Cutting Edge: Characterization of Allorestricted and Peptide-Selective Alloreactive T Cells Using HLA-Tetramer Selection

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The vast majority of alloreactive T cells recognize foreign MHC molecules in a peptide-dependent manner. A subpopulation of these peptide-dependent alloreactive T cells is peptide-specific and contains T cells that are of interest for tumor immunotherapy. Allorestricted T cells (i.e., peptide-specific and alloreactive) specific for tumor-associated Ags can be raised in vitro. However, it is technically difficult to distinguish between peptide-specific and peptide-nonspecific alloreactive T cells by functional assays in vitro. Here we show for the first time that allorestricted T cells specifically bind HLA-peptide tetrameric complexes, as nominal Ag-specific T cells would do. In consequence, fluorescent HLA-peptide tetrameric complexes can be used for sorting and cloning of allorestricted CTLs specific for a peptide of interest. We also show by the mean of HLA-peptide tetramers the existence of peptide-selective alloreactive T cells that recognize a conformation on the foreign-MHC brought about by some but not all peptides bound. The Journal of Immunology, 2001, 166: 4818–4821.

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tolograft rejection and graft-vs-host disease (GVHD) are the clinical manifestations of T cell reactivity to foreign MHC molecules (1). The molecular basis of allogeneic recognition has been extensively studied (2, 3). The nature of the determinants involved in the alloreactive T cell recognition appears to be very diverse (2). The targets of alloreactivity, the MHC class I molecules, bind 8- to 10-aa peptides from intracellular sources and display them at the cell surface. CTL clones that seem to recognize allogeneic molecules in a peptide-independent fashion have been reported (2, 4, 5). Several studies have indicated the existence of peptide-dependent but not peptide-specific CTLs (2, 6). This fraction of alloreactive T cells might be sensitive to the conformation of the MHC that is adapted when particular, but unrelated, peptides are bound (3). The existence of peptide-specific alloreactive T cells has been clearly demonstrated (2, 7–10). These allorestricted CTLs can recognize specific peptide-MHC complexes just like nominal Ag-specific T cells do (11–13). By using peptide libraries, our group has recently shown that the mouse as well as the human allorestricted T cell repertoire is broad and diverse (11, 13).

Many tumors overexpress normal proteins thereby modifying the set of self-peptides associated with MHC class I molecules. This phenomenon allows triggering of tumor-specific CTLs. However, CTLs undergo negative selection and peripheral tolerance mechanisms that diminish the number or eliminate self-peptide-specific CTLs. This is an obvious limitation to generate in vitro tumor-specific cytotoxic T cells to be used in adoptive immunotherapy. The existence of the allorestricted repertoire raises the possibility of generating CTLs reactive against synthetic self-peptides bound to nonself-MHC molecules, because tolerance to self-Ags is self-MHC restricted (14). Thus, it should be possible to produce in vitro CTL against self-Ags that are expressed in tumor cells for adoptive immunotherapy (14, 15). Indeed, it has been shown recently that allorestricted CTLs specific for mdm-2 wild-type peptide can be a successful reagent for immunotherapy in mice (12). These CTLs can engraft and retain specificity in the host without causing GVHD (16). We and others (15) evaluate the possibility to isolate allorestricted CTLs that originate from HLA-A*02-negative donors and recognize specifically HLA-A2-peptide complexes. However, the in vitro generation of such allorestricted T cells remains problematic because of the difficulty to separate between the large pool of alloreactive (i.e., recognizing foreign MHC) and the small fraction of allorestricted (i.e., restricted for a particular peptide on foreign MHC) CTL activities. Here, we investigated whether allorestricted T cells can bind HLA-tetrameric complexes specifically, as Ag-specific T cells would do, and whether HLA tetramers can be used for sorting and cloning of allorestricted CTLs specific against a peptide of interest.
Materials and Methods

Cells

The human EBV-transformed lymphoblastoid B cell lines (LLC) 721 (HLA-A*0201, -A*01, -B*05, -Cw7) (17), the TAP-deficient cell line T2 (HLA-A*101, -B*58, -Cw6) (18), the β2-microglobulin-deficient Burkitt’s Lymphoma Daudi (HLA-A1, -B51) (19), and the breast carcinoma cell line KLE (HLA-A*02) (kindly provided by Dr. B. Gückel, Tübingen, Germany) were used in 125I release assays or for T cell stimulation.

