

Use of Transgenic HLA A*0201/Kb and HHD II Mice To Evaluate Frequency of Cytomegalovirus IE1-Derived Peptide Usage in Eliciting Human CD8 Cytokine Response

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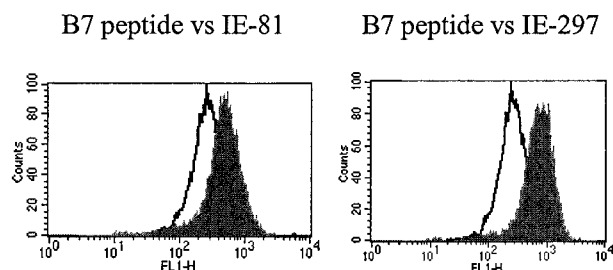
Unlike the pp65 protein of human cytomegalovirus (CMV), which has an immunodominant peptide, pp65₄₉₅₋₅₀₃, recognized by human CD8⁺ cells in the context of HLA A*0201, the fine peptide specificity for CMV IE1 has shown no such immunodominance. With the use of transgenic HLA A*0201/Kb and HHD II mice, a selected pool of IE1 peptides, including IE1_{p256-264}, IE1_{p297-304}, and IE1_{p316-324}, were shown to stimulate cytolytic T-lymphocyte lysis in the context of HLA A*0201. Based on an intracellular gamma interferon response, IE1_{p297-304}, a previously unrecognized CD8 epitope, triggered a prominent response to CMV IE1 in HLA A*0201 subjects.

Murine models to predict human cytomegalovirus (CMV) vaccine immunogenicity are available to detect the presence of a specific immune response, such as cytolytic T-lymphocyte (CTL) function (2, 5, 28), CD4⁺ gamma interferon (IFN- γ) helper response (15, 22), or antibody response to specific CMV proteins (18, 20, 21). Of interest is the use of the transgenic A2Kb mouse containing the human HLA A*0201 with the murine α 3 chain (Kb) (9, 19, 29). The murine model HHD II is also a transgenic HLA A*0201 mouse that contains a disrupted murine major histocompatibility complex (MHC) molecule, forcing the mouse to present all its immunological epitopes through the HLA pathway (10). Both have become useful models to investigate peptide recognition of specific proteins that can be extrapolated to human subjects.

The immunodominant HLA A*0201-restricted peptide (pp65₄₉₅₋₅₀₃) of CMV pp65 has been well studied (3, 6, 12, 30), whereas there is no immunodominant peptide recognized for the CMV IE1 protein, despite the prevalence of an IE1-specific CTL response (11, 14, 16, 17). Three reports have described the stimulatory effect of peptides IE1_{p315-323}, IE1_{p316-324}, and IE1_{p354-362} from CMV IE1 in the context of HLA A*0201 (11, 17, 24) in a cytokine flow cytometry or CTL assay. However, not all CMV-seropositive subjects respond to these peptides (17). This report describes the use of HLA A2 transgenic mice to identify a novel IE1 peptide, IE1-297, that is recognized by murine CTLs as well as human CD8 cells by cytokine flow cytometry.

T2 stabilization. T2 cells are defective for endogenous class I presentation, but the presence of peptide binding to the MHC molecule will stabilize its expression on the cell surface. The stabilized MHC molecule can be detected by flow cytometry with a monoclonal antibody to the HLA A*0201 molecule

(13). The peptide sequences used in this study were collected from two algorithms for HLA peptide predicted motifs: SYFPEITHI (23) and BIMAS. The first five peptides with the highest scores and common to both databases were synthesized at the Beckman Research Institute of City of Hope: IE1_{p81-89} (IE1-81) (LLSEFCRVL), IE1_{p256-264} (IE1-256) (ILDEERDKV), IE1_{p297-304} (IE1-297) (TMYGGISLL), IE1_{p304-312} (IE1-304)



	peak channel	Fold increase
IE-81	495	x2
IE-256	784	x3.3
IE-297	813	x3.4
IE-304	433	x1.8
(+)control pp65	1512	x6.4
(-)control B7 T10M	232	1
(-)control B7 R11L	261	1
(-) control no peptide	237	1

FIG. 1. Stabilization of HLA A2 expression by IE1-derived peptides. T2 cells incubated overnight with each peptide separately (100 μ M) were labeled with an HLA A*0201 monoclonal antibody. Plots show HLA A2 stabilization by the IE1-81 peptide and IE1-297. The clear area is the background signal detected by a mismatched HLA B7 peptide. Tabulated results are peak channel values derived for each peptide and control peptide.

