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CTL Response to Mycobacterium tuberculosis: Identification of an Immunogenic Epitope in the 19-kDa Lipoprotein

Nahid Mohagheghpour,2* Dawn Gammon,* Loyce Masae Kawamura,† Annika van Vollenhoven,‡ Claudia J. Benike,‡ and Edgar G. Engleman†

The successful resolution of infection with Mycobacterium tuberculosis (M.tb) is believed to involve the induction of CTLs that are capable of killing cells harboring this pathogen, although little information is known about the MHC restriction or fine specificity of such CTLs. In this study, we used knowledge of the HLA-A*0201-binding motif and an immunofluorescence-based peptide-binding assay to screen for potential HLA-A*0201-binding epitopes contained in the 19-kDa lipoprotein of M.tb (M.tb19). CD8+ T cells derived from HLA-A*0201+ patients with active tuberculosis (TB) as well as tuberculin skin test-positive individuals who had no history of TB were used as effector cells to determine whether these epitopes are recognized by in vivo-primed CTLs. An in vitro vaccination system using HLA-A*0201+ dendritic cells (DCs) as APCs was used to determine whether these epitopes can sensitize naive CD8+ T cells in vitro, leading to the generation of Ag-specific CTLs. The results show that an HLA-A*0201-binding peptide comprised of residues 88 to 97 of M.tb19 (P88–97) is recognized by circulating CD8+ CTLs from both healthy tuberculin skin-test-positive individuals and patients with active TB but not by tuberculin skin-test-negative subjects. Moreover, dendritic cells pulsed with this peptide induced class I MHC-restricted CTLs from the T cells of healthy unsensitized persons. Finally, CTL lines that were specific for P88–97 were shown to lyse autologous monocytes that had been infected acutely with the H37Ra strain of M.tb. These results demonstrate that M.tb19 elicits HLA class I-restricted CTLs in vitro and in vivo that recognize endogenously processed Ag. Epitopes of the type identified here may prove useful in the design of an M.tb vaccine.


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Materials and Methods

Study population

A total of 11 HLA-A*0201 individuals participated in this study. The protocol and consent forms relating to our use of human subjects were approved by the California Pacific Medical Center Research Institute Administrative Panel on Human Subjects in Medical Research. Informed consent for blood donation was obtained from all donors.

Our laboratory generated the following mAbs using hybridomas that were obtained from the American Type Culture Collection (ATCC, Manassas, VA): Leu-4 (CD3), Leu-3a (CD4), Leu-2a (CD8), Leu-11a (CD16), w6/32 (γ-chain specific) Ab were purchased from Zymed Laboratories (San Francisco, CA).

Antibodies

Our laboratory generated the following mAbs using hybridomas that were obtained from the American Type Culture Collection (ATCC, Manassas, VA): Leu-4 (CD3), Leu-3a (CD4), Leu-2a (CD8), Leu-11a (CD16), w6/32 (class I MHC), L243 (HLA-DR), and BB7.2 (HLA-A2). For blocking studies, mAbs were used as purified reagents at concentrations of 10 μg/ml. The HLA-A2.1/A2.2-specific mAb (CR11–351) was a gift of Dr. S. Ferrel from Columbia University (New York, NY). FITC-conjugated mAbs directed against CD3, CD4, CD8, CR3αβ, HLA-DR, class I MHC (w6/32), and CD14 (Leu-M3) were purchased from the Becton Dickinson Monoclonal Center (Mountain View, CA). FITC-conjugated goat anti-mouse IgG F(ab′)2 fragments and affinity-purified goat anti-mouse IgG (γ-chain specific) Ab were purchased from Zymed Laboratories (San Francisco, CA).

Synthetic peptides

A panel of 54 Mtb19-derived peptides (8–10 mer) containing HLA-A*0201-binding motifs were identified using a computer scoring program (25). A total of 28 peptides with a computed score between 48 and 69 were selected for testing in a T cell-binding assay. Two previously identified HIV-1-derived peptides, an HLA-A2-restricted HIV-1 gag peptide (amino acids (aa) 71–85, designated HIV gag A2) and an HLA-B8-restricted HIV-1 gag peptide (aa 253–267, designated HIV gag B26) (20) were used as controls, as was an HLA-A*0201-restricted IMP (aa 58–66) (24). Peptides were synthesized by F-moc chemistry at either University Hospital (Leiden, The Netherlands) or the Beckman Center at the Stanford University Medical Center. Before use, lyophilized, HPLC-purified peptides (>90% pure) were reconstituted at 40 mg/ml in DMSO and diluted to 1 mg/ml with Iscove’s modified Dulbecco’s medium (IMDM) (Life Technologies, Grand Island, NY).

