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Investigation of Peptide Involvement in T Cell Allorecognition Using Recombinant HLA Class I Multimers¹

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Alloreactive T cells are involved in injurious graft rejection and graft-vs-host disease. However, they can also evoke beneficial responses to tumor Ags restricted by foreign MHC molecules. Manipulation of these alloreactivities requires information on the basis of T cell allorecognition. The vigorous T cell response to foreign MHC molecules may arise from peptide-independent recognition of polymorphic residues of foreign MHC molecules or peptide-specific recognition of novel peptides presented by foreign MHC molecules. We investigated CD8⁺ T cell allorecognition using recombinant HLA class I/peptide complexes. Peptide-specific allorecognition was examined using tetramers of HLA-A*0201 representing five peptides derived from ubiquitously expressed self-proteins that are known to bind endogenously to HLA-A*0201. Distinct subsets of CD8⁺ T cells specific for each HLA-A*0201/peptide combination were detected within four in vitro-stimulated T cell populations specific for foreign HLA-A*0201. Peptide-independent allorecognition was investigated using artificial Ag-presenting constructs (aAPCs) coated with CD54, CD80, and functional densities of a single HLA-A*0201/peptide combination for four different peptides. None of the four T cell populations specific for foreign HLA-A*0201 were stimulated by the aAPCs, whereas they did produce IFN- γ upon stimulation with cells naturally expressing HLA-A*0201. Thus, aAPCs did not stimulate putative peptide-independent alloreactive T cells. The results show that these alloreactive populations comprise subsets of T cells, each specific for a self-peptide presented by foreign class I molecules, with no evidence of peptide-independent components. *The Journal of Immunology*, 2005, 175: 1706–1714.

The vigorous T cell alloresponse poses a significant problem in clinical transplantation across HLA differences. Currently, this is curtailed by blanket immunosuppression, but the treatment leaves transplant patients susceptible to opportunistic infection and malignancy. Improved understanding of the nature of ligands recognized by alloreactive T cells is required for development of novel methods for monitoring and specifically suppressing the alloresponse. Likewise, a better understanding of T cell alloreactivity is required for exploitation of the alloresponse for tumor immunotherapy. Autologous T cell responses to tumor Ags presented by self-MHC are usually weak and ineffective. This is because tumor-derived peptides are often self-Ags, and T cells with high affinity for self-Ags presented by self-MHC are deleted from the repertoire. However, the T cell repertoire has not been selected to ignore self-Ags presented by foreign MHC molecules. Therefore, allorestricted T cells represent a potent source of tumor-specific T cells. It has been established that infusion of lymphocytes derived from an HLA-mismatched donor to leukemia patients after bone marrow transplantation induces a graft-vs-leukemia response that can eradicate residual malignant

cells (1). This approach has also been used to treat solid tumors (2). Recently, protocols have been developed to identify, isolate, and expand ex vivo tumor peptide-specific allorestricted T cells (3–6), anticipating their use for adoptive immunotherapy. However, the plan may be hampered by adventitious generation of peptide-independent alloreactive T cells (3, 6), which could induce immunopathology. Therefore, renewed calls have been made for a more extensive analysis of T cell alloreactivity before clinical application of these strategies (7).

Up to 10% of T cells recognize foreign MHC class I and class II molecules (8, 9). Two models have been proposed to account for the high frequency of alloreactive T cells (reviewed in Ref. 10). The peptide-independent model of alloreactivity proposes that T cells recognize polymorphic residues located on the surface of foreign MHC molecules and are indifferent to bound peptide. For these MHC structure-specific T cells, all of the MHC molecules on a foreign cell represent potential ligands, creating a high Ag density that could account for the vigorous alloresponse (11). The peptide-specific model of alloreactivity proposes that T cells exhibit specificity for peptides presented by foreign MHC molecules. The novel constellation of self-peptides bound by foreign MHC molecules represents numerous potentially antigenic peptides. Therefore, the cumulative effect of T cells specific for each peptide could account for the strength of the alloresponse (12).

Examples of both peptide-independent and peptide-specific T cell allorecognition have been described previously (reviewed in Ref. 10), but their relative contribution to the T cell alloresponse is unclear. Studies reporting instances of peptide-independent T cell allorecognition are predominantly based on observations that some alloreactive T cells recognize MHC molecules expressed by Ag-processing deficient cells without addition of exogenous peptide (13–18). However, these results are difficult to interpret because MHC molecules expressed by the cells are not totally devoid of

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Table I. HLA class I types of the volunteer donors and cell lines

	HLA-A	HLA-B	HLA-C
Donor 1	A*1101, A*6801	B*3501, B*3503	Cw*04
Donor 2	A*3001, A*3101	B*51, B*18	Cw*01, Cw*05
Donor 3	A*0203, A*2402	B*35, B*5502	ND
Donor 4	A*0201, A*0301	B*0702, B*1801	Cw*07
Donor 5	A*24, A*68	B*15, B*27	ND
721.221 cells	Negative	Negative	Negative
A*0201/221 cells	A*0201	Negative	Negative
B*0702/221 cells	Negative	B*0702	Negative
T2 cells	A*0201	B*5101	Cw*0102
H6 cells	A*0201	B*27, B*62	Cw*1, Cw*3
P3 cells	A*01	B*40	Cw*3

bound peptide. Circumstantial evidence indicates that most alloreactive T cells are peptide-specific, but successful attempts to identify the peptides recognized are surprisingly few (reviewed in Ref. 10). The most abundant self-peptides bound endogenously by MHC molecules would be expected to be among the epitopes recognized by peptide-specific alloreactive T cells. However, studies of alloreactive T cells specific for HLA-A*0201 or HLA-B*0702 failed to identify any T cell clones that recognized self-peptides known to be bound *in vivo* by these HLA class I allotypes (19, 20).

