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Human Alveolar T Lymphocyte Responses to *Mycobacterium tuberculosis* Antigens

Role for $CD4^+$ and $CD8^+$ Cytotoxic T Cells and Relative Resistance of Alveolar Macrophages to Lysis[1]

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T cell-mediated cytotoxicity against *Mycobacterium tuberculosis* (MTB)-infected macrophages may be a major mechanism of specific host defense, but little is known about such activities in the lung. Thus, the capacity of alveolar lymphocyte MTB-specific cell lines (AL) and alveolar macrophages (AM) from tuberculin skin test-positive healthy subjects to serve as CTL and target cells, respectively, in response to MTB (H37Ra) or purified protein derivative (PPD) was investigated. Mycobacterial Ag-pulsed AM were targets of blood CTL activity at E:T ratios of ≥30:1 (^{51}Cr release assay), but were significantly more resistant to cytotoxicity than autologous blood monocytes. PPD- plus IL-2-expanded AL and blood lymphocytes were cytotoxic for autologous mycobacterium-stimulated monocytes at E:T ratios of ≥10:1. The CTL activity of lymphocytes expanded with PPD was predominantly class II MHC restricted, whereas the CTL activity of lymphocytes expanded with PPD plus IL-2 was both class I and class II MHC restricted. Both $CD4^+$ and $CD8^+$ T cells were enriched in BL and AL expanded with PPD and IL-2, and both subsets had mycobacterium-specific CTL activity. Such novel cytotoxic responses by $CD4^+$ and $CD8^+$ T cells may be a major mechanism of defense against MTB at the site of disease activity. ***The Journal of Immunology,*** **1997, 159: 290–297.**

Tuberculosis (TB)[3] continues to be a major health problem worldwide for which no effective protective vaccine exists (1, 2). While host defense against intracellular pathogens such as *Mycobacterium tuberculosis* (MTB), the etiologic agent of TB, involves natural resistance, the acquired immune response conferred by Ag-specific T cells and macrophages is critical for control of MTB infection. Thus, for improvement in control and prevention of TB through immunotherapy and vaccines, it is essential that the acquired human immune response to MTB be better understood.

Ag-specific T cells contribute to antimycobacterial defenses either through activation of macrophages for better killing of microbes via secreted lymphokines or cell contact or through lysis of the infected macrophage by CTL. The protective immune response to MTB in mice is conferred by $CD4^+$ and $CD8^+$ T cells (reviewed in Ref. 3). In humans, traditionally $CD4^+$ T cells are thought to have the major role in antimycobacterial immunity, including activation of or cytotoxicity for macrophages (3). Furthermore, human γδ T cells are activated by MTB, although their role in the protective immune response to MTB is less well defined (4).

Most studies of the human immune response to mycobacterial Ags have focused on blood cells. TB, however, is acquired primarily through inhalation of MTB bacilli, and the lung is a major site of disease manifestation. Alveolar macrophages (AM), which line the epithelial surface of pulmonary alveoli, are believed to be important in the initial containment of the micro-organisms through nonspecific or natural immune mechanisms. AM also phagocytose particles and microbial organisms, and carry them via lymphatics to regional hilar lymph nodes, where specific immune responses are believed to be generated. Thus, AM may be involved in specific immune responses to MTB both as accessory cells in induction of responses and as targets of CTL in the regional lymph nodes. Alternatively, AM may be targets of CTL during reinfection with MTB when tuberculous Ag-specific T cells have already been acquired. AM differ substantially from their precursors, blood monocytes (MN), for a wide spectrum of immune reactivities, including effector and immunoregulatory functions (5–7). The capacity of MTB-infected AM to serve as target cells of Ag-specific CTL is unknown, however.

Lymphocytes are the other major immune effector cell found in lung alveoli (8, 9). Trafficking of these lymphocytes also occurs between hilar lymph nodes, where immune responses are generated, and alveolar spaces. The responses of sensitized alveolar lymphocytes (AL) might, therefore, be expected to be protective in the immunized or in the naturally exposed and infected host. Little is known, however, about the ability of AL to serve as Ag-specific immune effector cells in antimycobacterial immunity.

This study was undertaken to determine the ability of AM to serve as target cells for mycobacterium-specific CTL and the ability of lymphocytes from alveoli to serve as a source of mycobacterial

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[2] Address correspondence and reprint requests to Dr. Elizabeth Rich, Department of Medicine, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4984. E-mail ear7@po.cwru.edu

[3] Abbreviations used in this paper: TB, tuberculosis; MTB, *Mycobacterium tuberculosis*; AM, alveolar macrophages; MN, monocytes; AL, cytotoxic T lymphocyte lines from alveolar lymphocytes; BL, cytotoxic T lymphocyte lines from blood lymphocytes; PPD, purified protein derivative of *Mycobacterium tuberculosis*; BAL, bronchoalveolar lavage; PHS, pooled human serum; ICAM-1, intracellular adhesion molecule-1; LAK, lymphokine-activated killer.

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Ag-specific CTL. Although mycobacterial Ag-pulsed AM could be specifically lysed by CTLs, they were significantly more resistant to such lysis than were autologous MN. AL served effectively as mycobacterial Ag-specific CTL against MN. Surprisingly, both $CD4^+$ and $CD8^+$ T cells derived from blood lymphocyte (BL) and AL cell lines expressed MTB-specific CTL activity.

Materials and Methods

Subjects

Healthy adult volunteers were recruited for this study ($n = 11$). These individuals were 36 ± 8 yr of age (mean ± SD). Seven subjects were male, and four were female. Two of the volunteers were smokers, and the others had never smoked. All subjects had positive skin test reactions against purified protein derivative of MTB (PPD). Each subject underwent venipuncture and fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) after giving written consent, and the protocols were approved by the institutional review board of University Hospitals of Cleveland.

Preparation of MN and AM

PBMC were prepared from whole blood by sedimentation over Ficoll-Paque (Pharmacia-Biotech, Uppsala, Sweden). MN were isolated by adherence of PBMC to plastic tissue culture petri dishes precoated with pooled human serum (PHS) as described previously (10). Briefly, nonadherent cells were removed from petri dishes after incubation at 37°C for 1 h. Plastic adherent cells were covered with HBSS without calcium or magnesium (BioWhittaker, Walkersville, MD) and were incubated at 4°C for 30 min. Cells then were scraped with a plastic cell scraper, washed, and resuspended to 10^6 cells/ml in 10% heat-inactivated PHS in RPMI supplemented with L-glutamine and penicillin (supplemented RPMI). Adherent cells were >95% viable as determined by exclusion of trypan blue and were 88 to 92% MN as determined by Wright's, peroxidase, and nonspecific esterase stains of cytocentrifuged preparations.

