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Mycobacterium tuberculosis-Reactive CD8$^+$ T Lymphocytes: The Relative Contribution of Classical Versus Nonclassical HLA Restriction[1]

David M. Lewinsohn,[2]* Andria L. Briden,† Steven G. Reed,† Kenneth H. Grabstein,† and Mark R. Alderson†

Previous studies in mice and humans models have suggested an important role for CD8$^+$ T cells in host defense to *Mycobacterium tuberculosis* (Mtb). In humans, CD8$^+$ Mtb-reactive T cells have been described that are HLA-A2-, B52-, as well as CD1-restricted. Recently, we have described Mtb-specific CD8$^+$ T cells that are neither HLA-A-, B-, or C- nor group 1 CD1-restricted. At present, little is known about the relative contribution of each of these restriction specificities to the overall CD8$^+$ response to Mtb. An IFN-γ enzyme-linked immunospot assay was used to determine the frequency of Mtb-reactive CD8$^+$ T cells directly from PBMC. The effector cell frequency among five healthy purified protein derivative-positive subjects was 1/7,600 ± 4,300 compared with 1/16,000 ± 7,000 in six purified protein derivative-negative controls. To determine the frequencies of classically, CD1-, and nonclassically restricted cells, a limiting dilution analysis was performed. In one purified protein derivative-positive subject, 192 clones were generated using Mtb-infected dendritic cells (DC). Clones were assessed for reactivity against control autologous DC, Mtb-infected autologous DC, and HLA-mismatched CD1$^+$ targets (DC), as well as HLA-mismatched CD1$^-$ targets (macrophages). Of the 96 Mtb-reactive CD8$^+$ T cell clones, four (4%) were classically restricted and 92 (96%) were nonclassically restricted. CD1-restricted cells were not detected. Of the classically restricted cells, two were HLA-B44 restricted and one was HLA-B14 restricted. These results suggest that while classically restricted CD8$^+$ lymphocytes can be detected, they comprise a relatively small component of the overall CD8$^+$ T cell response to Mtb. Further definition of the nonclassical response may aid development of an effective vaccine against tuberculosis. *The Journal of Immunology,* **2000, 165: 925–930.**

It is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb).[3] Consequently, tuberculosis is a leading cause of infectious mortality worldwide, accounting for over 8 million new cases and 2.9 million deaths annually (1). Mtb is an intracellular pathogen and thus the control of infection relies on the recognition and destruction of infected cells.

In tuberculosis, there is abundant evidence to support an important role for CD4$^+$ T cell-mediated immunity (2, 3). However, several lines of evidence support a role for CD8$^+$ CTL as well. MHC class I-deficient mice, and thus CD8$^+$ T cell-deficient mice, in which the gene for β_2-microglobulin has been disrupted, are more susceptible to Mtb infection than their wild-type littermates (4), as are mice deficient in TAP (5). Silva et al. found that CD8$^+$ CTL clones generated to the Mtb heat shock protein (hsp65) could confer partial immunity to Mtb infection in mice (6). Immunization of mice with plasmids expressing Mtb Ags such as hsp65 (7), Ag 85a (8), or the 38-kDa (9) Ag have resulted in protection from subsequent challenge with Mtb and have been associated with the generation of Ag-specific CD8$^+$ CTL. In addition, Stenger et al. have demonstrated that human CD1b-restricted CD8$^+$ CTL are able to inhibit the growth of Mtb in vitro (10). Finally, CD8$^+$ T cells have been shown to localize preferentially to the mouse lung following infection with Mtb (11, 12).