Generation of CTLs

PBL from healthy donors registered in the Blood Bank (Tübingen, Germany) were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). PBLs (10⁷) were stimulated with (10⁵) irradiated T2 cells (200 Gy) pulsed with peptide. Peptide loading was performed for 4 h at room temperature with 10 μM peptide in serum-free medium (20). After 5 days of culture, IL-2 (20 U/ml) was added to the culture medium (Proleukin; Chiron, Ratingen, Germany). After 10 days of culture, the bulk cultures were analyzed by FACS using tetrarmers and restimulated with (10⁵) irradiated T2 cells pulsed with peptide in IL-2-containing medium. After sorting, the cells were seeded at 1 cell/well in 96-well plates (Costar, Bodenheim, Germany) with 4 × 10⁴ irradiated syngeneic PBL (30 Gy) and 2 × 10⁵ irradiated T2 cells pulsed with peptide. The cultures were restimulated weekly in the same fashion and tested in 125I release assays against T2 and 721 target cells. Wells containing CTLs preferentially recognizing peptide-loaded targets or binding tetrarmers were expanded. All T cell cultures were performed in IMDM (Life Technologies, Eggenstein, Germany), 10% human serum (Pel Freez; Biotest, Dreieich, Germany), 2 mM glutamine (BioWhittaker, Rockville, Md.), 10% human serum (Pel Freez; Biotest, Dreieich, Germany), 2 mM glutamine (BioWhittaker), and 50 U/ml penicillin/50 μg/ml streptomycin solution (BioWhittaker).

HLA-peptide tetrameric complexes and flow cytometry

HLA-peptide tetrameric complexes were produced as previously described (21). In brief, the HLA heavy chain was modified by deletion of the transmembrane domain and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site (21). The HLA-A2 heavy chain and β2-microglobulin were produced using a prokaryotic expression system (pET/HLA-A*0201, pETβ2m) plasmids, and bacteria kindly provided by Dr. Vincenzo Cerundolo), purified and refolded in vitro by limiting dilution with the HLA-A*201 binding peptides. The HLA-A*201 binding peptide used were influenza matrix protein (MP58-66), HLA-A*201 (ILGKCFGIL), and GILGFVFTL (22), carcinoembryonic Ag (CEA) (22), GILGFVFTL (22), and myosin (22). The refolded complexes were purified by gel filtration (Superdex 75; Pharmacia, Uppsala, Sweden) using fast protein liquid chromatography, biotinylated by BirA (Avityd, Denver, CO) in the presence of biotin (Sigma, Deisenhofen, Germany), ATP (Sigma), and Mg²⁺ (Sigma). The biotinylated product was separated from free biotin by gel filtration and ion exchange (MonoQ; Pharmacia) using fast protein liquid chromatography. Tetramers were assembled by mixing biotinylated protein complexes with streptavidin-PE (Molecular Probes, Eugene, OR) at a ratio of 4:0:8. A total of 4 × 10⁵ cells from the in vitro allostimulations or 2 × 10⁶ CTL clones were incubated on ice or 37°C with 10 μg/ml tetrameric complexes. After 15 min of incubation, cells were washed extensively with PBS containing 1% FCS. CD8 Ab (Caltag Laboratories, Burlingame, CA) and CD4 Ab (Immunotech, Marseilles, France) were added, and the samples were incubated on ice for further 15 min. After extensive washing, samples were fixed with PBS containing 2% formaldehyde. Triple-color analysis was performed with tetramer-PE, CD8-Tricolor, and CD4-FITC using a FACScalibur (Becton Dickinson, Heidelberg, Germany) and CellQuest software (Becton Dickinson). Sorting was performed without fixation and using a FACS Vantage (Becton Dickinson).

Cytotoxicity assay

Targets were labeled with 1.85 MBq of Na251CrO4 for 1 h at 37°C, with or without preincubation with peptide (50 μM) for 1–2 h at room temperature in serum-free medium. Labeled targets were incubated for 4 h with the CTLs in RPMI 1640 (Life Technology), 10% FCS (Sigma), 2 mM glutamine (BioWhittaker), and 50 U/ml penicillin/50 μg/ml streptomycin solution (BioWhittaker). Subsequently, 50 μl of the supernatant was harvested. Percent specific lysis was calculated as (cpm experimental counts − cpm media control)/cpm detergent − cpm media control) × 100%. Medium controls were between 10 and 15% of detergent samples.

Results and Discussion

PBLs from an HLA-A*02-negative healthy donor (HLA-A*01/*24, -B*08, -Cw7) were stimulated in vitro with the TAP-deficient cell line T2 loaded with the MP58–66 peptide as a model Ag (22). The low level of peptides in this TAP-mutant T2 cell line causes most MHC class I molecules to remain empty or to associate with low-affinity peptides (25). By the external addition of peptides, empty molecules can be stabilized and low-affinity peptides replaced (20), leading to stimulation of the PBLs with a high copy number of a single peptide-MHC complex.