BAL was performed to obtain AM. The naso-oropharynx was anesthetized with 2% lidocaine. A flexible bronchoscope (type P30, Olympus BF, New Hyde, NY) was introduced through the nose, throat, and trachea with further instillation of 1% lidocaine to prevent coughing. The bronchoscope was wedged into the right middle lobe or the lingula, and 180 ml of 0.9% sterile saline was instilled in 30-ml aliquots into two different subsegments. Approximately 80 to 90% of the saline was retrieved. Lavage fluid was centrifuged at 350 × *g* for 15 min at 4°C. The cell pellet was suspended to 10^6 cells/ml in 10% PHS in supplemented RPMI. Bronchoalveolar cells were 90 to 95% AM and 6 to 10% alveolar lymphocytes by cytochemical criteria as previously described (7).

Mycobacterial Ag-specific CTL

PBMC were stimulated in 24-well plates with PPD (Lederle, Cambridge, MA; 10 μg/ml) or with the live H37Ra strain of MTB at 2.5×10^6 CFU/ml bacteria. Culture medium consisted of supplemented RPMI plus 10% PHS. After 7 to 10 days of culture at 37°C, cells were harvested, washed, then further expanded with IL-2 (Cellular Products, Buffalo, NY; 10%, v/v) for 2 to 4 days. In some experiments, IL-2 was added at the initiation of culture with PPD. PPD-stimulated T cell lines were washed to remove IL-2 and rested overnight before use in the cytotoxicity assay. The term BL will refer to mycobacterium-specific lymphocyte cell lines derived from the blood.

To prepare MTB-specific T cells from the lung, bronchoalveolar cells were obtained by BAL, washed, and suspended in 10% PHS in supplemented RPMI. The cells then were stimulated with PPD (10 μg/ml) and IL-2 (10%, v/v) for 7 to 10 days. The nonadherent cells were washed and rested overnight before being used as effector cells in the cytotoxicity assay. Alternatively, bronchoalveolar cells were adhered to nylon wool columns three times and eluted with RPMI plus 10% FCS to deplete AM. The eluted cells were resuspended in 10% PHS in supplemented RPMI to 10^6 cells/ml and were cultured with PPD (10 μg/ml) and IL-2 (10%, v/v) for 7 to 10 days, then washed and rested overnight. The term AL will refer to mycobacterial Ag-specific lymphocyte cell lines derived from lymphocytes from alveoli. The PPD-stimulated CTL populations from both blood and alveoli were >90% $CD3^+$ by FACS analysis.

Preparation of $CD4^+$ and $CD8^+$ T cells

$CD4^+$ and $CD8^+$ CTL were obtained by positive selection from the bulk CTL populations generated from PPD-stimulated PBMC or alveolar cells. Briefly, cultured lymphocytes were incubated with either anti-CD4- or anti-CD8-coated magnetic beads (Dynal, Oslo, Norway) at a ratio of 3:1 beads to cells. Cells bound to Ab-coated beads were removed by a magnet and then detached from the beads with DETACHABEADS (Dynal). By flow cytometry, the purified cells were >95% $CD4^+$ or $CD8^+$.

Cytotoxicity assay

MN and AM were incubated overnight (14 ± 2 h) at 37°C in microfuge tubes at 10^6 cells/ml in supplemented RPMI plus 10% PHS with or without PPD (20 μg/ml) or live H37Ra at various bacteria to macrophage ratios (5:1, 10:1, and 20:1). MN and AM then were washed and labeled with 100 μCi of ^{51}Cr for 1.5 h at 37°C. Target cells were washed four times and resuspended in 10% FCS at 10^5 cells/ml before being used in the cytotoxicity assay at 5000 cells/well. CTL were added as effector cells to 96-well round-bottom plates at various E:T ratios (1:1, 10:1, 30:1, and 50:1). The plate was centrifuged at 400 × *g* for 10 min, and then incubated at 37°C for 4 h. Supernatants (50 μl) were harvested, and ^{51}Cr release was measured using a gamma counter.

Spontaneous release was measured in wells containing target cells (in RPMI plus 10% FCS) alone. Maximum release was determined from target cells that had been lysed with 1% SDS. The percent specific ^{51}Cr release was calculated for each experimental group by the equation % specific release = [(cpm experimental − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)] × 100.

Specific release of 10% was considered significant cytotoxicity. Statistical analysis of data was performed using the paired *t* test for differences between Ag-pulsed vs no Ag within the same group of cells (e.g., percent specific release with Ag at one E:T ratio vs release without Ag at the same ratio) or for differences between the same variable among autologous cell types (e.g., percent peak-specific release at one E/T ratio for AM vs MN). Statistical significance was considered as $p < 0.05$.

Source of mAbs for cytotoxicity assays

Monoclonal Abs were obtained from the following sources: 9.3 F10 and L243 (anti-HLA-DR) and W6/32 (anti-HLA A, B, and C framework determinant) as hybridomas from the American Type Culture Collection (Rockville, MD), and anti-ICAM-1 and anti-LFA-1 from Boehringer Ingelheim (Ridgefield, CT) as purified Abs. IgG2a mAbs (Becton Dickinson, San Jose, CA) were used as isotypic controls. The concentration of Abs used was ~1 μg/ml.

Direct immunofluorescence and flow cytometric analysis

T cell lines/CTL were assessed by two-color flow cytometry. Cells were incubated with saturating concentrations of FITC-labeled anti-CD3 and phycoerythrin-conjugated anti-CD4, -CD8, or -γδ TCR or isotypic control mAbs and then fixed in 1% paraformaldehyde. Monoclonal Abs were purchased from Becton Dickinson. All data shown are the percentage of $CD3^+$ cells positive for a particular mAb minus the percentage of cells binding to the appropriate isotypic control.