We used recombinant HLA class I/peptide complexes to re-evaluate the role of peptide in T cell allorecognition. Peptide specificity was explored using a panel of HLA-A*0201 tetramers representing five self-Ags derived from ubiquitously expressed proteins known to be bound endogenously by this allotype. Peptide independence was explored using a panel of four different artificial Ag-presenting constructs (aAPCs)⁴ that represent functional densities of HLA-A*0201 molecules displaying a single peptide. Use of these reagents enabled stringent control of the Ags presented to alloreactive T cells. Our results demonstrate that peptide-specific recognition by alloreactive T cells predominates, supporting the proposal that T cell alloreactivity is primarily due to a diverse response to the novel set of peptides presented by foreign MHC molecules.

Materials and Methods

Preparation of PBMCs

PBMCs were isolated from the blood of healthy volunteer donors by Ficoll-Hypaque density gradient centrifugation. Donor 1 has had one prior episode of exposure to alloantigen, donor 2 is allonaive, and the alloimmune status of donor 3 was not known. HLA class I genotyping was performed using sequence-specific oligonucleotides (SSO) (RELI SSO was from Dynal Biotech), PCR sequence-specific primers (Olerup SSP; Geno-Vision, Alpha Helix), or reference strand-mediated conformational analysis (21). The SSO methodology provides low to medium resolution results at the two-digit level. PCR sequence-specific primers and reference strand-mediated conformational analysis provide high-resolution allelic level typing results represented by four digits. HLA class I genotypes are shown in Table I.

Ex vivo CD8⁺ T cell culture

Alloreactive CD8⁺ T cell lines specific for HLA-A*0201 or HLA-B*0702 were established by stimulating PBMCs from HLA-A*0201- and HLA-B*0702-negative donors with irradiated HLA-A-, -B-, and -C-negative lymphoblastoid 721.221 (abbreviated to 221) cells transfected and expressing HLA-A*0201 or HLA-B*0702, using a protocol described previously (22). To establish alloreactive CD8⁺ T cell populations biased to individual peptides presented by HLA-A*0201, PBMCs were stimulated with irradiated TAP-deficient T2 cells loaded with synthetic versions of HLA-A*0201 binding peptides as described previously (23). Alloreactive CD8⁺

T cell populations specific for multiple HLA mismatches were established by stimulation with allogeneic dendritic cells. Dendritic cells were isolated and cultured from PBMCs using a method described previously (24). After 6 days, dendritic cells were matured by overnight culture with 10 ng/ml TNF- α (R&D Systems) and 15 μ g/ml poly(I:C) (Sigma-Aldrich). Enrichment of responder CD8⁺ lymphocytes from PBMCs was performed using anti-CD8 Ab-coated magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Mature irradiated (30 grays) dendritic cells (0.3×10^6 /ml) were used to stimulate responder CD8⁺ lymphocytes (3×10^6 /ml) in the presence of irradiated (30 grays) CD8-depleted responder PBMCs (3×10^6 /ml) and 10 ng/ml IL-7 (R&D Systems). After 7 days, IL-2 (R&D Systems) was added at 20 U/ml. On day 12, cells were stimulated at 1×10^6 /ml with 3×10^6 /ml irradiated allogeneic PBMCs (30 grays) from the same individual used to prepare the dendritic cells. All alloreactive T cell cultures were maintained in IMDM (Invitrogen Life Technologies) supplemented with 10% FCS, 2% human serum, and 20 U/ml IL-2, and stimulated with the appropriate irradiated cells every 7 days.

Induction of CD8⁺ T cells specific for minor histocompatibility Ags was performed as described previously (25). In brief, CD4-depleted PBMCs were stimulated with irradiated autologous peptide-pulsed dendritic cells at a 10:1 responder to stimulator ratio in RPMI 1640 supplemented with 10% autologous serum, 1 U/ml IL-12 (R&D Systems), 1 U/ml IL-2 (Cetus), and an additional 10 U/ml IL-2 at day 5. T cells were restimulated every 7 days with irradiated autologous peptide-pulsed monocytes, and 10 U/ml IL-2 was added 24 h after each restimulation. T cells were cloned by limiting dilution as described previously (26) and expanded in the presence of irradiated allogeneic PBMCs (5×10^4) and minor histocompatibility Ag-positive EBV-transformed lymphoblastoid cell lines (5×10^3), in RPMI 1640 (Cambrex) supplemented with 15% human serum, 10 U/ml IL-2, and 1% leukoagglutinin (Sigma-Aldrich).

Ag specificity of the T cells was assessed by cytolytic activity in a 4-h chromium-51 release assay as described previously (22), and results were expressed as percent-specific lysis (experimental cpm - spontaneous cpm)/(total cpm - spontaneous cpm) \times 100. Assays were performed using 25,000 T cells at an E:T ratio of 5:1 with 221 cells and 1:1 with PBMCs.

HLA class I tetramers and flow cytometry

PE-conjugated HLA-A*0201 tetrameric complexes (listed in Table II) with five peptides derived from self-proteins known to be bound endogenously by this allotype (27-30) and a minimal HLA-A*0201-binding peptide (31) were produced as described previously (23). The HLA class I tetramers are referred to by the first three letters of the peptide sequence (for example, A*0201/SLL). A minimum of 1×10^6 T cells were labeled with tetramer and Abs against CD8 conjugated to FITC and CD3 conjugated to PerCP (BD Biosciences) as described previously (23).

Functional properties of tetramer-binding CD8⁺ T cells were assessed by intracellular flow cytometry. To measure cytokine production, cells were labeled with tetramer before stimulation with allogeneic cells (0.5×10^6 /ml) for 6 h, with monensin (1.5 μ M) added during the second hour of incubation. Cells were then labeled with PerCP-conjugated anti-CD8 Ab followed by intracellular cytokine labeling performed with FITC-conjugated anti-IFN- γ or anti-TNF- α Abs (BD Biosciences) according to the manufacturer's instructions. Cytolytic potential was measured by intracellular labeling with FITC-conjugated anti-perforin Ab (BD Biosciences) using a similar procedure, except stimulation with allogeneic cells was omitted.

Samples were analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences), and data was collected for at least

⁴ Abbreviations used in this paper: aAPC, artificial Ag-presenting construct; SSO, sequence-specific oligonucleotide.