In the host response to tuberculosis infection, the role of CD8$^+$ T cells may be in the recognition and destruction of heavily infected macrophages or in the elimination of infected MHC class II-negative cells such as endothelial cells and fibroblasts. CD8$^+$ CTL may play a protective role through several mechanisms. First, these cells produce potent anti-bacterial cytokines such as IFN-γ and TNF-α in response to antigenic stimulation. The importance of these cytokines has been illustrated by the susceptibility of mice to Mtb challenge in which the genes for IFN-γ (13) and TNF-R have been disrupted (14). Additionally, CD8$^+$ CTL may play a unique role in host defense to Mtb either by virtue of preferential presentation of HLA-I-associated Ags in heavily infected cells or through the enhanced release of granular constituents. Because it has been suggested that apoptosis selectively inhibits mycobacterial growth (15, 16), CTL may play an important role by inducing apoptosis in infected cells. However, mice deficient in the expression of perforin, granzyme, or CD95 (Fas) are still able to contain infection with Mtb (17, 18). These data have been used to argue that in the mouse model, CD8$^+$ CTL may play a role in host defense to Mtb through the secretion of macrophage-activating cytokines such as IFN-γ and TNF-α. Alternatively, other components of the cytotoxic granule such as granulysin may play a direct role in the inhibition of Mtb growth (10, 19, 20).

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[3] Abbreviations used in this paper: Mtb, *Mycobacterium tuberculosis*; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; LDA, limiting dilution analysis; PPD, purified protein derivative; HS, human serum; MOI, multiplicity of infection.

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At present, the role of MHC class I-restricted $CD8^+$ CTL in human immunity to tuberculosis remains largely unexplored. We have previously demonstrated the existence of human Mtb-reactive $CD8^+$ CTL to Mtb. These cells are present preferentially in persons infected with Mtb, are cytolytic, and are capable of IFN-γ production in response to Mtb-infected targets. While partially inhibited by anti-MHC class I Ab, they are not restricted to the MHC class I A, B, or C alleles. We demonstrated that these cells recognize a protein Ag that is generated in the proteasome, but that does not require transport through the Golgi endoplasmic reticulum. Our data suggested the possible use of nonpolymorphic MHC class Ib Ag-presenting structures other than the group 1 CD1 Ags (21). Interestingly, Canaday et al. (22) have described Mtb-reactive $CD8^+$ T cells that while inhibited by anti-class I Ab were not inhibited by brefeldin A. Whether or not these cells were classically or nonclassically restricted was not investigated (22).

Monocyte-derived dendritic cells (DC) pulsed with mycobacterial chloroform/methanol extract (23, 24) have been used to elicit Mtb-reactive CD1-restriced $CD8^+$ T cells. Similarly, HLA-B44-, B52-, and A*0201-predicted binding peptides for ESAT-6 (25) or an HLA-A*0201-predicted binding peptide for the 19-kDa Ag (26) have been used to elicit $CD8^+$ T cells that are reactive with Mtb-infected DC in an HLA-Ia-restricted manner. One limitation of these studies is that they have relied upon T cells stimulated in vitro with synthetic Ag. Hence, the relative contribution of these restriction specificities in the human host response to mycobacterially infected cells is unknown.

In this report, an IFN-γ enzyme-linked immunospot (ELISPOT)-based limiting dilution analysis (LDA) is described and is used to determine the distribution of both classically and nonclassically restricted Mtb-reactive $CD8^+$ T cells in two healthy purified protein derivative (PPD)-positive subjects. This method will allow for the detailed analysis of Mtb-specific T cell responses in a wide variety of subjects. Healthy, Mtb-infected individuals have developed a successful host response to ongoing mycobacterial infection. It is hoped that a more detailed understanding of this response in comparison to those with active disease may elucidate the immunopathogenesis of tuberculosis and hence allow for more rational vaccine design.

Materials and Methods

Human subjects

Subjects were recruited from employees at Harborview Medical Center, The Fred Hutchinson Cancer Research Center, Corixa Corporation, and Oregon Health Sciences University. PPD responses were determined by the employee health service at the respective institutions. Protocols for venipuncture and apheresis were Institutional Review Board approved. HLA typing was performed on PBMC by the Puget Sound Blood Center.

Monoclonal Abs and reagents

Culture medium consisted of RPMI 1640 supplemented with 10% FBS (BioWhittaker, Walkersville, MD), 50 μg/ml gentamicin sulfate (BioWhittaker), 5×10^{-5} M 2 ME (Sigma, St. Louis, MO), and 2 mM glutamine (Life Technologies, Grand Island, NY). For the elicitation of Mtb-reactive T cell clones, RPMI 1640 was supplemented with 10% human serum (HS). Mtb (H37Rv) was obtained from American Type Culture Collection (Manassas, VA) and grown in modified Middlebrook 7H9 media (Difco, Detroit, MI). After the preparation of glycerol stocks, aliquots were frozen and subsequently titered on Middlebrook 7H10 plates (Becton Dickinson Microbiology Systems, Cockeysville, MD).

