HLA-A2-Restricted CD8\(^+\)-Cytotoxic-T-Cell Responses to Novel Epitopes in *Mycobacterium tuberculosis* Superoxide Dismutase, Alanine Dehydrogenase, and Glutamine Synthetase

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Cellular immunity is essential for protection against infections caused by intracellular pathogens, including *Mycobacterium tuberculosis* (20). Major histocompatibility complex (MHC) class I-restricted CD8\(^+\) cytotoxic T lymphocytes (CTL) contribute to the control of *M. tuberculosis* infection by mediating specific effector functions, including lysis of infected cells, direct antimicrobial activity, and release of Th1 cytokines that activate antimycobacterial mechanisms in macrophages (7, 19, 23). Still, only a small number of MHC class I-restricted CTL epitopes have been identified within a few mycobacterial proteins. The human CD8\(^+\) CTL specific for immunodominant epitopes derived from *M. tuberculosis* proteins lyse infected cells and readily release gamma interferon (IFN-\(\gamma\)) upon recognition of their targets (3, 4, 8, 11, 12, 15, 17, 21). Several mycobacterial enzymes including SodA, AlaDH, and GlnS were previously identified in culture fluids of in vitro-growing bacilli (1, 2, 22). These enzymes may have important functions in pathogenicity of mycobacteria. Their extracellular expression reflects intracellular abundance and stability and may result from bacterial autolysis (26). Whether mycobacterial superoxide dismutase (SodA), \(\alpha\)-alanine dehydrogenase (AlaDH), and \(\alpha\)-glutamine synthetase (GlnS) can induce human CD8\(^+\)-CTL responses has not been previously investigated.

First, the relative affinities and stabilization capacities of synthetic peptides selected from the *M. tuberculosis* proteins SodA, AlaDH, and GlnS for the presence of HLA-A*0201* allele-binding motifs were measured in stabilization assays with T2 cells (16, 24) (Table 1). Binding of exogenous peptides to surface HLA-A2 molecules increased levels of expression of peptide-ligand complexes in T2 cells, as detected by immunostaining with conformation-specific monoclonal antibody (MAb) MA2.1 and flow cytometry, and allowed for selection of potential CTL epitopes. Next, memory CTL responses against peptide-pulsed T2 target cells were measured in peripheral blood mononuclear cells (PBMC) obtained from HLA-A*0201*-positive tuberculosis (TB) patients (\(n = 8\)) and healthy individuals with positive tuberculin skin tests (TST\(^+\) individuals; \(n = 8\)) or negative tuberculin skin tests (TST\(^-\) individuals; \(n = 2\)). The protocol and consent forms describing the involvement of human subjects were approved by the New York University School of Medicine Institutional Review Board for the Bellevue Hospital. Whole PBMC were stimulated with each peptide for 10 to 14 days in the presence of interleukin-2 and interleukin-7 (4, 12, 21). Responding CTL were amplified by restimulation rounds with autologous or allogeneic PBMC as feeder cells. Overall, there was no correlation between relative affinities of peptides for HLA-A*0201* and their abilities to amplify specific CTL responses (Table 1). In contrast, the stability of peptide-ligand complexes on the cell surface appears to be important because complexes with half-lives of less than 6 h were not recognized by CTL. CTL specific for the peptide comprising amino acids 160 to 168 of SodA (SodA*160-168*) were found in three of eight TB patients and in two of eight TST\(^+\) individuals, while AlaDH*160-169* and GlnS*308-316* specific CTL were absent in TB patients but present in two of eight and three of four TST\(^-\) individuals, respectively. No CTL specific responses for these peptides were detected in TST\(^-\) individuals.

CTL assays demonstrated that the SodA*160-168* AlaDH*160-169* and GlnS*308-316* peptides are novel CTL epitopes as peptide-pulsed T2 cells were specifically and efficiently lysed by effectors present in stimulated PBMC from TB patients and TST\(^-\) individuals (Fig. 1A to C). The efficiency of cytolysis was clearly increased after repeated stimulations with the peptides, thus suggesting a continuous enrichment with specific effectors in these cultures. The MHC class I restriction of generated CTL was confirmed with a panel of derivatives of the MHC class I-negative 721.221 cells expressing the HLA-G, HLA-B*2705*, or HLA-A*0201* allele. As shown, only cells which expressed the HLA-A*0201* allele and were capable of presenting SodA*160-168* AlaDH*160-169* and GlnS*308-316* peptides were lysed.

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TABLE 1. CTL epitopes identified within HLA-A*0201-binding peptides from mycobacterial SodA, AlaDH, and GlnS proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptidea</th>
<th>Sequence</th>
<th>RAa</th>
<th>DC30c</th>
<th>CTL activityd</th>
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<tbody>
<tr>
<td>SodA</td>
<td>160–168</td>
<td>DMWEAFYLF</td>
<td>15.6</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>160–169</td>
<td>DMWEAFYFLQ</td>
<td>20.0</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>AlaDH</td>
<td>160–169</td>
<td>VLMGGVPGE</td>
<td>1.8  &gt;8   Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>344–353</td>
<td>GLSTHEGALL</td>
<td>11.0</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>352–361</td>
<td>LLFSDYATLD</td>
<td>20.0</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td>GlnS</td>
<td>308–316</td>
<td>GLLHHAHSPSL</td>
<td>11.6</td>
<td>&gt;8   Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>316–324</td>
<td>LLAFTNPMTV</td>
<td>4.0  &gt;8   No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>422–430</td>
<td>QLSVDIDRL</td>
<td>5.6  6     No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The sequence numbers of the first and last amino acids are shown. CTL epitope predictions were made using SodA, AlaDH, and GlnS proteins (P17670, P30234, and Q0377 or w3846, r2780, and r2220, respectively). The peptides with the highest scores in the EpiMatrix program (5) were then analyzed by the SYFPEITHI and AMPLI programs (14, 18).