Peptide-binding assay

T2, an HLA-A*0201 Ag-processing defective cell line (22) which was a gift of Dr. M. Cheever (University of Washington, Seattle, WA), was propagated in RPMI 1640 containing nonessential aa, sodium pyruvate, and 10% FBS. Before use, the cells were incubated for 6 h at 37°C in serum-free IMDM; next, they were washed once, suspended in serum-free IMDM containing 20 μM of 2-ME and 15 μg/ml of human β2-microglobulin (β2m) (Calbiochem, La Jolla, CA), and pulsed with 0 to 200 μM of M.tb19 peptide. Control cells were pulsed with either HIV gag A2 or HIV gag B8 peptide. After a 24-h incubation at 37°C, T2 cells were washed 3 times with cold PBS containing 0.5% BSA and 0.02% NaN3. They were then stained directly with FITC-conjugated w6/32 mAb or indirectly with anti-HLA-A2.1 mAb as first Ab and with goat anti-mouse FITC-labeled F(ab′)2 fragments as a second-step Ab.

The percentage of FITC-positive cells as well as their staining intensity (mean fluorescence intensity [MFI]) was determined on an Epics Profile II (Coulter, Hialeah, FL). The ΔMFI for a particular mAb was calculated by subtracting the MFI of either the isotype-matched control mAb or the second-step Ab from each MFI value. The fluorescence ratio (FR) was calculated using the following formula: FR = ΔMFI of peptide-treated T2 cells/ΔMFI of nontreated T2 cells.

Culture medium

T cells were cultured in IMDM that had been supplemented with 10% pooled heat-inactivated human serum, 2 mM of l-glutamine, 100 μg/ml of streptomycin, 100 U/ml of penicillin, and 2.5 μg/ml of fungizone (hereafter designated complete medium (CM)). Monocytes were cultured in antibiotic-free RPMI 1640 containing 10% pooled human serum and 2 mM of l-glutamine (antibiotic-free CM).

Generation of CD8+ CTL lines from PBMCs

PBMCs from either patients with TB or healthy individuals were suspended in CM containing 10 μg/ml β2m and stimulated with 6 μM of each M.tb peptide or IMP using a previously described procedure with some modifications (27). A total of 4 × 105 cells/well were incubated at 37°C in 24-well plates containing 2 LiU/ml of tetanus toxoid (Department of Public Health, State of Michigan, East Lansing, MI) in 1 ml of CM. PBMCs from individuals that did not respond by proliferation to tetanus toxoid received 0.2 μg/ml of PHA (Wellcome Diagnostics, Research Triangle Park, NC) at the initiation of culture. After 3 days, 1 ml of CM supplemented with 10 U/ml of rIL-2 (Life Technologies) was added in each well. On day 7, the cultures were restimulated with the 6 μM of peptide in CM containing 10 μg/ml β2m and 10 U/ml rIL-2 in the presence of 1 × 106 irradiated (3000 rad), pooled, HLA-A2+ allogeneic PBMCs as feeder cells. On day 14, viable cells were recovered on Ficoll-Hypaque gradients, and CD8+ T cells were isolated by positive-selection panning using anti-CD8 mAb. After 1 day, the resulting cells (>95% CD8+, TCRαβ+ by flow cytometric analysis) were tested for CTL activity against autologous B-LCLs that had been pulsed with the stimulating peptide (see below).