Results

AM as targets of mycobacterial Ag-specific CTL

To determine whether MTB- or PPD-pulsed AM could serve as targets of CTL, AM from tuberculin-positive healthy subjects were infected with the H37Ra strain of MTB at various ratios of MTB to cells (5:1, 10:1, and 20:1) or pulsed with PPD (20 μg/ml) for 16 h at 37°C. CTL used in the cytotoxicity assay were autologous BL that had been prestimulated for 7 to 10 days with H37Ra or PPD. Figures 1 and 2 show the mean susceptibility of mycobacterial Ag-pulsed AM as targets for MTB (Fig. 1*A*)- or PPD (Fig. 2*A*)-stimulated BL as CTL ($n = 11$). In the absence of mycobacterial Ag, no significant target cell lysis was observed. AM prepulsed with MTB at a 5:1 MTB:AM ratio or with PPD were targets of cytotoxicity by MTB-stimulated BL (Fig. 1*A*) at E:T ratios of 30:1 and 50:1 ($p < 0.01$). Likewise, AM prepulsed with 5:1 MTB:AM ratios or with PPD were targets of cytotoxicity of PPD-stimulated BL (Fig. 2*A*) at E:T ratios of 50:1 ($p < 0.01$). Both PPD and MTB were effective inducers of CTL activity in BL. Furthermore, CTL populations were cross-reactive, since both recognized PPD- and MTB-pulsed AM.

Next, AM and MN were compared for their level of susceptibility to the cytotoxic action of mycobacterium-stimulated BL. The percent cytotoxicity against MN by MTB- or PPD-stimulated BL

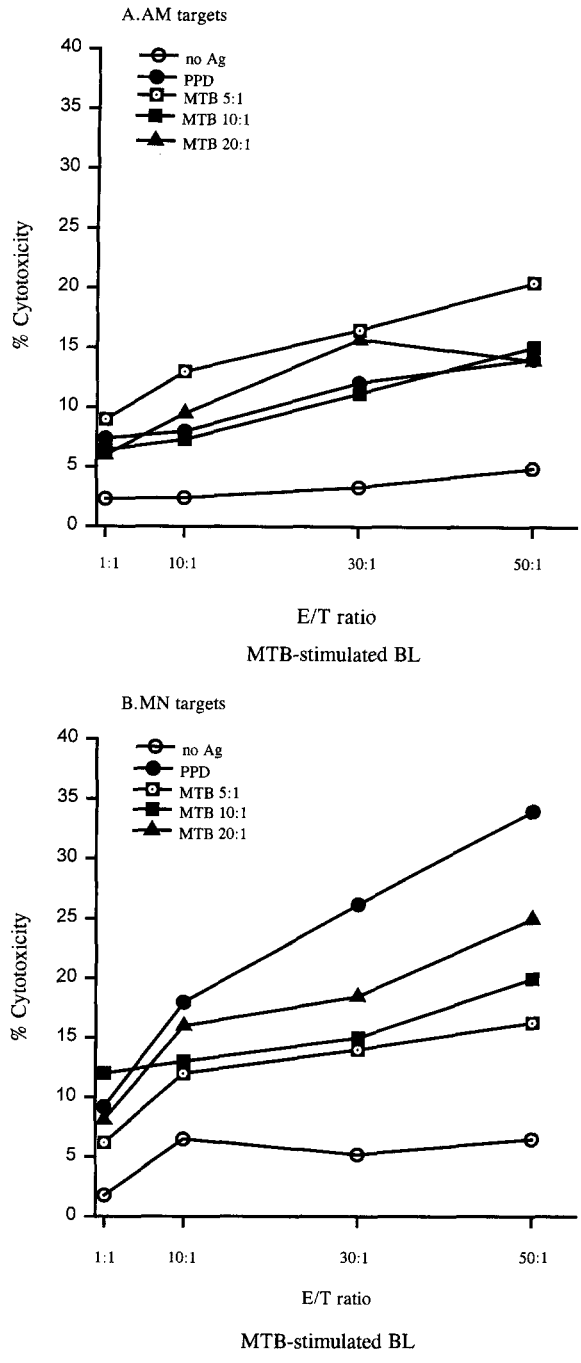

FIGURE 1. Mycobacterium-pulsed AM and MN as targets of Ag-specific cytotoxicity by BL stimulated with MTB. AM and MN were incubated overnight with no Ag, PPD (20 μg/ml), or MTB H37Ra at varying bacteria to macrophage cell ratios (5:1, 10:1, and 20:1). Targets were washed and labeled with ^{51}Cr. BL were added at varying E:T ratios (1:1, 10:1, 30:1, and 50:1), and ^{51}Cr release was measured after 4 h. *A* demonstrates AM as targets of Ag-specific cytotoxicity by BL stimulated with MTB; *B* represents cytotoxicity against MN. Data shown represent a mean of values from experiments with 11 different donors. The SD was <15% of the mean (not shown).