Generation of peripheral blood DCs and macrophages

Monocyte-derived DCs were prepared according to the method of Romani et al. (27). Briefly, PBMC were isolated from heparinized blood by centrifugation over Ficoll-Hypaque (Sigma) and washed three times with culture medium. Alternatively, PBMC were obtained via leukapheresis. Cells were resuspended in AIM-V medium (BioWhittaker) and allowed to adhere to a T-75 (Costar, Cambridge, MA) flask at 37°C for 1 h in the presence of 10 ng/ml of GM-CSF (Immunex, Seattle, WA). After gentle rocking, nonadherent cells were removed, and 30 ml of AIM-V containing 10 ng/ml of IL-4 (Immunex) and 30 ng/ml of GM-CSF (Immunex) was added. After 18 h, the media was removed and centrifuged, and the cell-conditioned media was placed on the adherent cells. After 5–7 days, cells were harvested with cell-dissociation media (Sigma). To generate macrophages, PBMC were adhered to a T-75 flask as described above and cultured in the absence of cytokine. When prepared in AIM-V, peripheral blood-derived DC were CD1a positive and CD14 negative, whereas macrophages were CD14 positive and CD1a negative.

Flow cytometry

Cells to be analyzed for cell-surface marker expression were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum (Sigma), 2% normal goat serum (Sigma), and 2% human serum to prevent nonspecific binding of mouse Ig. Cells were washed in FACS buffer (PBS containing 0.5% FBS and 0.02% sodium azide) and incubated with either FITC-conjugated anti-CD1a, anti-CD1b, CD4, anti-CD8, anti-CD14, anti-CD56 Abs (5 μg/ml), or an FITC-IgG1 control (Becton Dickinson Immunocytometry Systems, San Jose, CA; 5 μg/ml) for 30 min at 4°C in a total volume of 50 μl. Cells were then washed, flow cytometry was performed using a FACSCalibur (Becton Dickinson), and data were collected on 10^4 viable cells.

IFN-γ ELISPOT assay

Mtb-specific effectors were detected from purified $CD8^+$ T cells by ELISPOT, as described with minor modifications (28). Briefly, 96-well nitrocellulose-backed plates (MAHA S4510; Millipore, Bedford, MA) were coated as recommended by the manufacturer with 10 μg/ml capture mouse anti-IFN-γ mAb (1-D1K; Mabtech, Nacka, Sweden) overnight at room temperature. Plates were then washed six times with PBS/0.05% Tween 20 (Sigma), blocked with RPMI 1640/10% HS for 1 h at room temperature. Irradiated autologous Mtb-infected DC (2×10^4) were used as APC. Autologous purified $CD8^+$ T cells were then added, and the plate was incubated overnight at 37°C. After washing with PBS/0.05% Tween 20, 100 μl of 1 μg/ml biotinylated secondary anti-IFN-γ mAb (7B6-1; Mabtech) was added. After 2 h of incubation at room temperature, plates were washed six times, 100 μl avidin/biotinylated enzyme (HRP) complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) was added to wells, and the plates were incubated for a further 2 h. Then, plates were washed six times, and 100 μl 3-amino-9-ethylcarbazole substrate (Vectastain AEC substrate kit, Vector Laboratories) was added. After 4–7 min, the colorimetric reaction was stopped by washing with distilled water, and plates were air dried. Spots were quantitated using a Zeiss Axioplan 2 microscope with 3200 K incident illumination equipped with a Epiplan Neofluar 5×/0.15 objective, Sony DXC 950 CCD camera, Märzhäuser scanning stage, MCP4 control unit, Pentium PC computer, and KS ELISPOT software (Carl Zeiss Vision, Hallbergmoos, Germany).