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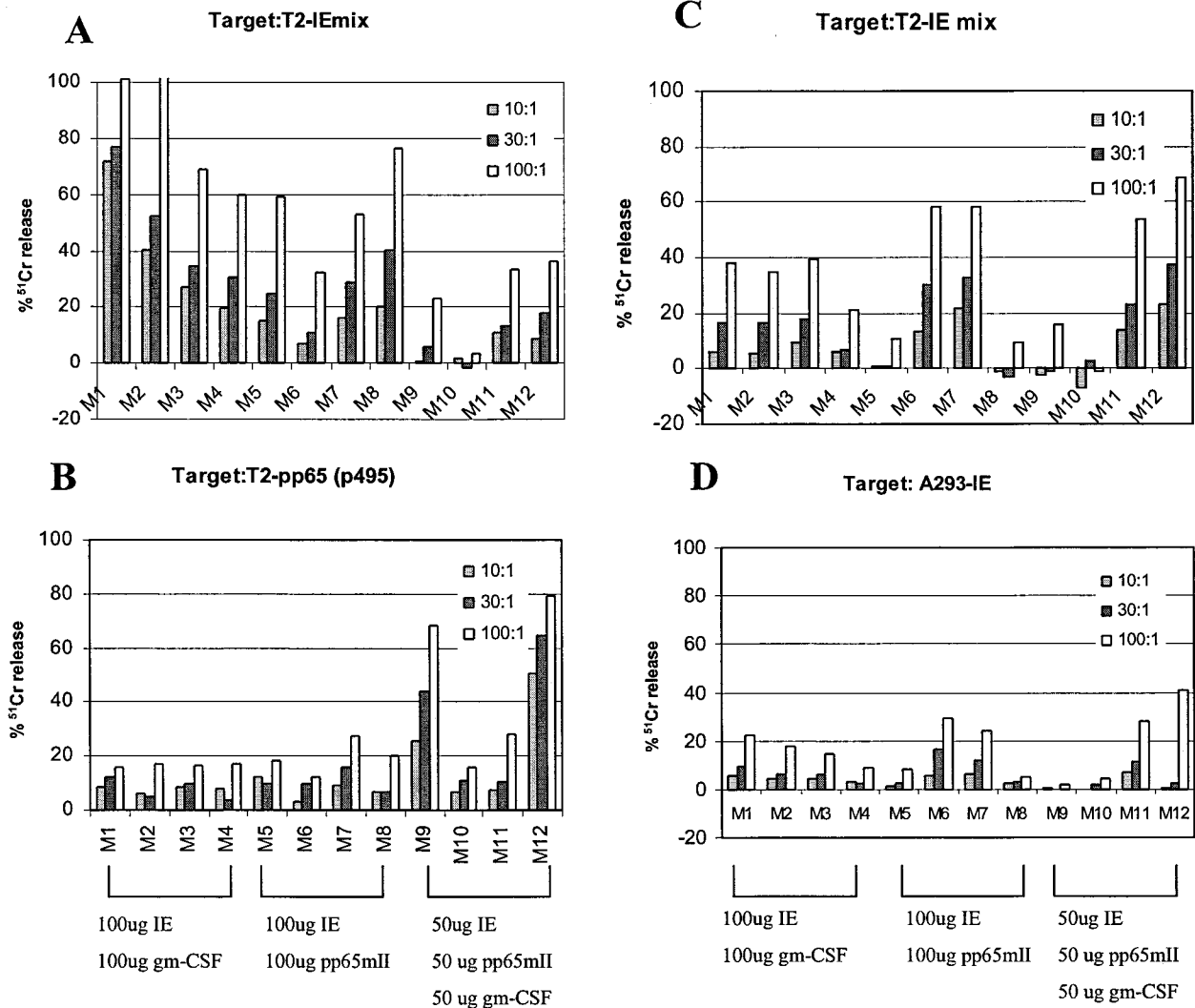