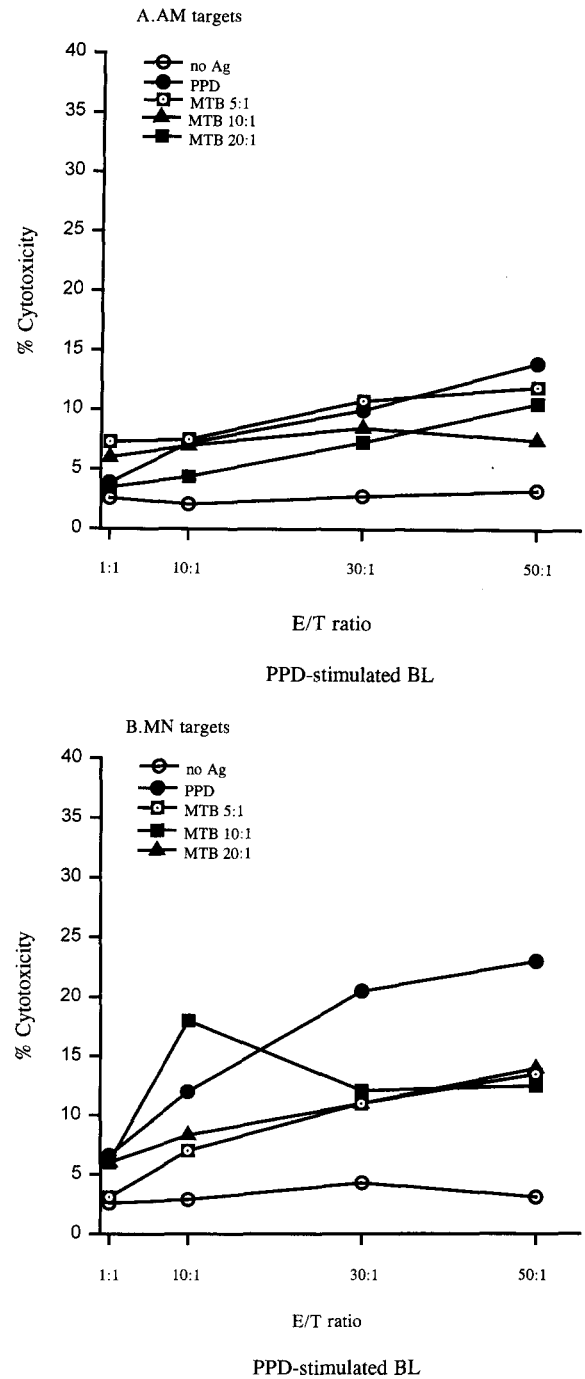

FIGURE 2. Mycobacterium-pulsed AM and MN as targets of Ag-specific cytotoxicity by BL stimulated with PPD. In studies performed concurrently with those shown in Figure 1, BL were stimulated with PPD, and the percent specific cytotoxicity of mycobacterium-stimulated AM (*A*) and MN (*B*) was determined as described in Figure 1.

is shown in Figures 1*B* and 2*B*, respectively. Unpulsed MN were not targets of cytotoxicity by stimulated BL. MN prepulsed with MTB at 5:1, 10:1, and 20:1 MTB:MN ratios ($p < 0.02$) or with PPD ($p < 0.01$) were targets of cytotoxicity by MTB-stimulated BL at E:T ratios of 10:1 and higher. Likewise, MN prepulsed with MTB at 5:1 and 20:1 MTB:MN ratios or with PPD were targets of cytotoxicity by PPD-stimulated BL at E:T ratios of 10:1 and higher ($p < 0.005$). MN (Figs. 1*B* and 2*B*) were more susceptible to cytotoxicity than AM (Figs. 1*A* and 2*A*), in that the lowest E:T ratio resulting in significant cytotoxicity for MN (for any stimulus) was 10:1, which was lower than that for AM (30:1 or 50:1) by MTB- or PPD-stimulated BL ($p < 0.03$). Furthermore, the percentage of mycobacterial Ag-specific cytotoxicity against MN at

30:1 and 50:1 E:T ratios was significantly higher than that against AM ($p < 0.03$).

AL as effector cells in mycobacterial Ag-specific cytotoxicity

The next series of experiments was aimed at determining whether mycobacterial Ag-specific T cell lines with CTL activity could be derived from lymphocytes within alveoli, which comprise 6 to 10% of bronchoalveolar cells in health. Since the number of lymphocytes available in bronchoalveolar cells is small, bronchoalveolar cells were stimulated with PPD plus IL-2 from day 0 of culture to generate a sufficient number of Ag-specific CTL to study. After 7 to 10 days of culture, nonadherent cells were >80% $CD3^+$ by flow cytometry. These AL T cell lines were used in cytotoxicity assays with MTB- or PPD-pulsed autologous MN (Fig. 3*A*). Mycobacterial Ag-specific cytototoxicity of AL occurred at E:T ratios of 10:1 and higher for PPD-pulsed MN ($p < 0.05$) and at E:T ratios of 1:1 and higher for MN pulsed with MTB at infection ratios of 10:1 and 20:1 MTB:MN ($p < 0.02$). Autologous BL prestimulated with PPD and IL-2 from day 0 in culture also were cytotoxic for PPD-pulsed MN at E:T ratios of 1:1 and higher (Fig. 3*B*), and there was no significant difference in the capacity of mycobacterial Ag-specific BL and AL to function as CTL for mycobacterium-pulsed MN. These results suggest that resident AL have the same CTL precursor distribution as circulating blood cells. Neither BL nor AL stimulated with PPD plus IL-2 had CTL activity for unpulsed MN, demonstrating that PPD plus IL-2 did not induce nonspecific cytotoxic cells, such as lymphokine-activated killer (LAK) cells, in this system.

Adhesion molecules in mycobacterial Ag-specific CTL activity against AM and MN

In earlier studies, we demonstrated that MTB-specific CTL activity for MN was dependent on cell contact and could be blocked by Abs against the cell adhesion molecules, ICAM-1 and LFA-1 (11). Table I shows that mAbs against ICAM-1 inhibit PPD-specific BL CTL activity against AM as well as MN ($p < 0.001$). Anti-LFA-1 also inhibited cytotoxicity of PPD-specific BL for AM and MN ($p < 0.04$). The CTL activity of PPD-specific AL also was inhibited by both anti-ICAM-1 and anti-LFA-1 ($p < 0.01$). Thus, cell contact is required for effective mycobacterial Ag-specific CTLs from the blood and lung against AM and MN.

MHC restriction of mycobacterial Ag-specific CTL activity against AM and MN

Previous studies have demonstrated that in humans, PPD-specific CTL activity of BL against MN is primarily mediated by class II MHC-restricted CD4 T cells (3). We next determined the MHC restriction of mycobacterial Ag-specific CTL for AM. As shown in Table II, CTL activity for both AM and MN required interaction of autologous CTL and targets. Next, the effect of blocking Abs against class I and class II MHC molecules on the cytotoxic activity of autologous, PPD-specific CTL from the blood against AM and MN was studied. The CTL activity of PPD-specific BL for both AM (Fig. 4*A*) and MN (Fig. 4*B*), prepulsed with PPD, was inhibited by anti-class II HLA-DR Abs L243 and 9.3F10 ($p < 0.0004$), but not by anti-class I MHC Ab (W6/32) or by IgG2a mAb, which served as an isotypic control. Thus, most of the mycobacterial Ag-specific cytotoxicity of BL for AM and MN was class II MHC restricted. For these studies with BL CTL, IL-2 was not added to mycobacterial Ags at the initiation of culture.