Cell separation

Populations that had been enriched for either dendritic cells (DCs) or monocytes were separated from PBMCs on the basis of their differential density (28, 29). Briefly, the PBMCs that were obtained from Ficol-Hypaque gradient centrifugation were separated into low-density (28, 29) were purchased from the Becton Dickinson Monoclonal Center (Mountain View, CA). FITC-conjugated goat anti-mouse IgG F(ab′)2 fragments and affinity-purified goat anti-mouse IgG (γ-chain specific) Ab were purchased from Zymed Laboratories (San Francisco, CA).
were refloated on a 14% mitomycin solution. The DC-enriched population, which stained brightly with anti-HLA-DR mAb, was used as APCs in the in vitro vaccination system, as described below. CD8+ T cells were obtained from the high-density mitomycin fraction by positive-selection panning (31) using anti-CD8 mAb. The resulting cells were >95% CD8+ and TCRαβ+ cells by flow cytometric analysis.

**Priming of naive CD8+ T cells with synthetic peptides**

Purified human CD8+ T cells (105 cells suspended in 100 μl of CM) from healthy HLA-A*0201+, M.tb-unresponsive (stimulation index (SI) < 2; Table I) subjects were added to 102 autologous DCs; the DCs had been pulsed with 6 μM of M.tb peptide by incubation for 2 h in 100 μl of CM supplemented with 20 μg/ml β2m and 1 μM of rIL-1 (R&D Systems, Minneapolis, MN). The total volume per well was 200 μl, and plates were incubated at 37°C in a humidified atmosphere containing 10% CO2. On day 3, a mixture of rIL-2 and rIL-4 (R&D Systems) at 5 U/ml was added to each well. On day 7, and weekly thereafter, T cells were restimulated with 6 μM of original peptide in the presence of irradiated (3000 rad) autologous monocytes in CM supplemented with 5 U/ml of rIL-2 and rIL-4. After 4 to 6 wk of expansion, the CD8+ T cells that had been recovered by positive panning with anti-CD8 mAb were tested for their CTL activity against autologous B-LCLs that had been pulsed with the stimulating peptides. Cultures that displayed peptide-specific cytolytic activity were expanded by weekly restimulation and restetted against autologous monocytes that had been infected with M.tb.

**Generation of B-LCLs**

To generate B-LCLs, PBMCs from each participant were transformed by EBV-containing supernatants from the marmoset line B-958 that was provided by Dr. S. H. Foung (Stanford University). Cells were incubated in 24-well plates (5 × 105 cells/well) in IMDM that had been supplemented with 30% heat-inactivated FBS, 2 mM of L-glutamine, 100 μg/ml of streptomycin, and 100 U/ml of penicillin. After 14 days, the transformed cells were expanded in IMDM containing 10% FBS, and an aliquot was stained with CR11–351 mAb. The percentage of HLA-A2.1/A2.2 cells ranged between 82 and 98 by flow cytometry. Before their use as targets, B-LCLs were expanded in IMDM containing 10% FBS, and an aliquot was stained with CR11–351 mAb. The percentage of HLA-A2.1 (BB7.2) mAb. The five peptides with high HLA-A*0201 affinity (FR > 2) as well as two peptides with low HLA-A*0201 affinity are shown.

**Results and Discussion**

**Identification of HLA-A*0201-binding peptides**

Using the MHC-binding motif for HLA-A*0201 (21), 28 peptides derived from M.tb19 were selected and synthesized for screening in a cell-based binding assay. Two previously described CTL epitopes, HIV gag A2 and HIV gag B8, were used as positive and negative controls, respectively. HLA-A*0201+ T2 cells, which have a defect in the assembly and transport of class I molecules (33–35), were used in these assays, because exogenously added HLA-A*0201-binding peptides can increase the number of properly folded HLA-A2 molecules on the cell surface. The increase in the surface expression of HLA-A2 molecules was measured by flow cytometry using mouse mAb to class I MHC and HLA-A2 (BB7.2) mAbs.

A total of 5 of 28 candidate peptides stabilized HLA-A*0201 expression on T2 cells (FR > 2.0 at 100 μM peptide) (Table II and Fig. 1). Two peptides, corresponding to residues 14 to 22 and 88 to 97, bound to the cells with affinities that were comparable with those of the control HIV gag A2 peptide. As expected, HIV gag B8 had no effect on HLA-A*0201 expression by T2 cells (FR = 1.1) (Fig. 1).