Table II. Recombinant HLA class I complexes

HLA Class I Complex	Peptide Sequence
Tetramers	
A*0201/Ser/Thr protein phosphatase 2A 402–410	SLL PAIVEL ^a
A*0201/RNA-dependent helicase 148–156	YLL PAIVHI
A*0201/BTG protein 103–111	TLW VDPYEV
A*0201/IFN- γ -inducible lysosomal thiol reductase leader sequence-11 to -3	LLD VPTAAV
A*0201/calreticulin leader sequence-17 to -8	MLL SVPLLLG
A*0201/minimal requirement for HLA-A*0201 binding	GLF GGGGGV
aAPCs	
A*0201/HA-1	VLHDDLLEA
A*0201/HA-2	YIGEVLVSV
A*0201/HY	FIDSYICQV
A*0201/Wilms' tumor transcription factor (WT-1)	RMFPNAPYL
B*0801/BZLF-1 (EBV-derived peptide)	RAKFKQLL

^a Tetramers are referred to by the first three letters of the peptide sequence.

200,000 live CD8⁺ T cells per sample. Data was evaluated using FlowJo software (Tree Star). The frequency of tetramer-binding cells is shown as a percentage of total CD8⁺ T cells, and cytokine-producing cells are expressed as a percentage of the CD8⁺ tetramer-binding population.

Preparation of aAPCs

The aAPCs coated with HLA class I/peptide complexes, CD80 and CD54 were prepared as described previously (32). In brief, polystyrene sulfate latex beads (Interfacial Dynamics) were incubated sequentially with streptavidin (10 $\mu\text{g}/10^7$ beads) (Molecular Probes), recombinant human CD54/Fc-chimera (0.5 $\mu\text{g}/10^7$ beads) and CD80/Fc-chimera (0.25 $\mu\text{g}/10^7$ beads) (both from R&D Systems), 1% human albumin (Sanquin), and biotinylated HLA class I/peptide complexes (0.5 $\mu\text{g}/10^7$ beads). The biotinylated recombinant HLA class I/peptide complexes were generated as described previously (33), using the peptides listed in Table II. The aAPCs were only used for functional assays if ligand densities were within the range previously established to be optimal for T cell stimulation, as established by FACS analysis (32).

Cytokine secretion assay

T cells ($2 \times 10^6/\text{ml}$) were stimulated with natural APCs ($2 \times 10^6/\text{ml}$) or aAPCs ($2 \times 10^6/\text{ml}$) for 4 h at 37°C in IMDM supplemented with 5% human serum. Responding CD8⁺ T cells were identified using the IFN- γ Secretion Assay Cell Enrichment and Detection Kit (PE) (Miltenyi Biotec) according to the manufacturer's instructions. In brief, after stimulation, cells were sequentially incubated with IFN- γ Catch Reagent, PE-conjugated IFN- γ Detection Ab, and FITC-conjugated anti-CD8 Ab (BD Biosciences), and stained with propidium iodide (Sigma-Aldrich) before FACS-analysis. The manufacturer's guidelines stipulate that this assay is optimized for cell samples containing <5% of total IFN- γ -secreting cells. Values are exaggerated at higher concentrations of responding cells due to nonspecific staining of cells not secreting cytokine.

Results

Detection of peptide-specific alloreactive CD8⁺ T cells using HLA class I tetramers

Alloreactive CD8⁺ T cells specific for HLA-A*0201 or HLA-B*0702 were generated by in vitro stimulation of PBMCs from three HLA-A*0201/HLA-B*0702-negative healthy volunteer donors with HLA-A*0201/221 or HLA-B*0702/221 cells, respectively. Specificity of the T cell lines for the stimulating HLA class I allotype was demonstrated by cytolytic assay (data not shown). Analysis of the three anti-HLA-A*0201 alloreactive T cell lines with the HLA-A*0201 tetramers revealed binding to small subsets (maximum 0.54%) of CD8⁺ T cells within each of the three pop-

ulations (Fig. 1). The tetramers did not bind unstimulated CD8⁺ T cells from peripheral blood (data not shown) or the three anti-HLA-B*0702 alloreactive T cell lines generated from the same donors (Fig. 1). Staining with an HLA-A*0201 tetramer was designated positive if the percentage tetramer-binding CD8⁺ T cells in the anti-HLA-A*0201 population was >4-fold above that of the control anti-HLA-B*0702 population established from the same individual. Each T cell line was analyzed on at least two separate occasions, and consistent patterns of tetramer binding were observed.

Patterns of tetramer staining were different for each anti-HLA-A*0201 alloreactive T cell line, suggesting that each tetramer binds a distinct subset of T cells. Anti-HLA-A*0201 alloreactive T cells from donor 1 bound the two tetramers A*0201/SLL and A*0201/TLW (Fig. 1A). Anti-HLA-A*0201 alloreactive T cells from donor 2 bound the four tetramers A*0201/SLL, A*0201/YLL, A*0201/TLW, and A*0201/LLD (Fig. 1B). Anti-HLA-A*0201 alloreactive T cells from donor 3 bound the three tetramers A*0201/SLL, A*0201/YLL, and A*0201/TLW (Fig. 1C). Staining with some tetramers produced profiles in which positive T cells were tightly clustered (for example, anti-HLA-A*0201 T cells from donor 1 with the A*0201/SLL tetramer), whereas others produced diffuse profiles (for example, anti-HLA-A*0201 T cells from donor 2 with A*0201/YLL tetramer). This variation in the staining profiles further supports the interpretation that each tetramer binds a different subset of T cells and, therefore, that these alloreactive T cells are peptide-specific.