In contrast to the studies with CTLs derived from peripheral blood, the CTL activity of PPD-specific AL, which were expanded with IL-2 from the initiation of culture, for PPD-pulsed MN was significantly inhibited by both anti-class II and anti-class I MHC

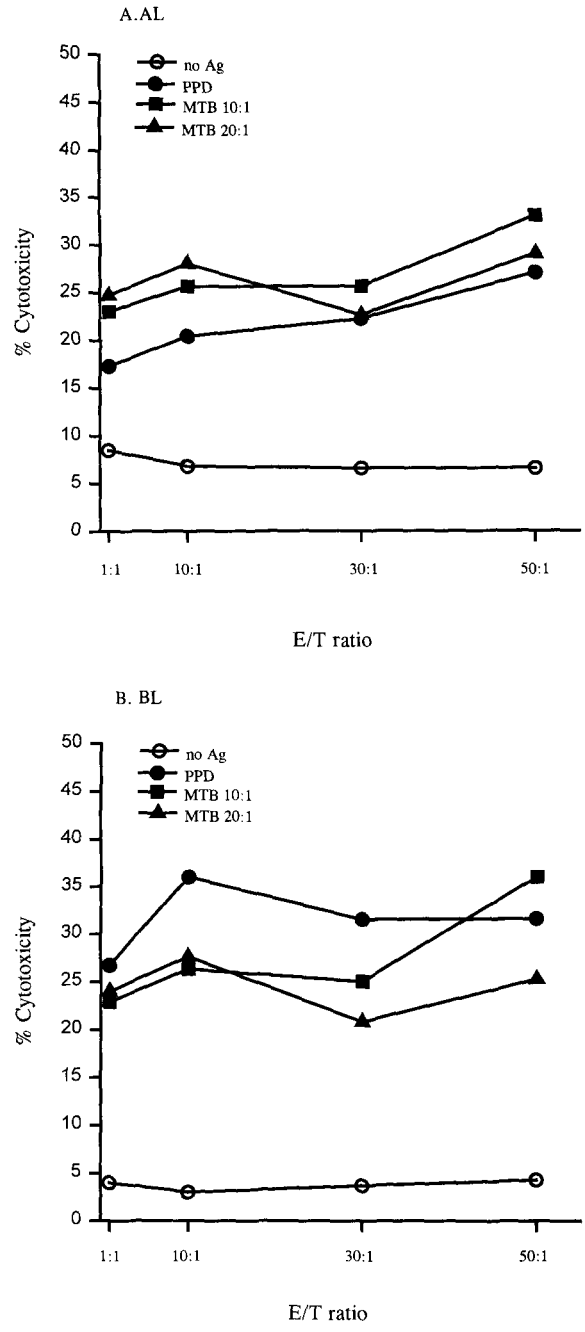

FIGURE 3. AL and BL as cytotoxic cells against MTB- or PPD-pulsed MN. MN were incubated overnight with no Ag or with PPD (20 μg/ml) or MTB H37Ra at 10:1 and 20:1 mycobacteria to macrophage cell ratios. MN were washed and labeled with ^{51}Cr. AL (*A*) or BL (*B*) stimulated with PPD and IL-2 for 7 to 10 days were rested overnight and added at varying E:T ratios to target cells. ^{51}Cr release was measured after 4 h. Data shown represent the mean ± SD ($n = 7$).

Abs (Fig. 5; L243, $p < 0.001$; 9.3 F10, $p < 0.01$; W6/32, $p < 0.01$). The results suggested that within AL both CD4 and CD8 CTLs were effector cells of mycobacterial Ag-specific lysis of autologous MN.

Role of CD4 and CD8 T cells as mycobacterial Ag-specific CTL

To assess the role of $CD4^+$ and $CD8^+$ T cells as CTLs for mycobacterial Ag-pulsed macrophages, lymphocytes from the blood

Table I. *Adhesion molecules in mycobacterial-Ag-specific CTL activity against AM and MN*[a]

Ab Added	E/T (% Cytotoxicity)		
	BL/AM	BL/MN	AL/MN
None	14 ± 7	21 ± 9	26 ± 10
Anti-ICAM-1	4 ± 3	4 ± 2	9 ± 1
Anti-LFA-1	4 ± 2	6 ± 2	1 ± 1
IgG2a	6 ± 3	26 ± 12	28 ± 8

[a] AM or MN targets pulsed with PPD were preincubated with anti-ICAM-1, anti-LFA-1, or IgG2a mAbs for 30 to 60 min. BL (prestimulated with PPD) were added to AM or MN at an E:T ratio of 30:1. In separate experiments, AL prestimulated with PPD plus IL-2 were added as effector cells to PPD-pulsed MN at an E:T ratio of 30:1. Percent release of ^{51}Cr was determined. Data represent the mean ± SD, $n = 7$. Isotype-matched IgG2a did not inhibit cytotoxicity for BL/MN and AL/MN.

Table II. *Lack of cytotoxicity of allogenic BL against MN or AM*[a]

Target Cell	Ag	Effector Cells (% Specific Cytotoxicity)	
		Autologous BL	Allogenic BL
MN	None	3	0
	PPD	23	7
	MTB	11	5
AM	None	1.5	2
	PPD	15	5
	MTB	11	5

[a] MN and AM were incubated overnight with medium alone, PPD (20 μg/ml), or MTB (MTB:macrophage ratio, 20:1). Target cells were washed and labeled with ^{51}Cr. Autologous or allogenic BL from another tuberculin-positive donor were added as effector cells at an E:T ratio of 30:1. ^{51}Cr release was measured after 4 h of incubation. The PPD-specific allogenic BL were cytotoxic for their own MN (data not shown).

and alveoli were expanded with PPD plus IL-2 from day 0, and FACS analysis was performed after 7 to 10 days. As shown in Table III, substantial numbers of both $CD4^+$ T cells and $CD8^+$ T cells were expanded by PPD plus IL-2. Furthermore, $CD8^+$ AL were not preferentially expanded compared with BL.

To directly prove that $CD8^+$ as well as $CD4^+$ T cells exerted mycobacterial Ag-specific CTL activity for mycobacterial Ag-pulsed targets, $CD8^+$ and $CD4^+$ cells were purified by positive selection from AL expanded with PPD and IL-2. Purified cells were >95% $CD4^+$ or $CD8^+$ as determined by flow cytometry. As shown in Figure 6, both $CD8^+$ and $CD4^+$T cells were cytotoxic in an Ag-specific manner for MTB-infected MN. In three experiments, mycobacterial Ag-specific cytotoxicity mediated by $CD8^+$ lymphocytes at an E:T ratio of 10:1 and an MTB:macrophage ratio of 10:1 was 20, 18, and 16% (mean, 18%; $p < 0.05$).