FIG. 2. Cytotoxicity on transgenic A2Kb mice immunized with 100 μ g of pcDNA-IE1 and 100 μ g of pcDNAGM-CSF (M1 to M4); 100 μ g of pcDNA-IE1 and 100 μ g of pcDNApp65mII (M5 to M8); and 50 μ g of pcDNA-IE1, 50 μ g of pcDNApp65mII, and 50 μ g of pcDNAGM-CSF (M9 to M12). (A and B) Responses in spleen cells collected at day 10 after the fourth immunization. (C and D) Long-term memory responses in spleen cells collected at day 60 after the third immunization. The percent chromium release is reported at an effector/target ratio of 10:1, 30:1, or 100:1. Blasts and target cells were incubated with a mixture of five nonapeptides (25 μ M each) derived from the IE1 protein for all panels except panel B, in which pp65_{p495} was used.

(LLSEFCRVL), and IE1_{p316-324} (IE1-316) (VLEETSVML). The control peptides were CMV A2pp65₄₉₅₋₅₀₃ (NLVPMVATV) (positive) and HIV₄₆₈₋₄₇₆ (ILKEPVHGV), B7 pp65₄₁₇₋₄₂₆ (TPRVTTGGGAM), and B7 pp65₂₆₅₋₂₇₅ (RPHERNGFTVL) (negative). Four IE1-derived peptides (IE1-81, IE1-256, IE1-297, and IE1-304) were tested individually for binding and stabilizing effect of the MHC molecule on T2 cells. Figure 1, upper panel, shows the displacement of the peak fluorescence to the right in cells incubated with representative IE1-81 (left) and IE1-297 (right) peptides compared to the background signal on T2 cells treated with mismatch (B7-restricted) peptide. All four IE1-derived peptides stabilized the HLA A2 molecule with varied binding affinities as tabulated in Fig. 1 where the peak values of IE1-297 (3.4-fold) and IE1-256 (3.3-fold) were highest and yet still lower than that for the positive control, pp65₄₉₅₋₅₀₃ (6.4-fold). Therefore, all four peptides (IE1-81, IE1-256, IE1-297, and IE1-

304) and later IE1-316 were used together, each at a 25 μ M concentration (IE1 mix) to bind to autologous blast cells for in vitro stimulation. The IE1 mix was also used to sensitize target T2 cells for cytotoxicity recognition.

DNA immunization with IE1, pp65mII, and GM-CSF combinations. Six- to eight-week-old A2Kb mice (1) were immunized three times at 4-week intervals with various combinations of DNA expressing CMV pp65mII, CMV IE1, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The genes for pp65mII, IE1, and murine GM-CSF were inserted into the mammalian expression vector pcDNA3.1⁺ (Invitrogen, San Diego, Calif.) containing the intron A as described previously (12). Mice M1 to M4 were inoculated intramuscularly with 100 μ g of pcDNA-IE1 and 100 μ g of pcDNAGM-CSF, M5 to M8 were inoculated with 100 μ g of pcDNA-IE1 and 100 μ g of pcDNApp65mII, and M9 to M12

