to rAg85A. These results support the immunodominance of Ag85 proteins which has been shown by other investigators, primarily by using cells from PPD⁺ asymptomatic individuals (6, 15, 26). Of interest, a

comparison of the immunogenicities of rAg85 proteins done with four Mtb lines revealed no significant differences in the ability of the three proteins to elicit strong IFN- γ production from Th1 responders. However, in three other Mtb lines, rAg85C elicited much higher levels of IFN- γ production, suggesting a differential response to these proteins in some individuals.

Work from several laboratories has shown that IL-12 contributes to the magnitude of anti-*Mycobacterium* Th1 responses (39, 40). We demonstrated that Mtb T-cell lines sensitized in the presence of IL-12 showed dramatically increased IFN- γ production in response to challenge with mycobacterial antigens, including rAg85B. These data support work by other investigators showing that IL-12 increased IFN- γ production in cultures from PPD⁺ individuals (25). Furthermore, in patients with multidrug-resistant tuberculosis, the addition of IL-12 to in vitro cultures of PBMC restored IFN- γ production (25). However, when we generated Mtb lines in the presence of antibody to IL-12, IFN- γ production was not reduced to levels below those seen in Mtb lines generated in the absence of IL-12. Also, endogenous IL-12 production by MIM in the presence or absence of PBMC did not correlate with the amount of IFN- γ production seen in Mtb lines. These data support observations demonstrating that inherent resistance to *Mycobacterium* in BCG-resistant mice does not depend on optimal IL-12 levels (35). Together, the results suggest that other cytokines (e.g., IL-18 and MIP-1 β) also contribute to the development of *M. tuberculosis*-specific Th1 responses (11, 34; N. Kozlova, D. L. Lakey, T. Winn, D. Kernodle, and D. M. Russo, Abstr. 34th Tuberculosis-Leprosy Conf. Tuber. Lung Dis., p. 262, 1999).

Human T-cell responses to secreted Ag85 proteins appear to be immunodominant in PPD⁺ latently infected individuals (6, 15, 26) and are downregulated in those with active tuberculosis (3, 15, 26). These findings suggest an association with protective responses. Furthermore, strong Th1 responses have been elicited in vitro from PPD⁺ asymptomatic individuals using native, recombinant, or synthetic peptide forms of Ag85 proteins (15, 23, 26). In addition, Ag85 proteins have been shown to induce partial protection in murine models of infection (17, 18). These findings have provided hope that an efficacious subunit vaccine can be developed. However, recombinant proteins expressed in bacterial systems can be problematic in terms of immunogenicity due to improper processing and folding. We used an in vitro sensitization system to derive important information regarding a vaccine candidate (gp63) for *Leishmania* (30). We showed that the immunogenic potential of *E. coli*-expressed recombinant gp63 was limited due to its inability to prime T cells which could subsequently recognize native antigen. This result provided one explanation as to why recombinant gp63 failed to protect mice (14, 24), even though native gp63, other recombinant forms of gp63, and peptides of gp63 had all induced some degree of protection (8, 19, 29, 37, 38).

Since naive T-cell sensitization studies with rAg85 proteins had not been reported previously, we were concerned that a similar problem might be observed with tubercular rAg85. Therefore, we tested the immunogenicity of rAg85B in a human vaccine model where naive T cells were immunized with rAg85B in the presence or absence of IL-12. We found that rAg85B-specific T-cell lines generated in the presence of IL-12 responded to challenge with MIM by proliferation and, more importantly, by enhanced production of IFN- γ . Thus, rAg85B expressed in *E. coli* can prime *Mycobacterium*-specific human Th1 cells which recognize the naturally expressed ligand on MIM. This result suggests that rAg85B in combination with an appropriate adjuvant (IL-12 or an IL-12-inducing substance)

can induce the human immune responses required of a potential candidate vaccine for *M. tuberculosis*.

In summary, we conclude that naive human T cells react with whole *M. tuberculosis* cells and defined subunit antigens in vitro, reproducibly generating predominantly Th1 responses, although some variability based on the individual T-cell donor was observed. T-cell lines generated using rAg85B subsequently responded to live tubercle bacilli, validating the concept that humans vaccinated with a single mycobacterial antigen might develop beneficial T-cell responses. Further definition of the antigens and conditions required to drive naive human T cells to differentiate into Th1 effectors should facilitate the development of an improved tuberculosis vaccine.

ACKNOWLEDGMENTS

We thank Frank Hatcher for help analyzing the flow cytometry data and James M. Burns, Jr., and Radiah Corn for critically reviewing the manuscript.

This work was supported by the Department of Veteran Affairs.

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Editor: S. H. E. Kaufmann