To determine whether the $CD8^+$ CTL population in AL was unique to AL or a result of costimulation with PPD plus IL-2, experiments were performed with PBMC stimulated with PPD plus IL-2 from the beginning of culture. Sorted PPD- plus IL-2-stimulated $CD8^+$ T cells from the blood served as Ag-specific CTL for autologous MN; in two experiments Ag-independent CTL activity was 12 and 16%, and mycobacterial Ag-specific CTL activity was 23 and 30%, respectively (E:T ratio, 30:1). $CD8^+$ T cells from a PPD skin test-negative donor showed no CTL activity either for MTB-infected or for mycobacterial Ag-pulsed MN (percent specific cytotoxicity at 30:1 MTB:MN, 7%; for mycobacterial Ag, 2%). These results suggest that the expansion and the presence of $CD8^+$ CTL were not unique to AL, but the result of stimulation with PPD plus IL-2 and that $CD8^+$ CTL may be involved in specific responses to MTB both in the lung and in the periphery.

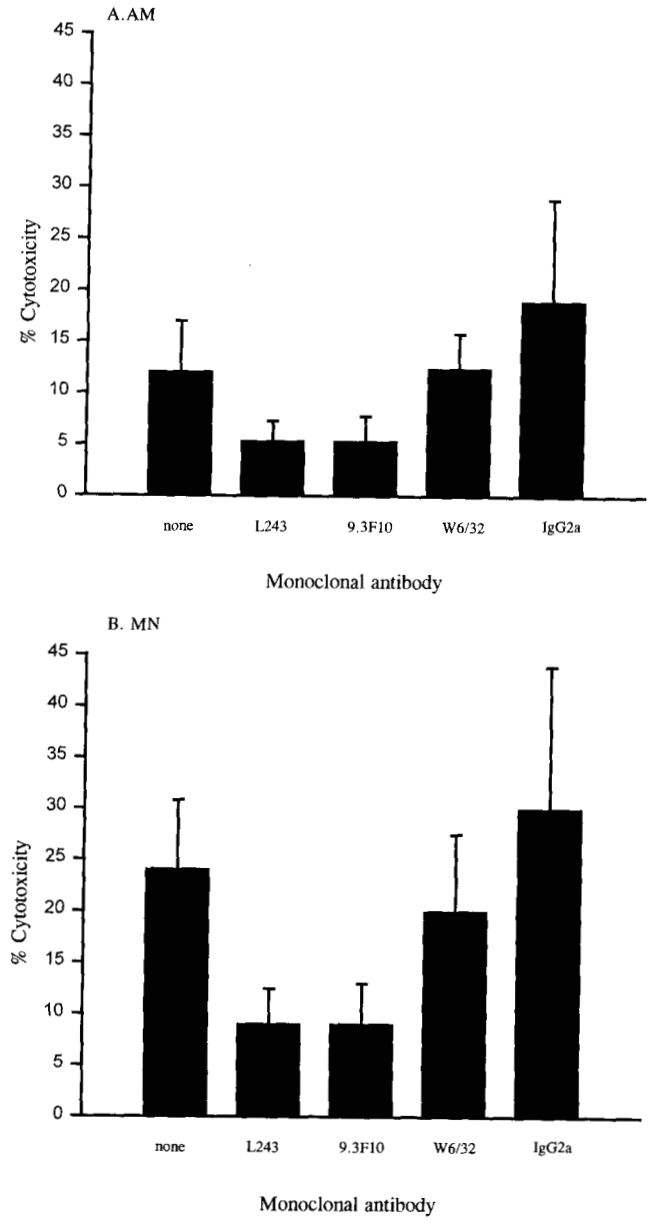

FIGURE 4. Class II MHC restriction of BL CTL activity for AM and MN. ^{51}Cr-labeled AM (*A*) or MN (*B*) targets pulsed with PPD were preincubated for 30 to 60 min with mAbs L243 and 9.3 F10 (anti-HLA-DR) and W6/32 (anti-HLA-A, -B, and -C) or with IgG2a mAb as an isotypic control. BL CTL were added at a 30:1 E:T ratio. The percent specific ^{51}Cr release was determined after incubation at 37°C for 4 h. Data shown represent the mean ± SD ($n = 10$).

Next, we assessed whether the mycobacterial Ag-specific reactivity of $CD8^+$ T cells was class I MHC restricted. MN were preincubated with or without W6/32 (5 μg/ml) or isotypic control IgG2a, then added to $CD8^+$ T cells, purified by positive selection from mycobacterial Ag-stimulated T cell lines. As shown in Figure 7, anti-class I MHC Ab specifically inhibited the reactivity of $CD8^+$ T cells in response to MTB Ag presented by MN.

Discussion

Little is known about specific immune responses to mycobacterial Ags in the lung. The results of our study demonstrate that AM can

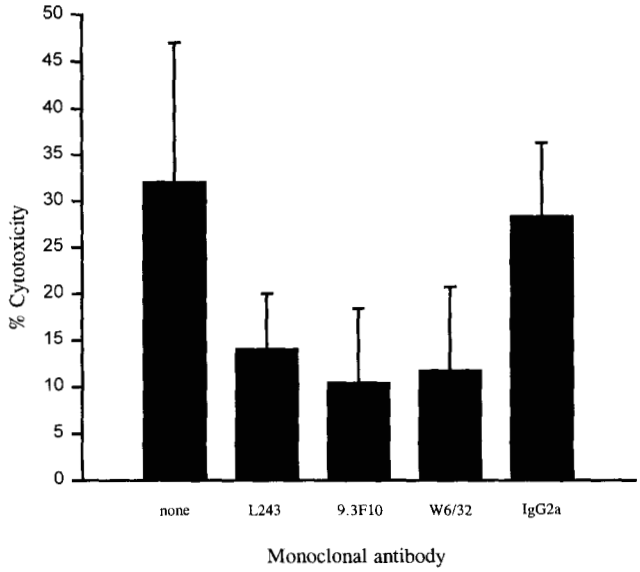

FIGURE 5. Class I and II MHC restriction of AL CTL activity. ^{51}Cr-labeled MN pulsed with PPD were preincubated for 30 to 60 min with L243 (anti-HLA-DR), 9.3 F10 (anti-HLA-DR), and W6/32 (anti-HLA-A, -B, and -C) or with IgG2a mAb as an isotypic control. AL prestimulated with PPD and IL-2 for 7 to 10 days were added at a 30:1 E:T ratio for the cytotoxicity assay. Data represent the mean ± SD of seven experiments.