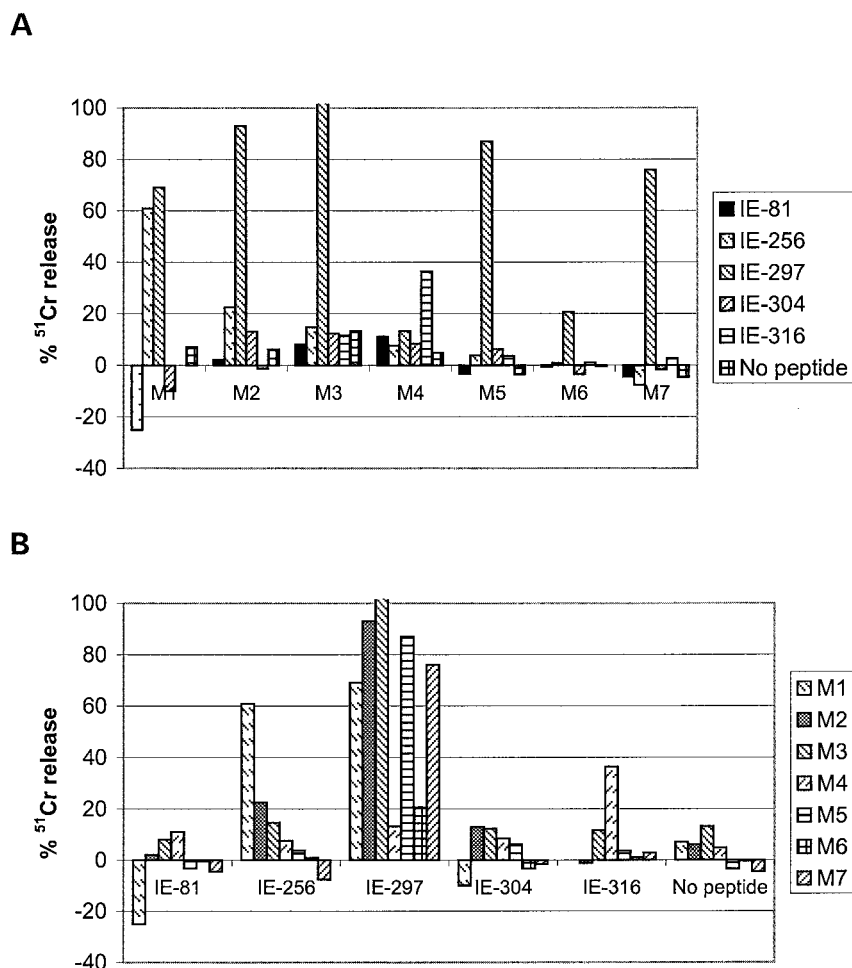


FIG. 3. Immunodominant IE1 epitopes in A2Kb mice. Splenocytes from mice immunized with IE1 and GM-CSF DNA were stimulated in vitro with a peptide mixture and used as effector cells. (A) For preferential recognition of peptide in individual mice (M1 to M7), the specificity of the stimulated population was determined by Cr release with T2 cells sensitized with each individual peptide (100 μ M) as targets (effector/target ratio of 100). (B) For preferential evaluation of each peptide, the IE1-297 peptide is shown as the most frequently recognized epitope in the mixture.

were inoculated with 50 μ g of pcDNA-IE1, 50 μ g of pcDNApp65mII, and 50 μ g of pcDNAGM-CSF. Figure 2 shows the results of CTL killing mediated by splenocytes collected 10 days after the last immunization (Fig. 2A and B) or collected 60 days after the last immunization (C and D). The specific targets used in this experiment were T2 cells incubated with IE1 mix or pp65₄₉₅₋₅₀₃ or A293 cells constitutively expressing the IE1 gene. The control target T2 cells (no peptides) or nontransfected A293 cells were not lysed by the effector cells (data not shown). The pp65mII DNA was considered a positive control that resulted in an immune response in 50% of immunized mice (12). The GM-CSF DNA was added as a cytokine adjuvant to increase the immune response to DNA immunization in mice (25, 27).