Rapid generation of Mtb-reactive $CD8^+$ T cell clones

A total of 1×10^6 monocyte-derived DCs were cultured overnight in the presence of Mtb (H37Rv; multiplicity of infection (MOI), 5) in low-adherence 16-mm wells (no. 3473; Costar). After 18 h, the cells were harvested and resuspended in RPMI 1640/10% HS. A total of 2×10^4 Mtb-infected DC were seeded into each cloning well.

$CD8^+$ T cells were purified from PBMC first by negative selection using $CD4^+$ Ab-coated magnetic beads and then positive selection using CD8 Ab-coated magnetic beads per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Flow cytometric analysis confirmed that the cells were >98% CD8 positive. Ag-specific T cells were seeded by limiting dilution at 25 cells per well in the presence of 1×10^5 irradiated (3500 rad using a ^{137}Cs source) autologous PBMC and rIL-2 (10 ng/ml). Cell culture media consisted of 200 μl of RPMI 1640 supplemented with 10% HS. Wells were assessed for growth between 10–14 days. Wells exhibiting growth were selected and transferred to a new plate for further analysis.

Evaluation of clonal responses to Mtb-infected APCs

DC and macrophages were prepared in AIM-V, harvested, and incubated in the presence of Mtb (MOI, 50). After 18 h, cells were harvested, washed, and seeded at $2–4 \times 10^4$ cells per well in RPMI 1640/10% HS in ELISPOT plates coated with IFN-γ mAb. Aliquots of each clone (25 μl) were then added and the ELISPOT assay completed after 18 h incubation at 37°C. Assays were performed in the presence of IL-2 (1 ng/ml).

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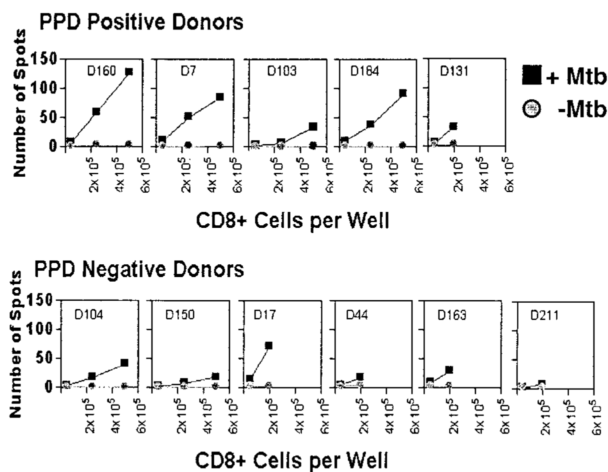

FIGURE 1. Mtb-reactive CD8$^+$ effectors can be detected from PBMC of PPD$^+$ and PPD$^-$ individuals. Monocyte-derived DC were infected with Mtb and used as APC in an IFN-γ ELISPOT assay where autologous CD8$^+$ lymphocytes that had been positively selected from CD4$^+$ lymphocyte-depleted PBMC using immunomagnetic beads as the effectors. After 18 h of coincubation, ELISPOT plates were developed, and spots were enumerated. Results of two experiments are presented. These experiments are representative of at least two separate effector frequency determinations for each donor. For example, three independent determinations for D160 CD8$^+$ effector cell frequency have yielded frequencies of 1/4000, 1/3800, and 1/4500 (mean, 1/4100 ± 400).

Table I. *Effector frequencies of Mtb-reactive CD8$^+$ T cells*[a]

	Donor	Frequency
PPD positive	160	1/4,000
	7	1/5,900
	103	1/16,000
	184	1/5,600
	131	1/6,300
Average ± SD		1/7,600 ± 4,300
PPD Negative	104	1/13,000
	150	1/29,000
	17	1/2,900
	44	1/13,000
	163	1/7,200
	211	1/29,000
Average ± SD		1/16,000 ± 11,000

[a] Effector cell frequencies are calculated using linear regression analysis from the data shown in Fig. 1.