### Table II. HLA-A*0201 binding peptides derived from M.tb19

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Computed Score</th>
<th>Affinity (FR)</th>
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</thead>
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<tr>
<td>4-12</td>
<td>G-L-L-T-V-A-V-A-G-A</td>
<td>52</td>
<td>2.5</td>
</tr>
<tr>
<td>14-22</td>
<td>I-L-V-W-A-G-L-S-G-C</td>
<td>54</td>
<td>4.6</td>
</tr>
<tr>
<td>88-97</td>
<td>V-L-T-D-G-N-P-P-E-V</td>
<td>69</td>
<td>5.3</td>
</tr>
<tr>
<td>101-108</td>
<td>G-L-G-N-V-N-G-V</td>
<td>60</td>
<td>3.5</td>
</tr>
<tr>
<td>131-139</td>
<td>K-I-T-G-T-A-T-G-V</td>
<td>63</td>
<td>2.4</td>
</tr>
<tr>
<td>53-61</td>
<td>V-I-D-G-K-D-Q-N-V</td>
<td>55</td>
<td>0.8</td>
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</table>

* A total of 28 peptides that had both primary and secondary residues conforming to the consensus A*0201binding motifs and a computed score of ≥48 were analyzed for their binding affinity to HLA-A*0201+ T2 cells by flow cytometry using anti-HLA-A*0201 (BB7.2) mAb. The five peptides with high HLA-A*0201 affinity (FR > 2) as well as two peptides with low HLA-A*0201 affinity are shown.

* The sequence numbers of the first and last aa are shown.

* The single-letter code for aa is used. A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Try.
PBMCs from an HLA-A*0201+ TSP subject were stimulated separately with each peptide; after 2 to 4 wk of expansion, CD8+ T cells that had been isolated by positive selection were analyzed for their cytotoxic activity against peptide-pulsed autologous B-LCLs. After 2 wk of stimulation, a CTL response (33% specific lysis at a 40:1 E:T ratio) was detected against B-LCLs that had been pulsed with P88-97 (Fig. 2A), and the intensity of lytic activity increased after each round of restimulation with the peptide, indicating a time-dependent enrichment of Ag-specific effectors (Fig. 2, B and C). The cultured effectors lysed P88-97 pulsed B-LCLs in a dose-dependent manner at all three timepoints. Only weak lysis was observed for nonpulsed B-LCLs or for target cells that had been pulsed with either HIV gag A2 (Fig. 2) or two other 19-kDa-derived peptides, P14-22 or P101-108 (data not shown). The cytotoxic activity of these Ag-specific CTLs was markedly inhibited by Abs to CD8 or class I MHC (Fig. 2D) but was not inhibited by Abs to CD4 (Fig. 2D) or HLA-DR (data not shown). These results were confirmed in two other experiments that tested the responsiveness to P88-97 of PBMCs that were obtained 11 mo later from the same donor (TSP-1) (Fig. 2E) or PBMCs from a second TSP individual (donor TSP-2; 38% specific lysis at an E:T ratio of 40:1). In parallel experiments, CD8+ T cells isolated from the PBMCs of TSP-1 and TSP-2 subjects were incubated with an IMP. The cells from both subjects exhibited 42% lysis (Fig. 2F) and data not shown) of autologous targets that had been pulsed with IMP at an E:T ratio of 40:1. By contrast, P14-22 and P101-108 did not elicit CTL activity when tested repeatedly on PBMCs that had been derived from the same TSP participants (data not shown).

To determine whether P88-97-specific CTLs are present in M.tb-infected persons, PBMCs from four HLA-A*0201+ patients with active TB were expanded in vitro in the presence of P88-97 peptide. After 4 wk of culture, CD8+ T cells from three patients lysed autologous B-LCLs that were pulsed with P88-97 peptide. After 4 wk of culture, CD8+ T cells from three patients lysed autologous B-LCLs that were pulsed with P88-97 (Fig. 3). The specificity of the response was established by the absence of cytotoxic activity against B-LCLs that were pulsed with HIV gag A2 (Fig. 3), and the MHC restriction was indicated by ~64% inhibition of CTL-mediated lysis by Ab to either class I MHC or CD8 molecules (data not shown). T cells from one patient (TB-4) did not yield a strong CTL response against peptide-pulsed autologous B-LCLs or a vigorous proliferative response against M.tb (SI = 4) (Table I).