For Fig. 2A, immunized A2Kb mice were boosted 10 days prior to splenocyte collection. The splenocytes were incubated with IE1 mix-blasts during a 6-day in vitro stimulation and then subjected to a chromium release assay in the presence of T2 target cells labeled with IE1 mix peptides. Of 12 mice, 11 showed various levels of CTL activity for the mixture of IE1 peptides ranging from 20 to 100% ⁵¹Cr release, demonstrating

a strong CTL immune response to the CMV IE1 immunization. Figure 2B represents the same spleen cells, stimulated with the peptide specific to CMV pp65mII (p₄₉₅₋₅₀₃) instead of IE1 mix. Only the mice immunized with the pp65mII DNA (M5 to M12) showed some CTL activity in the presence of T2-p₄₉₅, demonstrating specificity of response with the respective peptides. Mice M9 to M12, receiving lower doses of the combination regimen, showed a higher lytic response to pp65 than to IE mix stimulation. Without knowing the relative expression of pp65 and IE1 after DNA immunization, it is difficult to interpret differences in percent lysis between these two CMV proteins.

In Fig. 2C, the presence of memory T cells directed to the CMV IE1 gene was detected in immunized splenocytes collected at day 60 after the last injection, stimulated with IE1 mix as described above, and incubated in the presence of target T2-IE1 mix cells. In Fig. 2D, memory effector cells can also recognize endogenously processed IE1 in human A293 targets (A293-IE1), implying that the pool of IE1 peptides may include peptides that are naturally processed through the proteasome of the cell. These data indicate, therefore, that stim-