To separate between alloreactive CTL and allorestricted CTL activities, we constructed HLA-A*0201-peptide tetrameric complexes based on the MP58–66 peptide. HLA-tetrameric complexes have been developed by Altman et al. (21) to study peptide-specific CD8⁺ T cells. They have been used to follow the fate of the immune response after viral or bacterial infections (26, 27). They allowed characterization of cell surface Ags expressed by autoantigen-specific T cells in autoimmune disorders and in tumor patients (26, 28). HLA-peptide tetrameric complexes bind to Ag-specific CTLs with high specificity and show no cross-reactivity on CTLs specific for an irrelevant peptide. Tetramer binding is known to correlate with both peptide-specific cytolytic functions and cytokine secretions (26). Furthermore, even down to very low frequencies of Ag-specific T cells, HLA-peptide tetramers allow direct isolation of tetramer-positive cells by FACS (29).

By analysing the bulk alloreactive culture with an HLA-A*02-MP tetramer that stained specifically a MP58–66-specific CTL clone (Fig. 1A), we observed a very low but significant frequency of allorestricted CTLs specific for the MP58–66 peptide (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D). This specific staining was seen only among the T cell blast population (Fig. 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Fig. 1D).
cells (Fig. 1C) and in the fresh PBLs of the same donor (data not shown, see also Ref. 29). Furthermore, using an irrelevant HLA-A*02-peptide tetrameric complex folded with the CEA694–702 peptide, no staining was detectable in both the unstimulated and the blast T cell population (data not shown and Fig. 1B, respectively). A striking observation in this experiment is that the vast majority of alloreactive T cells can not bind the HLA-peptide tetramers (Fig. 1), although such bulk cultures kill T2 cells, as we know from other experiments (data not shown). Among mouse and human alloreactive CTLs, a dominance of peptide-dependent recognition has been described (30, 31). The determinant recognized by the majority of those alloreactive CTLs that do not bind the HLA-peptide tetrameric complexes in our culture could therefore be dependent on TAP-independent peptides presented by the HLA-A*02 molecule on T2 cells. An alternative explanation would be that these alloreactive CTLs are of low affinity and need a high density of MHC-peptide complexes to be activated in vitro (32) and that we are not providing enough HLA-peptide complexes to stain these low-affinity T cells. In any case, this experiment clearly shows that the bulk of alloreactive T cells directed against HLA-A2 is not stained by specific HLA-A2-peptide tetramers.

We further examined the peptide specificity of the allorestricted CTLs by sorting the tetramer-positive cells using FACS. Tetramer-sorted cells were plated at 1 cell/well and expanded in vitro using T2 cells loaded with peptide and autologous feeder cells in IL-2-containing medium. The ability of the CTL clones to lyse peptide-pulsed target cells was then assessed (Fig. 2). The CD8+ HLA-A*02-MP tetramer+ clones were able to lyse peptide pulsed HLA-A*02+ targets. In contrast, no significant activity was observed on T2 pulsed with an irrelevant peptide (Fig. 2). These data confirmed that the tetramer-binding cells in Fig. 1C were allorestricted CTLs specific for the MP56–66 peptide.

In a second experiment, PBLs from the same HLA-A*02-negative donor were stimulated in vitro with T2 cells as described before. As observed previously, CD8+ HLA-A*02-MP tetramer+ cells were detected after tetramer staining of the bulk culture (data not shown). Peptide specificity of these putative allorestricted CTLs was then assessed again by sorting the tetramer-positive cells using FACS and performing a chromium release assay on peptide-pulsed target cells, as described before (Fig. 3). A detailed analysis of one of these CTL clones, BC19-3, is described in Figs. 3 and -4. In contrast to what we observed previously, HLA-A*02+ peptide-pulsed T2 cells were lysed as well as the unpulsed cells (Fig. 3). To exclude that the CTL clones were exhibiting an NK cell-like activity, we performed a killer assay using the NK-sensitive HLA-deficient cell line Daudi and an HLA-A*02-negative target KLHE (Fig. 3B). None of the cell lines were recognized by the clones (Fig. 3), showing clearly that the lysis was MHC specific and correlates with the expression of HLA-A*02 on the target cells. Thus, these CTL clones are either peptide independent or dependent on TAP-independent peptides on T2. It is not obvious why the alloreactive T cells in this experiment are of different specificity than those in the first experiment (Fig. 1), where the majority of tetramer-sorted T cells was peptide specific. One explanation could be that the T cell repertoire of the donor has changed in the meantime, e.g., due to an immune response against an environmental Ag. Another explanation might be a subtle change in MHC expression or peptide loading in T2 cells between the two experiments. In contrast, it is likely that these CTL clones were not specific for a particular TAP-independent peptide on T2 because the wild-type LCL 721 was equally lysed irrespectively of the peptide pulsed (Fig. 3B).