Analysis of tetramer-binding alloreactive CD8⁺ T cells

The small populations of tetramer-positive cells were authenticated, and their peptide specificity was explored by several different approaches. First, we were able to expand individual subsets of tetramer-binding T cells by stimulation in vitro with specific peptides. TAP-deficient T2 cells alone or loaded with a synthetic peptide were used to stimulate alloreactive CD8⁺ T cells from donor 1. The alloreactive CD8⁺ T cell population produced by stimulation with T2 cells alone contained a relatively high percentage of T cells that bound the A*0201/LLD and A*0201/MLL tetramers (Fig. 2). These peptides are derived from proteins that reside in the endoplasmic reticulum and are known to be among the limited set of peptides presented by HLA-A*0201 expressed by T2 cells (29, 30). As anticipated, T cells stimulated with T2 cells alone did not bind tetramers A*0201/SLL, A*0201/YLL, or A*0201/TLW (Fig. 2), because these three peptides derive from cytoplasmic or nuclear proteins and are therefore dependent on TAP for transport into the endoplasmic reticulum. Alloreactive populations generated using T2 cells loaded with either the SLLPAIVEL, YLLPAIVHI, or TLWVDPYEV peptide contained significant numbers of T cells that bound the A*0201/SLL, A*0201/YLL, or A*0201/TLW tetramer, respectively (Fig. 2). There was no appreciable cross-reactivity with other members of the tetramer panel, indicating that these T cells were specific for the peptide bound by HLA-A*0201.

To further demonstrate the peptide specificity of alloreactive CD8⁺ T cells, binding by a mixture of six tetramers was analyzed anticipating that the total percentage of CD8⁺ T cells stained should be a summation of the tetramer-binding percentages of the individual specificities. Two alloreactive T cell lines were prepared using the same HLA-A*0201-negative responder (donor 1) stimulated with mature dendritic cells from HLA-A*0201-positive donor 4 and HLA-A*02-negative donor 5, respectively. Both T cell lines were analyzed with the HLA-A*0201 tetramers. Staining

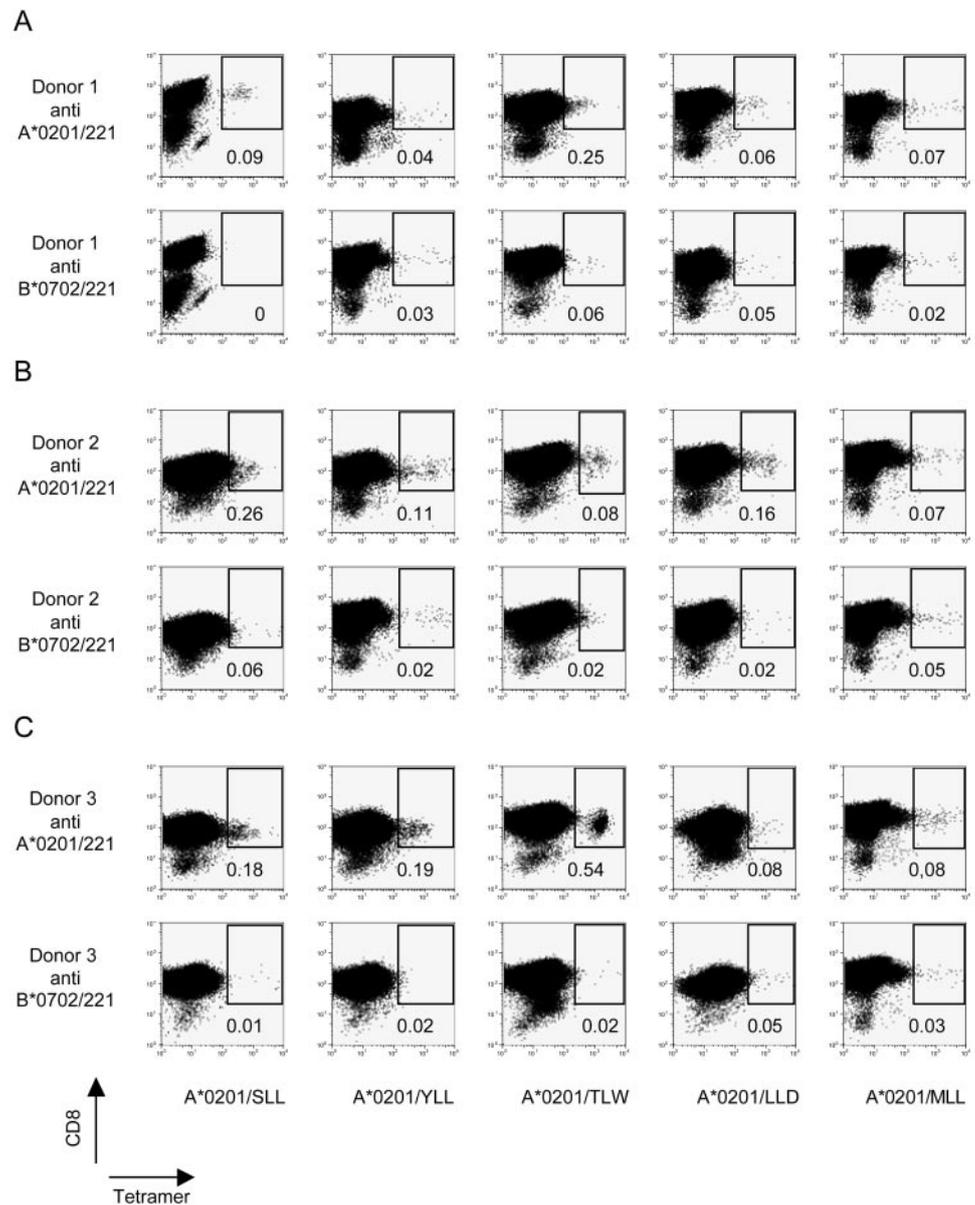


FIGURE 1. Alloreactive CD8⁺ T cells within anti-HLA-A*0201 populations bind HLA-A*0201 tetramers with several different self-peptides. Alloreactive CD8⁺ T cell lines specific for HLA-A*0201 or HLA-B*0702 from donors 1 (A), 2 (B), and 3 (C) were assessed for tetramer binding.

with an HLA-A*0201 tetramer was considered positive if the percentage of tetramer-binding CD8⁺ T cells in the antidonor 4 population was >4-fold above that of the control antidonor 5 population. Although the alloresponse to donor 4 was more complex, including responses to both HLA-A*0201 and HLA-B*0702 (Fig. 3A) due to multiple HLA class I mismatches, peptide-specific anti-HLA-A*0201 alloreactive T cells could still be detected. Subsets of T cells bound the A*0201/SLL, A*0201/YLL, A*0201/TLW, A*0201/MLL, and A*0201/GLF tetramers, and a pool of the tetramers stained 2.89% of the CD8⁺ T cells representing the combined subsets (Fig. 3B). Intracellular staining was performed to determine the functional characteristics of tetramer-binding T cells within the antidonor 4 population. Staining for intracellular perforin showed that the majority of A*0201/YLL tetramer-binding T cells possesses the potential for lytic activity (Fig. 3C). IFN- γ and TNF- α were produced by HLA-A*0201 tetramer-binding T cells in response to stimulation with cells that express HLA-A*0201, but not when exposed to cells expressing an irrelevant HLA class I type (Fig. 3C).