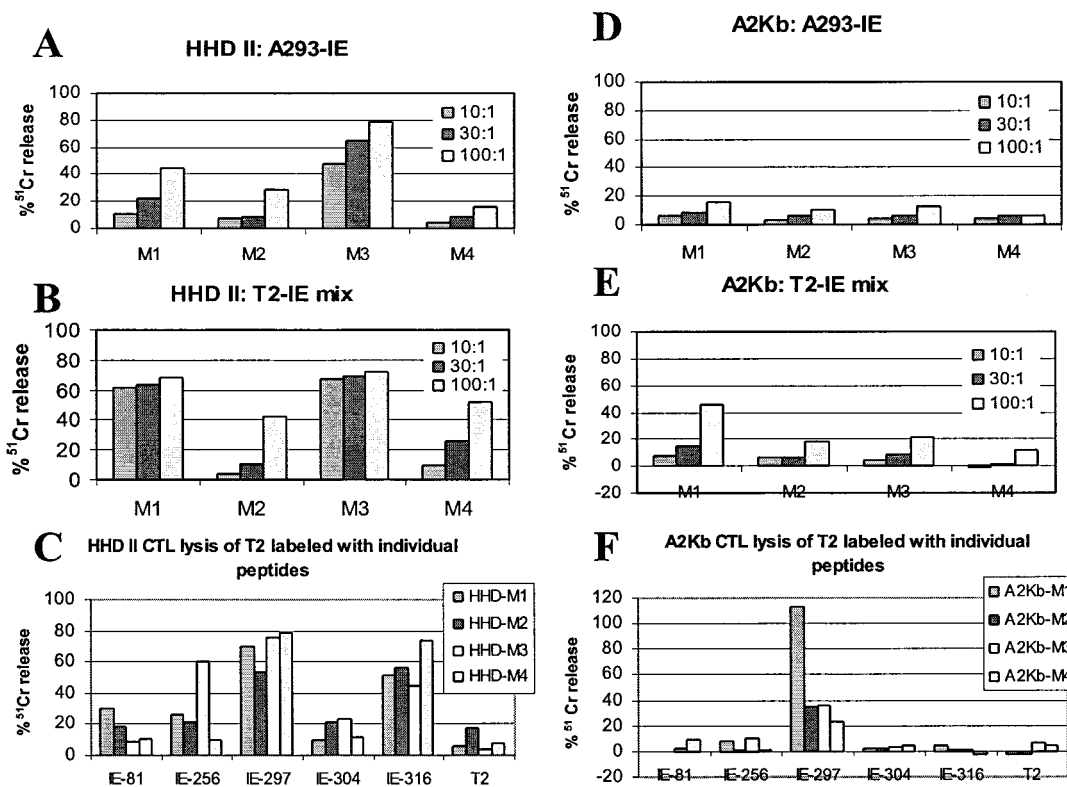


FIG. 4. Efficiency of IE1 epitope recognition in A2Kb and HHD II transgenic mice after recAAV vector immunization. HHD II mice (A to C) and A2Kb (D to F) mice were injected with 1.5×10^8 IU of recAAV-IE1. Splenocytes, collected 30 days postimmunization, were stimulated in vitro with a mixture of IE1 peptides. (A and D) Recognition of endogenously processed IE1; (B and E) cytotoxicity against T2 cells sensitized with the same peptide-IE1 mix; (C and F) recognition of individual peptides is shown for HHD II mice and A2Kb mice, respectively.

ulation with the IE1 mixture of peptides generates CTLs that can recognize T2 cells labeled with the IE1 mix and that the CTL can also recognize endogenously processed IE1 CMV gene.

CTL response to individual peptides. The DNA immunizations for individual peptide analysis consisted of one Sensorcaine-MPF (bivucaine HCl [USP], 0.05%) injection into the thigh followed 5 days later with one injection of 50 μ g of pcDNA-IE1 and 50 μ g of pcDNAGM-CSF (8, 26). The splenocytes were collected at day 20 postimmunization and stimulated for 6 days with IE1 mix-blasts. The splenocytes from responsive mice, as determined by lysis of IE1 mix-loaded T2 cells, were then tested with T2 cells loaded with individual peptides. The results of target cell lysis by splenocytes from seven IE1-immunized mice are shown in Fig. 3. Mouse M1 recognized T2 targets loaded with IE1-256 or IE1-297. M2, M3, M5, M6, and M7 splenocytes recognized IE1-297, and M4 splenocytes recognized IE1-316. M1 spleen cells were stimulated in vitro with the pool of IE1 peptides six times whereas the other splenocytes were stimulated only once. Figure 3B shows that the peptide most frequently recognized by A2Kb spleen cells was IE1-297, though IE1-256 and IE1-316 were also present. In this group of effector cells, no response to IE1-81 or IE1-304 was detected.

Comparison of CTL responses between HHD II and A2Kb mice. The immune response to full-length IE1 induced by recombinant adeno-associated virus (recAAV-IE1) was char-

acterized for preferential peptide presentation in HHD II and A2Kb mice. The HHD II mice (HLA-A-0201 α 1- α 2, H-2D^b α 3-transmembrane and intracytoplasmic domains) in which the H-2D^b and mouse β 2m genes have been disrupted by homologous recombination (10) were obtained from F. Lemonnier. The recAAV CWRSP plasmid was a gift from S. Chatterjee (4). The internal cassette was removed from the CWRSP plasmid backbone, leaving the internal transcription region from AAV2 intact, and replaced by the CMV promoter, intron A, multiple cloning site, and BGHpa cassette from pcDNA3.1⁺. The IE1 gene was then placed in the multiple cloning site at the *EcoRI/XbaI* site (CwCMV-IE1). The AAV Helper-Free system (Stratagene, Cedar Creek, Tex.) was used to encapsidate the AAV. The viral lysate was cleared by centrifugation, and the titer of the supernatant was determined on HT1080 cells. The viral vector recAAV containing the IE1 gene was used as another mode of immunization to check for preferential peptide presentation. Four HHD II and four A2Kb mice were immunized intramuscularly with a single dose of 1.5×10^8 IU of recAAV-IE1 per mouse. The splenocytes were collected 30 days after immunization, stimulated with autologous blast cells loaded with the IE1 mix peptides for 6 days, and assayed for IE1 CTL response. In Fig. 4, panels A to C show the results for HHD II mice and panels D to F show results for the A2Kb mice. After one IE1 mix stimulation, three out of four HHD II mice generated CTLs recognizing endogenously processed IE1 peptides (A293-IE) (Fig. 4A)