Expansion of T cell clones

To expand the CD8$^+$ T cell clones, a rapid expansion protocol using anti-CD3 mAb stimulation was used (29). T cell clones were cultured in the presence of irradiated allogeneic PBMC (25 × 10^6), irradiated allogeneic lymphoblastoid cell line (5 × 10^6) and anti-CD3 mAb (30 ng/ml; Chiron, Emeryville, CA) in RPMI 1640 media with 10% HS in a T-25 upright flask in a total volume of 30 ml. The cultures were supplemented with IL-2 (1 ng/ml) on days +1, +4, +7, and +10 of culture. The cell cultures were washed on day +4 to remove remaining soluble anti-CD3 mAb.

Results

Mtb-specific CD8$^+$ effector cell frequencies from PBMC from Mtb-infected and uninfected individuals

Previous work by others and ourselves has suggested that Mtb-specific CD8$^+$ T cells are found preferentially in persons infected with Mtb (21, 25, 26, 30, 31). These studies have relied on the generation of lines or on peptide-specific responses and thus may be limited either by their sensitivity or in their ability to reflect a diverse immune response. To determine the frequency of Mtb-specific CD8$^+$ effector cells, monocyte-derived DC were generated from a panel of five healthy Mtb-infected individuals and six PPD-negative controls. Monocyte-derived DC were infected with Mtb and used as APC in an IFN-γ ELISPOT assay using autologous magnetic bead-purified CD8$^+$ T lymphocytes as effectors. Results from two experiments are presented in Fig. 1, and the derived effector cell frequencies are presented in Table I. All five of the Mtb-infected individuals had strong Mtb-specific CD8$^+$ T cells responses, with effector frequencies ranging from 1:4,000 to 1:16,000 (1:7,600 ± 4,300). Interestingly, all six of the PPD-negative individuals also had demonstrable Mtb-reactive CD8$^+$ T cells (range, 1:2,900 to 1:29,000; mean, 1:16,000 ± 11,000; $p > 0.6$, Student's *t* test). These responses were all at least 2 SDs above the uninfected DC control and were within the reported sensitivity of the IFN-γ ELISPOT-based LDA (28). Thus, IFN-γ ELISPOT can be used to determine effector cell frequencies in both Mtb-infected and naive individuals and would suggest that an LDA-based approach can be used to further characterize the CD8$^+$ T cell response in both subject groups.

In two Mtb-infected donors, nonclassically restricted, non-CD1-restricted cells constitute the majority of the CD8$^+$ T cell response

To determine the frequencies of classically, CD1-, and nonclassically restricted cells, a modified LDA was performed. To avoid bias that might be introduced by repeated in vitro stimulation of T cells lines, we devised a strategy that would allow for the evaluation of clonal T cell responses after a single in vitro stimulation with Mtb-infected DC and would allow for subsequent expansion and characterization of these clones. In brief, magnetic bead-purified CD8$^+$ T cells were seeded at 25 cells/well into 96-well cloning plates containing Mtb-infected DC as APC (MOI, 5), irradiated autologous PBMC feeders, and IL-2. After 12 days, 192 wells exhibiting growth were transferred to a second 96-well plate to facilitate subsequent analysis. IFN-γ ELISPOT was chosen for the analysis because of its ability to detect small numbers of T cells (in our hands, <20 cells using both CD4 and CD8 T cell clones; data not shown). T cell clones were classified as either classically or nonclassically restricted based upon recognition of Mtb-infected autologous or HLA-A, B-mismatched targets (Fig. 2). To distin-

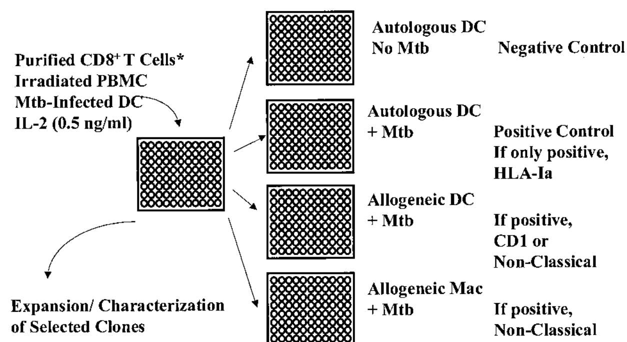

FIGURE 2. Strategy for analysis of classically vs nonclassically restricted T cell clones. A clone is defined as nonspecific if it reacts to all four target cells. A clone is defined as classically HLA restricted if it only recognizes autologous Mtb-infected APC. A clone is defined as nonclassically restricted if it recognizes all Mtb-infected APC. A clone is defined as CD1-restricted if it recognized Mtb-infected DC but not macrophages.