To determine whether the in vitro expansion of Ag-specific CD8+ CTLs reflects in vivo priming by exposure to mycobacteria rather than in vitro priming by the peptide in this experimental

FIGURE 1. Stabilization of HLA-A*0201 molecules by peptides from M.tb. T2 cells were analyzed by flow cytometry for their cell surface expression of HLA-A*0201 molecules after being pulsed with either 100 μM (A) or 0 to 200 μM (B) of the indicated peptide.

FIGURE 2. P88-97 is recognized by CD8+ T cells from a healthy TSP subject. PBMCs from a healthy HLA-A*0201+ TSP participant (TSP-1) were stimulated with P88-97 as described in Materials and Methods. After either 2, 3, or 4 wk of expansion (A, B, and C, respectively), CD8+ T cells that had been isolated by positive-selection panning were tested for cytolytic activity against peptide-pulsed autologous B-LCLs that had been incubated with either P88-97 or HIV gag A2 or maintained in medium alone. D, CTLs that had been expanded for 3 wk were assessed in the presence of either anti-CD8, anti-class I MHC (w6/32), or anti-CD4 mAb. The percentage of specific lysis at an E:T ratio of 40:1 was measured in a 5-h 51Cr release assay. E and F depict the CTL responses of PBMCs that were obtained from donor TSP-1 after 11 mo. CD8+ T cells were tested for their lytic activity at 3 wk after stimulation with either P88-97 (E) or IMP (F).
system, we measured the CTL response of CD8⁺ T cells that had been isolated from the PBMCs of two HLA-A*0201⁺ individuals (blood donors N-4 and N-5) following 3 wk of in vitro incubation with M.tb P₈₈₋₉₇. In parallel, we assessed the cytolytic activity of PBMCs that had been stimulated with IMP. As depicted in Figure 4, A and C, P₈₈₋₉₇-specific CD8⁺ CTLs were not recovered when PBMCs from these TSN subjects were cultured in the presence of M.tb P₈₈₋₉₇. In contrast, incubating the PBMCs with

FIGURE 3. P₈₈₋₉₇ is recognized by CD8⁺ T cells from patients with active TB. PBMCs from four HLA-A*0201⁺ patients were stimulated with P₈₈₋₉₇. After 5 wk of expansion, CD8⁺ T cells were tested for cytolytic activity against peptide-pulsed autologous B-LCLs as described in the legend to Figure 2.

FIGURE 4. CD8⁺ CTL precursors that are specific for P₈₈₋₉₇ are not detectable in the peripheral blood of healthy TSN individuals. PBMCs from two HLA-A*0201⁺ healthy TSN individuals (N-4 (A and B) and N-5 (C and D)) were cultured for 3 wk with either M.tb P₈₈₋₉₇ (A and C) or IMP (B and D) as described in the legend to Figure 2. Target cells were autologous B-LCLs that had been pulsed with either P₈₈₋₉₇, IMP, or HIV gag A2 or maintained in medium alone.
IMP resulted in an expansion of peptide-specific CTLs (Fig. 4, B and D). These results, taken together with those detailed above, indicate that the peptide-specific CTL precursors that expanded in this experimental system had been primed in vivo.

Finally, to determine whether P 88 –97 can induce a class I-restricted CTL response in naive T cells, CD8+ T cells isolated from the PBMCs of two HLA-A*02011, M.tb-unresponsive subjects (N-1 and N-2) were stimulated with peptide-pulsed autologous DCs. After 4 wk, the expanded CD8+ cells were enriched by positive-selection panning and tested for their cytolytic activity. When subjected to flow cytometric analysis, these lines were ≥92% CD8+ and TCRαβ+ (data not shown). Target cells were autologous B-LCLs that had been pulsed with either P 88 –97 or HIV gag A2 or maintained in medium alone. Cytotoxicity was measured at a 40:1 E:T ratio in a 5-h 51Cr release assay.