In summary, expansion of tetramer-positive populations by stimulation with relevant peptide, the lack of significant cross-

reactive tetramer binding, summation of the individual specificities by staining with a tetramer mixture, and HLA-A*0201-dependent functionality together conclusively demonstrate presence of peptide-specific alloreactive CD8⁺ T cells.

No detection of peptide-independent alloreactive CD8⁺ T cells using HLA class I/peptide-coated aAPCs

So far, our results suggest that the anti-HLA-A*0201 alloreactive populations contain small subsets of T cells, each specific for a peptide presented by HLA-A*0201. However, presence of peptide-independent alloreactive T cells within the populations cannot be excluded because they may possess low affinity for foreign HLA class I that could preclude binding to tetramers. To test for the presence of peptide-independent alloreactive CD8⁺ T cells, the anti-HLA-A*0201 alloreactive populations were stimulated with various aAPCs, each coated with a single HLA-A*0201/peptide combination (Table II), and production of IFN- γ was measured.

The aAPCs are efficient stimulators of Ag-specific CD8⁺ T cells (32). Fig. 4A illustrates that T cell responses to aAPCs are comparable to those obtained with natural APCs. We detected production of IFN- γ by HLA-A*0201/HA-1-specific clonal T cells

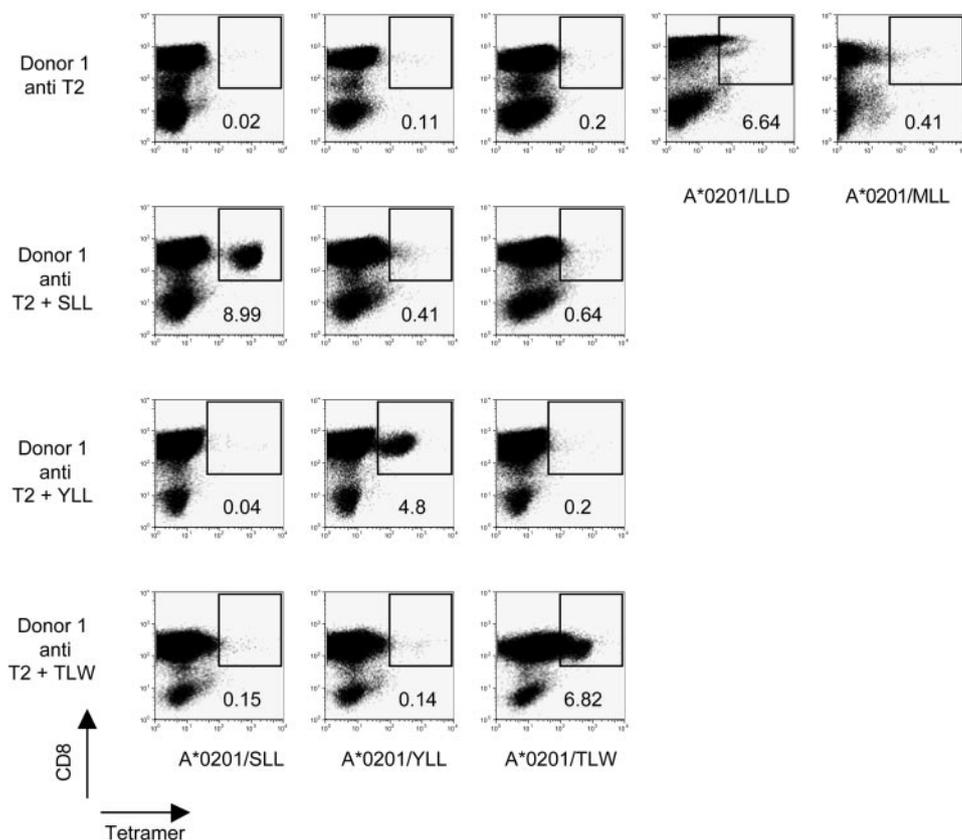


FIGURE 2. Alloreactive CD8⁺ T cells exhibit precise specificity for peptides bound by foreign HLA-A*0201 molecules. CD8⁺ T cells from donor 1 stimulated with T2 cells alone or T2 cells pulsed with self-peptides SLLPAIVEL, YLLPAIVHI, or TLWDPYEV were assessed for tetramer binding.