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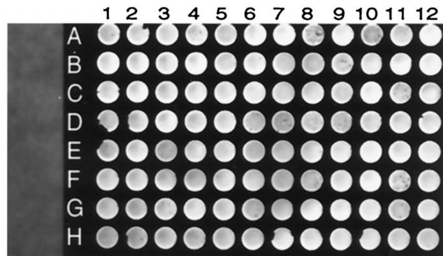

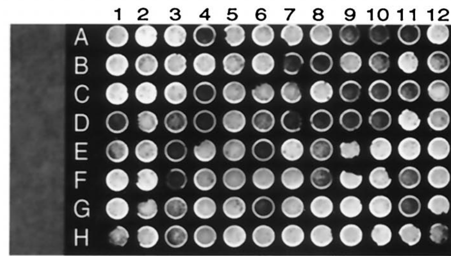

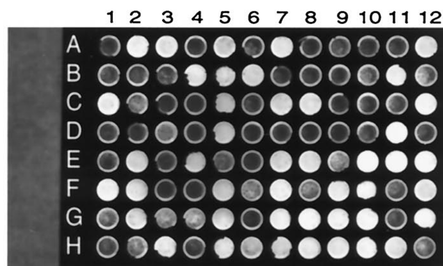

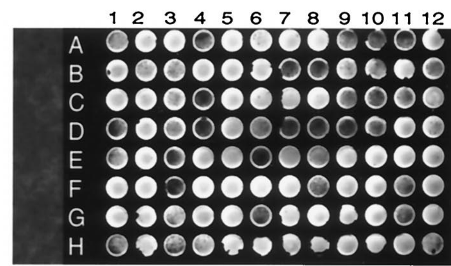

FIGURE 3. Discrimination of classically vs nonclassically restricted T cell clones using ELISPOT-based LDA. Lymphocytes from a healthy, PPD$^+$ donor were twice depleted of CD4$^+$ T cells using immunomagnetic beads and then positively selected for CD8$^+$ lymphocytes in the same fashion. Cells were seeded at 25 cells/well with Mtb-infected (MOI, 5) autologous DC and irradiated autologous feeders. After 12 days, clones (>95% confidence; Poisson analysis) were transferred to a new plate. Twelve percent of each clone was transferred to an ELISPOT plate containing either autologous (D160) or HLA-mismatched (D173) APC. Where indicated, APC were previously infected with Mtb (MOI, 50). After 18 h, the ELISPOT was developed and scored. Shown is one of two plates.

guish group 1 CD1-restricted from nongroup 1 CD1-restricted responses, peripheral blood-derived DC and 5-day adherent macrophages were generated from the HLA-A, B-mismatched donor in AIM-V serum-free media. AIM-V was used to ensure expression of group 1 CD1 Ags. Peripheral blood-derived DC were confirmed by flow cytometry to be CD1a and CD1b positive, whereas macrophages were CD1a and CD1b negative (data not shown). One-eighth of each clone was assessed for its ability to recognize HLA-matched and -mismatched Mtb-infected targets (Fig. 3). Of 192 clones tested, 70 did not produce IFN-γ. Six produced IFN- γ in response to uninfected DC and were termed nonspecific. Twenty-four were HLA restricted, in that IFN-γ was produced only in response to autologous Mtb-infected DCs. The remaining 92 were nonclassically, nongroup 1 CD1 restricted in that IFN-γ was produced in response to all Mtb targets, including the group 1 CD1-negative macrophages; representing 81% of the Mtb-reactive clones. As an example, the clone tested in position B2 (Fig. 3) responds only to D160 Mtb-infected APC and thus would be termed HLA restricted, whereas the clone tested in A4 responds to all Mtb-infected APC and is thus nonclassically restricted.