Table III. *Phenotype of AL and BL after stimulation with PPD and IL-2*[a]

Effector Cells	Ag	%CD4	%CD8	%γδ
BL	None	64	23	3
	PPD	55	37	7
AL	None	71	16	4
	PPD	59	38	10

[a] AL and BL were precultured for 7 to 10 days with PPD (10 μg/ml) plus IL-2 or no Ag. Cells were analyzed by two-color flow cytometry for $CD3^+$ lymphocytes expressing CD4, CD8, or γδ T cell receptors. Data represent the mean values for three subjects: SD was <10% of the mean (not shown). The total number of BL and AL increased 5- to 10-fold when stimulated with PPD and IL-2.

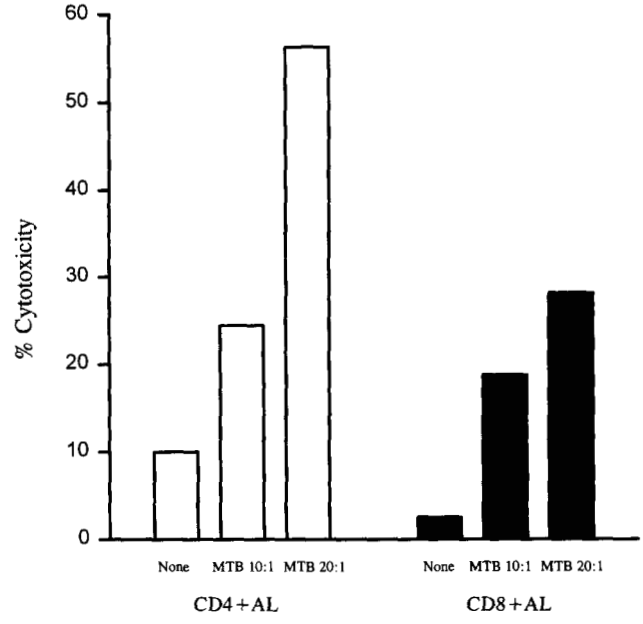

FIGURE 6. $CD4^+$ and $CD8^+$ AL as CTL against MTB-infected MN. MN were incubated overnight with no Ag or with MTB H37Ra at 10:1 and 20:1 bacteria to macrophage ratios. AL were stimulated with PPD and IL-2 for 7 to 10 days; $CD4^+$ and $CD8^+$ T lymphocytes were purified by positive selection using magnetic beads. Lymphocytes were added at a 10:1 E:T ratio. PPD-pulsed MN also were targets of $CD4^+$ and $CD8^+$ AL (data not shown). The experiment shown is representative of three for $CD8^+$ and two for $CD4^+$ T cells.

serve as targets for mycobacterial Ag-specific CTL, but are more resistant to lysis than are MN. CTL activity for AM and MN is cell contact dependent and requires MHC-matched CTL and targets. Lymphocytes from the lung contain mycobacterial Ag-specific CTL precursors, which can be expanded with mycobacterial Ag and IL-2. Surprisingly, while soluble mycobacterial Ags alone expanded class II MHC-restricted CTL, mycobacterial Ags plus IL-2 activated and expanded not only $CD4^+$ T cells, but also a class I MHC-restricted $CD8^+$ CTL population from the lung and blood that was specific for mycobacterial Ags.

In mice, the role of T cells in protective immunity was first demonstrated by North (12). Adoptive transfer of T cells from mycobacterium-immunized mice protects them from infection (13, 14). $CD4^+$ T cells are the predominant protective cells in mouse immunity to mycobacterial infections both through activation of macrophage killing of bacilli and as CTLs (3). $CD8^+$ cells from mice, however, also clearly confer protection. Adoptive transfer of cytotoxic $CD8^+$ T cell precursors induces protective immunity against TB (15). Selective depletion of $CD4^+$ or $CD8^+$ T cells renders mice more susceptible to infection with MTB (16). $CD8^+$ T cell clones from MTB-immunized mice show mycobacterial Ag-specific CTL activity (17). Furthermore, β_2 microglobulin knockout mice, which lack $CD8^+$ T cells, have enhanced susceptibility to MTB infection (18).

In humans, CD4 T cells are considered to be the primary T cell subset responsible for protective immunity both through activation of macrophages by cytokines and as CTLs for macrophages expressing mycobacterial Ags (3). In vitro, stimulation of lymphocytes from the blood with mycobacterial Ags results primarily in CD4 cell expansion (19–21). The expansion of soluble mycobacterial Ag-specific, class II MHC-restricted $CD4^+$ CTL has been demonstrated by several investigators (11, 22–27). In each of these studies, IL-2 was not used at the initiation of culture with mycobacterial Ag.

Our study is the first to demonstrate that $CD8^+$ T cells from humans can function as soluble MTB Ag-specific CTLs in a class I MHC-restricted manner, and that these cells are present among alveolar lymphocytes. Turner and Dockrell recently demonstrated that *M. bovis* Calmette-Guérin bacillus could activate human $CD8^+$ T cells in peripheral blood but this study did not show class I restriction of the CD8 cells (28). Kaleab et al. showed that Calmette-Guérin bacillus and *M. leprae* bacilli expanded CD4 and CD8 mycobacterium-specific CTL, but, like the studies of others, soluble MTB Ag (PPD) generated only CD4 T cells (26). In our study, PPD alone also expanded only $CD4^+$, class II MHC-restricted CTLs. Since lymphocytes in alveoli are limited in number, IL-2 was used to generate PPD-specific CTLs from the lung. Surprisingly, these mycobacterial Ag-specific AL were class I and class II MHC-restricted CTLs, suggesting that both $CD4^+$ and $CD8^+$ CTLs had been expanded. Flow cytometry, in fact, showed that when lymphocytes from the blood or alveoli were stimulated