diluted to 1% within an HLA-B*0702-restricted clonal T cell population when stimulated with A*0201/HA-1 aAPCs or with HLA-A*0201-positive cells presenting HA-1 peptide. No IFN- γ was produced after stimulation with aAPCs coated with other A*0201/peptide combinations or HLA-A*0201-positive, HA-1-negative cells. Values 4-fold or more above the background IFN- γ produced by unstimulated T cells were considered positive. To evaluate the capacity of aAPCs to stimulate alloreactive T cells, a population containing 7.59% A*0201/YLL tetramer-binding CD8⁺ T cells was generated from HLA-A*0201-negative donor 5 by stimulation with T2 cells loaded with YLL peptide (data not shown). IFN- γ production was detected after stimulation with A*0201/YLL aAPCs, but not aAPCs coated with other A*0201/peptide combinations or B*0801/BZLF-1 (Fig. 4B). The small percentage of CD8⁺ T cells that produced IFN- γ in response to A*0201/YLL aAPCs (0.63%) implies not all A*0201/YLL tetramer-binding T cells produce IFN- γ . This is because the A*0201/YLL tetramer-binding cells are unlikely to be clonal. Functional heterogeneity of our tetramer-binding CD8⁺ T cells is indicated by the results presented in Fig. 3C, which showed ~10% produce IFN- γ in response to specific Ag, and others exhibit TNF- α production or cytolytic potential. The high percentage of CD8⁺ T cells producing IFN- γ after stimulation with HLA-A*0201⁺ natural cells is attributed to the summation of responses by A*0201/YLL-specific T cells and other alloreactive T cells specific for TAP-independent peptides presented by HLA-A*0201 molecules on T2 cells (see Fig. 2). Consistent with this explanation, stimulation with HLA-A*0201-positive T2 cells alone or HLA-A*0201-positive T2 cells with synthetic YLL peptide induced similar percentages of IFN- γ -producing T cells (data not shown). Collectively, the results presented in Figs. 4, A and B, show that A*0201/peptide-coated aAPCs can stimulate detectable responses by IFN- γ -producing T cells present at frequencies down to ~1%.

Despite the stimulatory capacity of aAPCs and sensitivity of the assay to low frequencies of responding cells, the anti-HLA-A*0201 alloreactive T cell lines from donors 1, 2, and 3, and the donor 1 antidonor 4 population did not show responses above background to any of the aAPCs (Table III). All four alloreactive T cell lines produced significant amounts of IFN- γ when stimulated with HLA-A*0201-positive cells. There was also a small amount of IFN- γ produced after stimulation with HLA-A*0201-negative P3 cells, but this was likely due to recognition of HLA class II mismatches. In summary, although the aAPCs can induce responses by peptide-specific alloreactive CD8⁺ T cells, peptide-independent responses were not detected.

Discussion

We used recombinant HLA class I multimers to study T cell allorecognition. Alloreactive CD8⁺ T cell populations were shown to contain small distinct subsets of T cells that exhibit precise specificity for peptides bound endogenously by foreign HLA class I molecules. No peptide-independent alloreactive T cells were detected. Alloreactive populations stimulated with TAP-deficient T2 cells contained T cells specific for HLA-A*0201 with TAP-independent peptides. The proportion of T cells that bound the HLA-A*0201 tetramers comprising peptides from proteins present in the endoplasmic reticulum was relatively high (up to 6.64%), suggesting the alloresponse to Ag-processing deficient cells is focused to the limited set of peptides presented by these cells. MHC structure-specific T cells would be expected to respond to a foreign MHC molecule complexed with a variety of peptides because of indifference to the sequence of the bound peptide. However, subsets of anti-HLA-A*0201 alloreactive T cells that bound one tetramer combination did not cross-react with other members of the tetramer panel. Also, functional densities of HLA-A*0201 molecules