To further characterize the HLA-restricted clones, all twenty-four clones were expanded using anti-CD3, as were 20 of the nonclassically restricted clones. Of the 24 HLA-restricted clones, four were CD8$^+$ and 20 were CD4$^+$, while the nonclassically restricted clones were all CD8$^+$. All expressed the $\alpha\beta$ TCR and were negative for NK markers CD16 and CD56. All expanded clones were retested for specificity against HLA-matched and -mismatched DC and macrophages. None of the nonclassically restricted cells exhibited reactivity against uninfected mismatched DC.

To define a restricting MHC class I allele for classically restricted Mtb-reactive CD8$^+$ T clones, a panel of DCs was generated that matched the CD8$^+$ T cells at a single HLA-A or B locus. As shown in Fig. 4, clone 1-6F is HLA-B14 restricted because it is reactive with D44-infected DC who match D160 at HLA-B14. Clones 1-1B and 1-4F are HLA-B44 restricted because they are reactive with D131 and D184 DC, both of which share the HLA-B44 allele with D160. Interestingly, one of the clones is neither HLA-A nor HLA-B restricted.

To extend these results, an identical ELISPOT-based LDA was performed on a second PPD$^+$ donor (D184). In this analysis, 26% of the CD8$^+$ Mtb-reactive T cells were found to be HLA-Ia restricted, with the remaining 74% nonclassically restricted. Group 1 CD1-restricted T cells were not detected.

Discussion

In this report, we have used an IFN-γ ELISPOT-based analysis to estimate the frequency of Mtb-reactive CD8$^+$ T cells from peripheral blood and to define the relative frequencies of both classically and nonclassically restricted Mtb-reactive CD8$^+$ T cells. All of the Mtb-infected donors who were tested have strong CD8$^+$ T cell responses to Mtb-infected DCs, suggesting these responses may

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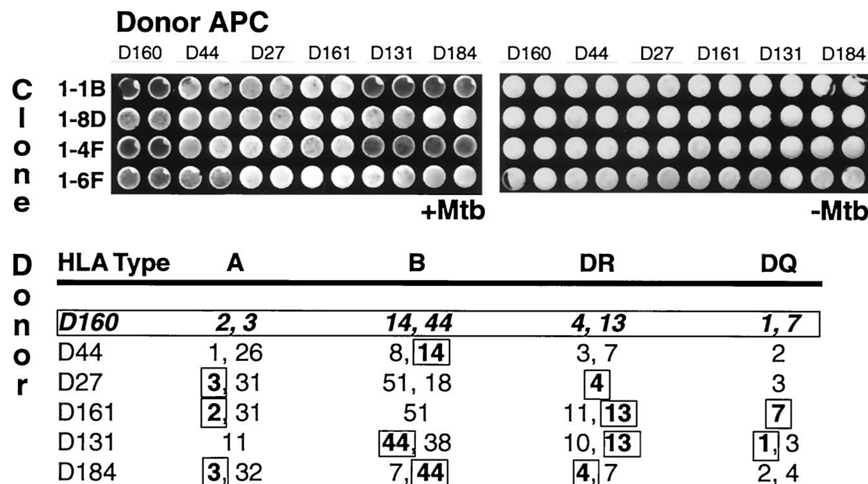

HLA Type	A	B	DR	DQ
D160	2, 3	14, 44	4, 13	1, 7
D44	1, 26	8, 14	3, 7	2
D27	3, 31	51, 18	4	3
D161	2, 31	51	11, 13	7
D131	11	44, 38	10, 13	1, 3
D184	3, 32	7, 44	4, 7	2, 4

FIGURE 4. Restriction specificity of HLA-restricted clones. All 24 HLA-restricted clones were expanded using anti-CD3 mAb. Of the 24, 20 were CD4$^+$. The remaining four were CD8$^+$. These were tested for reactivity against Mtb-infected DC that matched at a single HLA-A or -B locus. Clones 1-1B and 1-4F are HLA-B44 restricted, while clone 1-6F is HLA-B14 restricted. Where expanded and retested, all nonclassically restricted cells have been CD8$^+$.