We examined whether the CTL clone described in Fig. 3 would bind different MHC-peptide complexes using HLA-peptide tetrameric complexes folded with several peptides. As expected, the CTL clone could be stained by the HLA-A*02-MP tetramer that was used for the FACS sorting. However, this clone could also bind the HLA-peptide tetrameric complex folded with the CEA694–702 peptide. In contrast, it could not bind the HLA-peptide tetrameric complex folded with the TyrA369–377 Peptide (Fig. 4). These particular HLA-peptide tetrameric combinations were used because for each of them a positive control was available consisting either of HLA-A*02-transgenic mouse CTLs or human CTL clones specific for the HLA-A*02-CEA (data not shown) or the HLA-A*02-TyrA tetramers, respectively (data not shown and V. Teichgräber, A. Moris, M. Müller, B. Schitteck, C. Garbe, and H.-G. Rammensee, manuscript in preparation). This alloreactive CTL

![FIGURE 2](image)

**FIGURE 2.** Cytotoxic activity of HLA-A2-MP-sorted alloreactive CTL clones. The nine different clones were tested in a chromium release assay against the HLA-A*0201-positive LCL 721 pulsed with MP56–66 peptide (●) or a control peptide from the RNA-helicase p72 YLLPAIVHI (○).

![FIGURE 3](image)

**FIGURE 3.** Cytotoxic activity of the HLA-A2-MP-sorted alloreactive CTL clone BC19-3. A: Targets: TAP-deficient T2 cell line unloaded (○), loaded with MP56–66 (●), CEA694–702 (△) or TyrA369–377 (●). B: Targets: LCL 721 loaded with MP56–66 (●) or MelanA27–35 AAGIGILTV (○), unloaded β2-microglobulin-deficient Daudi cells (□) and the HLA-A*02-negative breast carcinoma cell line KLHE (△).

![FIGURE 4](image)

**FIGURE 4.** HLA tetramer staining of the alloreactive CTL clone BC19-3. Double staining was performed using the HLA-A*02 tetramers and CD8 Ab: HLA-A*02-CEA694–702 (TA2-CEA) (left panel), HLA-A*02-MP56–66 (TA2-MP) (middle panel), and HLA-A*02-TyrA369–377 (TA2-TyrA) (right panel).
clone is therefore peptide selective but not peptide specific (Fig. 4). It might be sensitive to the conformation of the HLA molecule that is adapted when particular, but unrelated, peptides are bound (2, 3). One or more peptides of this sort seem to be present at the surface of T2 and T21 as they are both recognized in a killer assay irrespectively of the peptide pulsed (Fig. 3). Our group speculated on the existence of such alloreactive T cells in a previous study where the recognition of HPLC-fractionated Kβ-extracted peptides by Kβ-specific CTLs was analyzed (2). In that study, a unique alloreactive CTL line that recognized multiple HPLC fractions was described. Different groups obtained analogous results, which suggested that some clones may be peptide dependent but not peptide specific inasmuch as a variety of different peptides could promote their recognition of target cells (2, 33, 34). Thus, the present study shows for the first time directly that such alloreactive peptide-selective CTLs exist.

Using HLA-peptide tetrameric complexes, we have described a new approach to generate peptide-specific CTL that could be useful for clinical tumor immunotherapy. Allogeneic bone marrow transplantation as treatment of leukemia patients is often associated with GVHD which is detrimental for the patient, but also graft-vs-leukemia (GVL), which is positively correlated with the prognosis (35, 36). Both activities are attributed to alloreactive donor T cells (36). The transfer of donor alloreactive CTLs specific for a leukemia tumor Ag at the same time as stem cell transplantation could be of particular interest to enhance the GVL without causing GVHD (36, 37). A better understanding of the nature of the determinants recognized by GVL-mediating alloreactive CTLs is of obvious interest to establish new transplantation protocols. Importantly, undetected crossreactivity of tumor-specific alloreacted T cells could induce immunopathology such as GVL. We have shown that HLA-peptide tetrameric complexes are a powerful tool that allows the characterization of recognition patterns by alloreactive T cells and new insights in the molecular basis of alloreactivity.

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