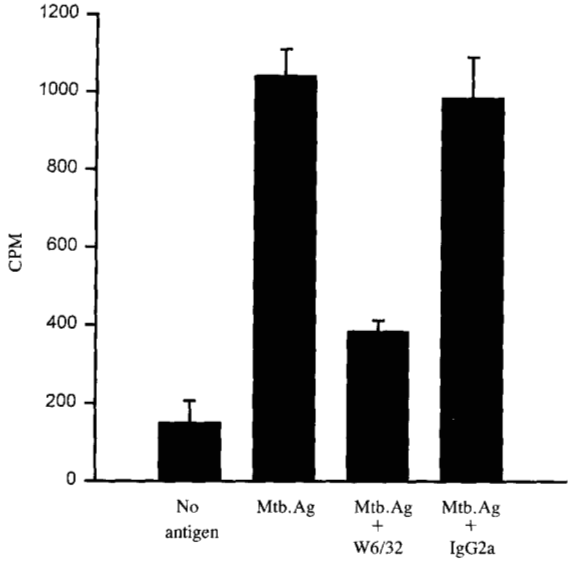

FIGURE 7. Class I MHC restriction of mycobacterial Ag-specific $CD8^+$ T cells. $CD8^+$ T cells were purified by positive selection using magnetic beads (>95% $CD8^+$ and $CD3^+$) from PBMC stimulated with soluble mycobacterial Ags for 10 days. Autologous MN (5×10^4 cells/well) as APCs were pretreated (2 h) with W6/32 (5 μg/ml), IgG2a isotypic control, or no Ab before adding $CD8^+$ T cells (2.5×10^4 cells/well) and MTB Ag. Cultures were incubated for 72 h before measuring [^{3}H]TdR incorporation (counts per minute). Results are expressed as the mean and SD of triplicate cultures and are representative of four experiments.

with both IL-2 and PPD, $CD4^+$ and $CD8^+$ T cells were expanded. Sorting experiments confirmed that not only $CD4^+$ but also $CD8^+$ T cells could function as mycobacterial Ag-specific CTLs.

In our study, no Ag-independent CTL activity was observed in cells expanded (7–10 days) in the presence of IL-2, indicating that the CTL activity was not conferred by LAK cells. Hancock et al., however, found that PPD plus IL-2 induced LAK activity at 4 days in mycobacterial-infected target cells (27). One possible explanation for this discrepancy is that their starting population of T cells was depleted of MN and had been purified with SRBC, whereas our starting population was PBMC. It is also possible that LAK cells were generated in the presence of IL-2 in our study at an earlier time point (before 7 days) and had subsequently decreased in number or activity before our assay.

Soluble Ags generally are believed to require processing and presentation through class II MHC pathways. Therefore, our finding that PPD, which is a mixture of soluble Ags of MTB, can stimulate class I MHC-restricted $CD8^+$ T cells does not fit the classical model for class I MHC Ag processing. There are two possible explanations for our results. PPD consists largely of degraded protein Ags, and thus, mycobacterial peptide fragments could bind directly to class I MHC without requirements for processing. Recent studies in murine models suggest alternative mechanisms. Soluble Ags taken up by murine macrophages through a macropinocytotic mechanism can enter the class I MHC processing pathway (29). Harding et al. have demonstrated that microbial Ags can be processed for presentation by class I MHC molecules via an alternative pathway that does not require delivery of Ags to the cytosol and bypasses the proteasome and TAP transporters (30). Additional experiments are required to determine the mechanism(s) by which MTB Ags are processed for class I MHC-restricted $CD8^+$ T cells.

The majority of people with recent MTB infection do not develop disease and presumably have developed protective immunity. The findings in the current study and that of others of CTL activity for mycobacterial Ags in healthy tuberculin-positive individuals therefore suggests that CTL may have a role in protective immunity against MTB both in the lung and peripherally. We recently showed that during active pulmonary TB, lymphocytes within alveoli are increased in number, and that the majority of these lymphocytes are T cells expressing the $\alpha\beta$ TCR (31). Both $CD4^+$ and $CD8^+$ T cells in alveoli were increased proportionately. Thus, during active disease, $CD4^+$ and $CD8^+$ T cells are involved in the local host defense response and in the immunopathogenesis of the disease. The relative protective vs immunopathogenic role of mycobacterium-specific CTL activity in the lung during active disease, however, remains to be determined. Interestingly, Kumaratne et al. demonstrated that the number of peripheral blood-derived CTLs was higher in tuberculous patients with more extensive tissue necrosis in the lung, suggesting a role for CTLs in immunopathology (24).

The alveolar spaces represent a unique microenvironment for nonspecific and specific immune responses that are largely controlled by the AM. Human AM are better able to contain the growth of MTB in vitro than their precursor blood MN, in part through increased expression of TNF (10). Thus, the natural resistance of AM against MTB exceeds that of MN. Natural resistance, however, is insufficient for protection, as demonstrated by our finding that the growth of MTB plateaus but does not decline in infected AM (10). Animal studies demonstrate that AM carry ingested particles, such as beads from alveolar spaces, to hilar lymph nodes, where presumably specific immune responses are generated (32). Indeed, hilar lymph node enlargement is a sine qua non of primary MTB infection.

Once specific responses are acquired, the capacity of AM to serve as targets of mycobacterium-specific CTL may be relevant to host defense after the primary immune response has developed within hilar lymph nodes, during activation of latent foci with release of bacilli to alveolar spaces, or upon reinfection with MTB. Human AM are potent suppressors of T cell responses to soluble stimuli (7). It is, therefore, of interest that AM did not prevent the expansion of mycobacterium-specific CTLs.

The importance of the finding that AM, while capable of being lysed by mycobacterial Ag-specific CTLs, resist such lysis compared with blood MN is speculative. Inordinate lysis of AM expressing mycobacterial Ags could be deleterious because mycobacteria could be spread quickly to other parts of the lung. On the other hand, lysis of MTB-infected macrophages in general may be advantageous to the host. We recently found that during active pulmonary TB there is an increase in immature, peroxidase-positive macrophages in the lungs (30). These macrophages are probably derived from the blood and become the cellular building blocks of granulomas. As first suggested by Kaufmann et al. (33), Ag-specific lysis by sensitized T cells of dying, infected macrophages may allow the release of bacilli to more activated and viable macrophages capable of better killing the organism.

In summary, AM and AL from PPD-reactive human subjects can function as targets and effector cells, respectively, of mycobacterial Ag-specific cytotoxicity. The lysis of AM, however, is relatively restricted. Both $CD4^+$ and $CD8^+$ CTLs can be expanded in response to mycobacterial Ag, suggesting that both these subpopulations of lymphocytes play a role in protective immunity against MTB. The relative importance of activation of macrophages vs cytotoxicity toward infected macrophages in specific protective immunity is uncertain. Nevertheless, this study suggests

that cytotoxic mechanisms against MTB-infected cells may be involved in host defense at the most common site of disease activity in TB, i.e., the lung.

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