represent a recall response to Mtb. Interestingly, the PPD$^-$ individuals had demonstrable Mtb-reactive CD8$^+$ T cells. It is possible that these responses represent exposure to atypical mycobacteria or that they are qualitatively different from those found in Mtb-infected donors. Although the Mtb-specific effector cell frequencies were not statistically different between the PPD-positive and -negative groups, larger subject groups will be assessed to determine whether or not CD8$^+$ T cell responses in those who are Mtb infected are distinct from naive individuals. The prevalence of Mtb-reactive CD8$^+$ lymphocytes in PPD$^-$ individuals may have been previously underestimated by others and ourselves due to the fact that ELISPOT-based estimates are more sensitive than those derived from the generation of T cell lines.

Mtb-infected DC were used in a modified LDA to generate a panel of T cell clones from peripheral blood from one of two PPD$^+$ donors. Using the IFN-γ ELISPOT, each clone was assessed for its ability to recognize HLA-matched and -mismatched targets and thus to infer its restriction specificity. In these individuals, the majority of the Mtb-reactive CD8$^+$ T cells were nonclassically and nongroup 1-CD1 restricted, suggesting that these cells may represent the immunodominant CD8$^+$ T cell response to Mtb. Therefore, if these results can be extended to additional Mtb-infected healthy individuals, then further characterization of these cells may facilitate the rational design of an effective vaccine.

For the classically restricted clones, and a subset of the nonclassically restricted clones from one donor, these results were confirmed by expansion and further characterization of these Mtb-specific T cell clones. While in the minority, Mtb-infected DC also stimulated HLA-Ia-restricted T cells. This represents the first report of the elicitation and cloning of MHC class Ia-restricted cells directly from Mtb-infected APC. One advantage of this approach is that we have allowed the immune system to select the Ag specificity and restricting allele, thus strengthening the argument that these responses reflect recall responses to Mtb. Interestingly, two clones were HLA-B44 restricted and one was HLA-B14 restricted, whereas none were restricted by HLA-A alleles. It is possible that the one remaining clone is HLA-C restricted. Whether or not HLA-B and possibly C restriction plays a unique role in the host response to Mtb will be the subject of future investigations, although HLA-A*0201-specific responses to Ags ESAT-6 and the 19-kDa Ag have been previously reported (25, 26).

Group 1 CD1-restricted T cell clones were not identified. In previous reports, CD1-restricted T cells have been identified from both Mtb-infected and PPD$^-$ individuals (32). Whether or not CD1-restricted responses represent a recall response to Mtb remains an important and unresolved question. Thus, it is possible that the individuals analyzed are deficient in group 1 CD1-restricted responses or that the effector frequency was below the limit of detection of this analysis. Alternatively, it is possible that the in vitro culture conditions were not favorable to the generation of group 1 CD1-restricted responses. Stenger et al. have recently reported that Mtb infection can decrease group 1 CD1 expression (33). Thus, there may have been inadequate CD1 expression on our Mtb-infected DC to stimulate CD1-restricted T cells. Finally, clones were analyzed initially on the basis of IFN-γ production. Thus, clones that grew in response to Mtb, but did not produce IFN-γ, would not have been detected.

The approach described in this report is well suited to the analysis of populations of Ag- or pathogen-stimulated T cells from peripheral blood with minimal in vitro manipulation. In this report, we were able to detect and characterize Mtb-specific HLA-Ia-restricted T cells that comprised a relatively small component of the overall CD8$^+$ T cell response to Mtb. Conventional bulk culture-based techniques are not well suited to such subpopulation analysis. With regard to Mtb, it is hypothesized that individuals who are healthy Mtb infected (PPD$^+$), Mtb infected with active tuberculosis (PPD$^-$), and those infected with bacillus Calmette-Guérin may have distinct CD8$^+$ T cell responses. Thus, this analysis will be extended to these subject groups in future studies. Alternatively, the approach is well suited to any situation where T cells can be distinguished on the basis of selective responses to APC. Examples include restriction specificity as described herein or antigenic specificity using protein or peptide pulsed APC.

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