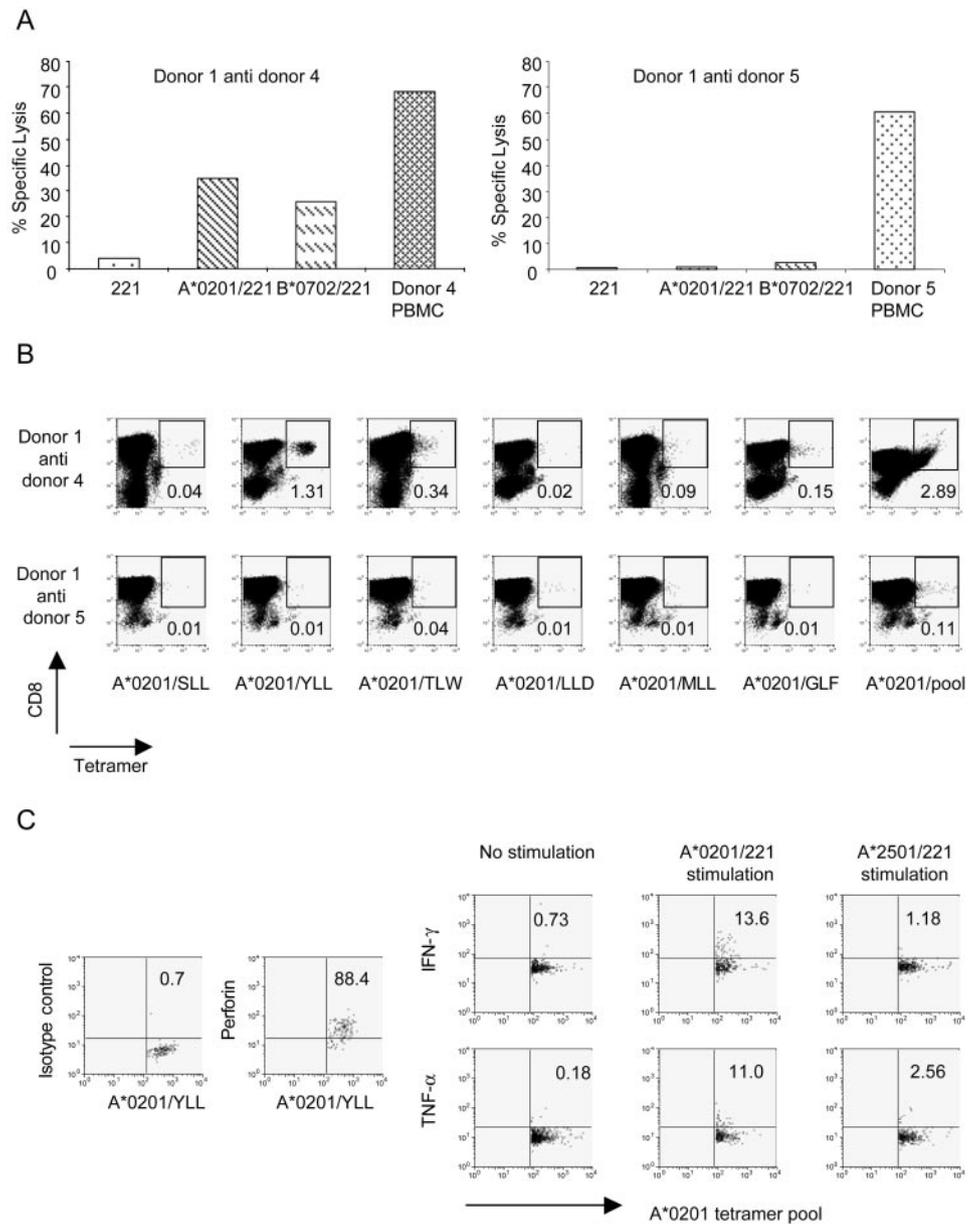


FIGURE 3. Alloreactive HLA-A*0201 tetramer-binding T cell populations do not overlap and are functional. Alloreactive CD8⁺ T cells were established from donor 1 by stimulation with mature dendritic cells from donor 4 (HLA-A*0201 positive) or donor 5 (HLA-A*0201 negative) and analyzed for cytolytic activity (A), tetramer binding (B), and perforin expression by A*0201/YLL tetramer-binding CD8⁺ T cells, and cytokine production by pooled HLA-A*0201 tetramer-binding CD8⁺ T cells within the donor 1 antidonor 4 alloreactive population (C).

complexed with single peptide presented on aAPCs did not stimulate anti-HLA-A*0201 alloreactive T cells. Our results indicate that peptide-independent alloreactive T cells, if they exist, are rare within the alloreactive T cell populations we analyzed.

All four of the anti-HLA-A*0201 alloreactive populations that we examined contained T cells specific for at least three of a panel of five self-peptides bound endogenously by HLA-A*0201. T cells specific for these peptides could not be generated from PBMCs of HLA-A*0201-positive individuals (data not shown), consistent with deletion of self-reactive cells from the repertoire during thymocyte maturation. Although circumstantial evidence has favored the proposal that alloreactive T cells are specific for the novel set of self-peptides presented by foreign MHC molecules, few of the peptides recognized by alloreactive T cells have been identified (reviewed in Ref. 10). The use of HLA class I tetramers enables detection of frequencies of individual peptide-specific alloreactive T cells as low as 0.04% within a complex mixture. The HLA-A*0201-binding self-peptides we studied are known to be present at relatively high density on the cell surface and may be candidate immunodominant alloligands (27). However, the most abundant of

the specificities detected with tetramers represented only 1.31% of the total CD8⁺ T cell population. The low frequency of individual specificities explains why previous studies using alloreactive T cell clones failed to detect responses to self-peptides known to be bound by HLA class I molecules *in vivo* (19, 20). The pool of HLA-A*0201 tetramers bound only a few percentage of T cells, but a significant number of tetramer-negative T cells also exhibited anti-HLA-A*0201 alloreactivity because they produced IFN- γ in response to stimulation with HLA-A*0201-positive cells (Table III). Because the tetramer-negative cells are not peptide independent, we assume that they comprise T cells specific for other peptides from among the many known to be bound endogenously by HLA-A*0201 (27). Two reports describe examples of alloreactive T cells that are peptide-dependent but not peptide-specific based on their ability to bind tetramers comprising several different peptide combinations presented by HLA-A*0201 (6, 34). However, in both studies, the T cells were produced by stimulation with high densities of a single HLA class I/peptide combination that may artificially promote MHC structure-specific recognition.

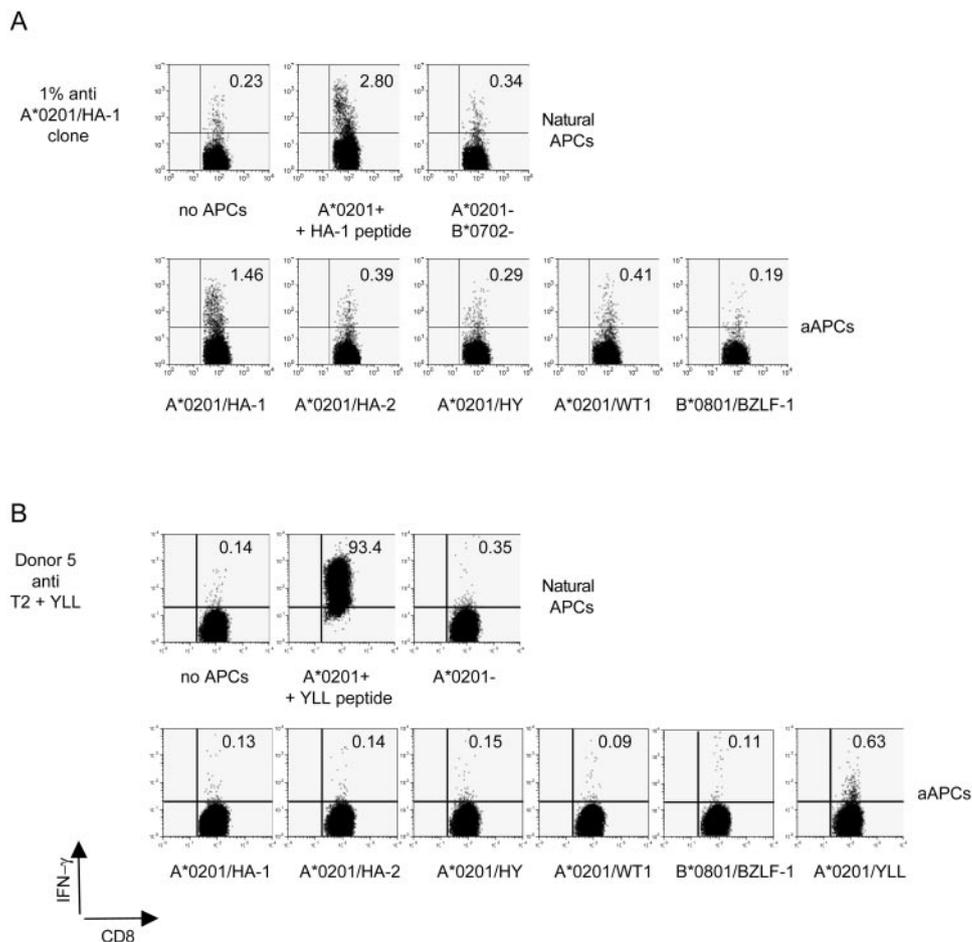


FIGURE 4. Alloreactive CD8⁺ T cells are not stimulated by aAPCs expressing single HLA-A*0201/peptide combinations. *A*, 1% HA-1-specific HLA-A*0201-restricted T cell clone diluted in a HLA-B*0702-restricted T cell clone. *B*, Donor 5 anti-T2 + YLL alloreactive T cell line were tested for IFN- γ production in response to EBV-transformed cells H6 and P3 or aAPCs.