TABLE 1. Cytokine flow cytometry with fresh blood from CMV-seropositive stem cell transplantation subjects stimulated with either the pp65 epitope or the IE1-297 epitope

UPN ^a	Day posttransplant	pp65 _{p495}		IE1-297		PHA, ^d % CD8 ⁺ /IFN- γ
		% CD8 ⁺ /IFN- γ	Total no. of cells/liter ^b	% CD8 ⁺ /IFN- γ	Total no. of cells/liter	
100	40	0.11	4.08×10^5	0	0	2.09
93	120	3.62	4.77×10^7	0.16	2.11×10^6	37.6
70	150	0.47	NA ^c	0.20	NA	33.15
70	180	1.26	6.97×10^6	0.01	5.53×10^4	19.2

^a Unique patient number.

^b Total number of IFN- γ -positive cells was calculated taking into account the percentage of total lymphocytes in the blood and the percent CD8⁺ cells from that fraction and expressed as number of cells per liter of blood. A total of 50,000 events were counted for each sample. The human immunodeficiency virus-negative controls (range, 0.03 to 0.31%) were subtracted from the reported numbers.

^c NA, total leukocyte count and percent lymphocyte count not available.

^d PHA, phytohemagglutinin.

whereas four out of four HHD II mice responded to the IE1 immunization with IE1 mix T2 target cells (Fig. 4B). When the same splenocytes were incubated with T2 cells labeled with individual peptides, IE1-297 and IE1-316 were the most frequently recognized peptides (four mice), followed by IE1-256 (one mouse) (Fig. 4C).

However, for the A2Kb mice the response to HLA A2-IE1 target cells was low (Fig. 4D), probably due to the fact that the murine CTLs can respond to either the murine MHC or HLA A2 presentation of the antigen. One out of four mice showed a substantial CTL recognition with IE1 mix-T2 targets after IE1 mix stimulation (Fig. 4E). However, the CTL response was specific for the IE1-297 peptide in all four A2Kb mice (Fig. 4F). This response probably reflects a preferential recognition of the peptide IE1-297 in the targeting of CTLs generated by CMV IE1 immunization.

Intracellular cytokine response to IE1-297 in human HLA A*0201 lymphocytes. Since IE1-297 peptide had been identified as an important epitope in the process of CTL response to CMV IE1 immunization in A2 transgenic mice, it was then used to determine if it was recognized by CD8 cells from CMV-seropositive human subjects. Cytokine flow cytometry was performed essentially as described in the work of Dunn et al. (7) with 200 μ l of fresh blood, stimulated with 100 μ M CMV-specific peptides for pp65 (P₄₉₅₋₅₀₃) or IE1 (P₂₉₇₋₃₀₅) for 6 h. Fixed cells were stained for intracellular IFN- γ with an anti-IFN- γ -antigen-presenting cell conjugate and analyzed on a FACSCalibur (Becton Dickinson, San Jose, Calif.) flow cytometer. Fresh whole-blood samples collected at days 40, 120, 150, and 180 post-stem cell transplantation from three subjects susceptible to CMV reactivation were stimulated with pp65₄₉₅₋₅₀₃, IE1-297, human immunodeficiency virus peptide as negative control, and phytohemagglutinin. Two out of four patient samples showed a cytokine response to the IE1-297 peptide whereas four out of four responded to the pp65₄₉₅₋₅₀₃ peptide (Table 1). These data indicate that HLA A*0201 CD8⁺ lymphocytes can respond to IE1-297 stimulation and that this peptide can be used to characterize the status of a CMV cellular response in human subjects.

In conclusion, a novel IE1 epitope, IE1-297 (IE1_{p297-304}), has been found to be a relatively strong stimulator for IE1 immune CTL response in human CD8⁺ lymphocytes. Moreover, the use of transgenic mice is an important tool to define immune responses to specific epitopes in the context of HLA.

The report here shows that it is possible to extrapolate the results to human application. The information derived from this murine model for elucidating CMV IE1 epitopes will be useful in more fully characterizing the CMV immune response in subjects at risk for CMV disease.

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