Recognition of endogenously synthesized Ag by peptide-specific CTLs

We examined the ability of peptide-specific CTLs to recognize endogenously synthesized epitopes by measuring their cytolytic activity against autologous monocytes that were acutely infected with tubercle bacilli. The results in Figure 6 show that CTLs that were derived from both in vivo- and in vitro-primed CD8+ T cells were able to recognize and lyse M.tb-infected monocytes in a class I MHC-restricted manner. As shown, only weak lysis was observed for uninfected monocytes. These results suggest that P 88 –97 is generated by natural processing within the infected cells.

In summary, we used 8 to 10 aa synthetic peptides with a relatively high affinity for HLA-A*0201 molecules (Fig. 1) and two independent experimental systems to identify epitopes that elicit class I MHC-restricted CD8+ T cell responses against tubercle bacilli. One system detected peripheral blood CD8+ CTL populations that were primed in vivo (27), while the other used peptide-pulsed DCs as APCs to sensitize naive CD8+ T cells in vitro (28, 36). The results establish that class I MHC-restricted CD8+ CTL precursors with a specificity for P 88 –97 are present in patients with active pulmonary TB as well as in TSP individuals without a history of TB. Moreover, peptide-specific CTLs that were expanded from in vivo-primed CD8+ T cells or generated from naive CD8+ restimulation, the expanded CD8+ T cells were tested for CTL activity against autologous peptide-pulsed B-LCLs. As shown in Figure 5, P 88 –97 induced a specific CTL response in both normal donors. These results confirm our earlier observation that human peripheral DCs, when appropriately pulsed with synthetic peptides, can sensitize naive CD8+ T cells and consequently enable the generation of Ag-specific CTL lines in vitro (36).

**FIGURE 5.** CTL activity of CD8+ T cells sensitized in vitro to P 88 –97. Purified CD8+ T cells from two healthy HLA-A*02011, M.tb-unresponsive subjects (N-1 and N-2) were primed using peptide-pulsed autologous DCs. After 4 wk, the expanded CD8+ cells were enriched by positive-selection panning and tested for their cytolytic activity. When subjected to flow cytometric analysis, these lines were ≥92% CD8+ and TCRαβ+ (data not shown). Target cells were autologous B-LCLs that had been pulsed with either P 88 –97 or HIV gag A2 or maintained in medium alone. Cytotoxicity was measured at a 40:1 E:T ratio in a 5-h 51Cr release assay.

**FIGURE 6.** Peptide-specific CTL lines lyse M.tb-infected monocytes in a class I MHC-restricted manner. CTL lines from N-1, N-2, and N-3 were generated using peptide-pulsed autologous DCs as described in the legend to Figure 5: the CTL line from TSP-2 was generated from PBMCs as described in the legend to Figure 2. Target cells were autologous monocytes that were either maintained in medium alone or acutely infected with M.tb. Infected monocytes contained 36 (N-1), 29 (N-2), 69 (N-3), and 17 (TSP-2) CFU/cell. Cytotoxicity was measured in an 18-h 51Cr release assay. The percentages of monocytes harboring acid-fast bacilli are shown in parentheses. For mAb-blocking studies, infected monocytes were pretreated with either anti-class I MHC (w6/32) or anti-HLA-DR (L243) mAb. The percentage of specific lysis at an E:T ratio of 40:1 is shown.
T cells by in vitro sensitization with peptide-pulsed DCs recognized and lysed autologous targets that were acutely infected with tubercle bacilli in a class I MHC-restricted manner. Peripheral blood DCs that have been pulsed with exogenous peptides are potent stimulators of quiescent T cells (28, 37). In natural infection, DCs residing in airway epithelium and lung parenchyma (38–40) are among the first cells to encounter the bacillus and, presumably, to acquire and process mycobacterial Ags for presentation to naïve T cells (41, 42). A recent report indicates that Mtb enters human DC progenitors in vitro, resulting in the activation and maturation of DCs (43).

These findings are potentially relevant for both vaccine development and adoptive immunotherapy. Epitopes that are generated by the intracellular processing of endogenously synthesized Ags are appropriate candidates for inclusion in the design of a peptide-based Mtb vaccine. The in vitro generation of M.tb-specific, biologically active effector cells potentially permits a large scale ex vivo expansion of CTLs for adoptive immunotherapy.

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References