b RA (relative affinity) is the ratio of the concentration of each peptide to the concentration (5 μM) of the reference peptide TAX11-19, LLGFPYPPVY, that induced 20% HLA-A*0201 expression on T2 cells in stabilization assays (24).

c DC30 (dissociation complex) is the time required for the loss of 50% of peptide-ligand HLA-A2 complexes stabilized at the T2 cell surface after overnight culture with 100 μM (each) peptides and 100 ng of β2-microglobulin/ml.

d The HLA-A*0201-positive TB patients and TST-positive donors had no MAb to CD4, thus corroborating the MHC class I restriction and CD8 dependency of these effectors (Fig. 1G to I and data not shown).

To control mycobacterial growth, MHC class I-restricted CD85 CTL are expected to recognize and respond to their target epitopes presented by M. tuberculosis-infected cells during the natural course of infection. We next examined the ability of CTL specific for SodA160–168, AlaDH160–169, or GlnS308–316 to recognize the endogenously generated epitopes and to exert effector functions against infected targets. Therefore, S1, A1, and G1 effectors were assayed for cytolysis of the HLA-A*0201-positive monocytic cell line THP-1 acutely infected with M. tuberculosis (4). All three peptide-specific effectors were able to recognize and lyse M. tuberculosis-infected THP-1 cells (Fig. 1K to M).

The importance of Th1 cytokines IFN-γ and tumor necrosis factor alpha (TNF-α) in immune response to M. tuberculosis infection is known (19). Accumulated data indicate that production of these cytokines is an essential effector function of CD85 T cells responding to the pathogen in humans and mice (10). We therefore investigated whether peptide-specific CTL can release IFN-γ and TNF-α upon recognition of their targets. First, using enzyme-linked immunosorbent assays (ELISA), we found that peptide-stimulated, CD8-enriched S1, A1, and G1 effectors released IFN-γ and TNF-α in the range of 0.8 to 1.5 ng/ml and 4.0 to 4.5 ng/ml, respectively (data not shown). Next, the frequencies of cytokine producers among effectors were determined by intracellular staining. Induction of IFN-γ release was significantly more robust in AlaDH-specific CTL, which showed a high frequency (40%) of producers, than in SodA- and GlnS-specific CTL, which showed lower frequencies (6 to 7%) (Fig. 2). TNF-α producers were detected...
FIG. 2. Production of IFN-γ and TNF-α by SodA-, AlaDH-, and GlnS-specific CTL. S1, A1, and G1 effectors were stimulated with target peptide-pulsed (pep) or nonpulsed (−) T2 cells in the presence of brefeldin A and then tested for the frequency (in percent) of cytokine producers by intracellular staining with MAb to IFN-γ and TNF-α and flow cytometry analysis: The CTL were 100% CD3+ and CD8+. The histogram profiles were obtained after gating of viable lymphocytes. The total cytokine-producing potential assessed after stimulation with phosphor myristate acetate and ionomycin was 43 to 51%; that is, 43 to 51% of these effectors were IFN-γ and TNF-α producers (data not shown). The data are representative of results from three experiments. FITC, fluorescein isothiocyanate; PE, phycoerythrin; FL, fluorescence channel; FSC, forward scatter.

among all effectors, with frequencies of about 2 to 10%. Differences in frequencies of cytokine producers may reflect the presence of effectors that have similar lytic capabilities but that bear T-cell receptors with differential avidities for the peptide-ligand complexes and/or the presence of functionally distinct CTL not secreting IFN-γ and TNF-α (13, 27).

We have identified three novel immunodominant CTL epitopes, SodA168-168, AlaDH160-165, and GlnS208-216, which are endogenously processed and presented by M. tuberculosis-infected cells. CTL precursors specific for these epitopes and possessing effector functions such as cytolysis and IFN-γ and TNF-α release can be isolated from peripheral blood of TB patients and/or TST+ individuals. The proteins expressing these CTL epitopes are enzymes with defined roles in the metabolic processes in mycobacteria. In addition, they may be involved in host-pathogen interactions. Thus, SodA detoxifies superoxide anion and is capable of neutralizing oxygen metabolites released from immune cells. This is likely to have a broad effect on infection pathogenesis. Indeed, SodA has been shown to inhibit innate immune responses in a mouse model (6). AlaDH is involved in nitrogen metabolism and has been implicated in the adaptation of mycobacteria to an anaerobic dormant state, a condition that may be related to the state of latent infection (9). Finally, GlnS is similarly involved in another pathway of nitrogen metabolism and is essential for intracellular growth of bacilli in macrophages (25). Therefore, these enzymes appear to be crucial for the survival of mycobacteria and may be the targets of strong cell-mediated host immune responses, as revealed in our studies. Further studies are under way to determine whether the response to these CTL epitopes in TB patients and TST+ individuals is a correlate of immunity to TB.

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REFERENCES


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