We easily detected peptide-specific anti-HLA-A*0201 alloreactive CD8⁺ T cells within populations stimulated by HLA mismatches ranging from only three residues (HLA-A*0203) up to 28 residues (HLA-A*2402). Owing to the difficulty finding HLA-matched transplants for patients from unrelated donors, there is interest in identifying potentially permissive HLA-mismatched combinations that do not induce strong alloreactivity (35). Tools are being developed to rank HLA mismatches and provide scores on which to base judicious selection of mismatches (36). At first glance, HLA-A*0203 might be viewed as a permissive mismatch with HLA-A*0201. The three polymorphic positions Thr for Ala at 149, Glu for Val at 152, and Trp for Leu at 156 located within the peptide binding site appear to have limited functional impact, because these allotypes bind very similar sets of peptides (37). However, we detected alloresponses to four of five peptides bound by HLA-A*0201 across the HLA-A*0203 mismatch. The ability of alloreactive T cells to distinguish between the same peptide presented by related HLA class I molecules has been described pre-

viously in the context of HLA-B*27 subtypes (38) and also the HLA-B*4402/HLA-B*4403 dimorphism (39). In the latter case, the single amino acid difference located in the peptide binding site induces sufficient *in vivo* alloreactivity to form a barrier to bone marrow transplantation (40). These alloresponses occur because identical peptides bound by different MHC molecules are presented in altered conformations that can be distinguished by T cells (39, 41). Demonstration that subtle changes in peptide presentation can profoundly influence T cell recognition highlights the need for detailed studies to establish whether definable and consistent differences in the strength of the T cell alloresponse actually exist.

The emerging picture is that peptides play an integral role in all forms of T cell recognition. MHC-bound self-peptides influence development of immature thymocytes (42, 43), and mature T cells interact with Ags from foreign pathogens presented by self-MHC or self-peptides presented by foreign MHC molecules. The alloreactive T cells can originate from both naive and memory T cell populations (44). However, involvement of memory T cells need

Table III. Alloreactive CD8⁺ T cell lines do not produce IFN- γ in response to aAPCs

T Cell Line	Stimulation with Natural Cells			Stimulation with aAPCs				
	No Stimulation	H6 (A*0201 ⁺)	P3 (A*0201 ⁻)	A*0201/HA-1	A*0201/HA-2	A*0201/HY	A*0201/WT1	B*0801/BZLF-1
Donor 1 anti-A*0201/221	0.53 ^a	16.00	0.87	0.25	0.13	0.36	0.37	0.60
Donor 2 anti-A*0201/221	0.21	36.71	2.01	0.25	0.08	0.24	0.03	0.06
Donor 3 anti-A*0201/221	0.22	54.42	1.92	0.16	0.14	0.18	0.32	0.10
Donor 1 anti-donor 4	0.46	96.77	4.50	0.50	0.48	0.50	0.52	0.59

^a Results shown are percentage live CD8⁺ T cells producing IFN- γ . Values 4-fold or more above background IFN- γ produced by unstimulated T cells are considered positive.

not imply previous alloimmunization by pregnancy, blood transfusion, or allogeneic tissue transplantation. Instead, memory T cells specific for viral peptides presented by self-MHC can exhibit cross-reactivity with allogeneic MHC molecules (45). T cells need to be cross-reactive because they have to interact with a self-peptide bound by self-MHC during thymic selection and subsequently recognize self-MHC presenting a foreign peptide. This is the minimum set of ligands. It is likely that a single T cell recognizes several different pathogen-derived peptides, otherwise estimates suggest the T cell repertoire is of insufficient size to provide effective pathogen surveillance (46). Alloreactivity seems to be an adverse consequence of the need for cross-reactivity.

Structural studies are revealing how a TCR cross-reacts with several different combinations of MHC and bound peptide. TCRs possess inherent MHC reactivity (47, 48) because the majority of contact points with a MHC molecule involve conserved residues (49). Cross-reactive peptide recognition is not due to fundamental differences in mode of interaction, because the structure of a TCR bound to allogeneic MHC/peptide shows a diagonal orientation very similar to the one used to contact self-MHC (50). However, the set of peptides recognized by a single TCR does not have to share obvious sequence homology (51–53). Evidence indicates that accommodation of structurally dissimilar peptides is achieved by flexibility of the CDR3 regions of the TCR that contact peptide (54).

Our demonstration that T cell alloreactivity primarily involves low frequency responses to individual peptides presented by foreign HLA molecules and does not seem to be directed at HLA molecules per se has several clinical implications. First, it renders the suggestion that alloresponses could be specifically controlled by donor Ag modification impractical (55). However, precise knowledge of the structure of alloantigens should facilitate development of improved methods for diagnosis of transplant rejection or graft-vs-host disease and monitoring for establishment of transplant tolerance. Furthermore, our findings address concerns raised earlier regarding the use of allorestricted T cells specific for tumor Ags for immunotherapeutic purposes (3–7). The absence of peptide-independent T cells within our alloreactive populations suggests that the adventitious generation of detrimental alloreactivities will be rare. The ease with which we detected peptide-specific responses restricted by foreign HLA class I and the absence of undesired alloreactivities illustrates the considerable potential that exists within the allorestricted T cell repertoire that could be exploited for adoptive immunotherapy.

Disclosures

The authors have no financial conflict of interest.

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