

The Role of Peptides in T Cell Alloreactivity Is Determined by Self-Major Histocompatibility Complex Molecules

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Abstract

By analyzing T cell responses against foreign major histocompatibility complex (MHC) molecules loaded with peptide libraries and defined self- and viral peptides, we demonstrate a profound influence of self-MHC molecules on the repertoire of alloreactive T cells: the closer the foreign MHC molecule is related to the T cell's MHC, the higher is the proportion of peptide-specific, alloreactive ("allorestricted") T cells versus T cells recognizing the foreign MHC molecule without regard to the peptide in the groove. Thus, the peptide repertoire of alloreactive T cells must be influenced by self-MHC molecules during positive or negative thymic selection or peripheral survival, much like the repertoire of the self-restricted T cells. In consequence, allorestricted, peptide-specific T cells (that are of interest for clinical applications) are easier to obtain if T cells and target cells express related MHC molecules.

Key words: peptide library • T cell repertoire • molecular basis of alloreactivity • limiting dilution • positive selection

Introduction

Alloreactive T lymphocytes respond to allelic MHC variants in the apparent absence of nominal antigen and are the cause for important clinical problems such as graft rejection and graft versus host disease. *In vitro*, alloreactive T cells are activated easily by foreign MHC molecules without previous *in vivo* priming and are detectable in amazingly high frequencies (1). The molecular basis for these phenomena has not been elucidated, despite extensive characterization of peptide requirements in alloreactivity and the solution of several molecular structures of the 2C and AHIII12.2 TCR allo- and xenomodels (2–5).

Several hypotheses have been proposed to explain alloreactivity (2). Matzinger and Bevan (6) suggested that alloreactive T cells recognize many different cellular antigens, e.g., peptides, together with the foreign MHC. This model explains the strong alloresponse by multiple binary interactions between allorestricted T cell clones and new peptides not presented by self-MHC. Later, Kaye and Janeway (7) and Bevan (8) proposed that alloreactive T cells see all cell

surface MHC molecules regardless of peptides as antigens, leading to a high determinant density per target cell. In the meantime, evidence for peptide-dependent and peptide-independent allorecognition has accumulated. On the one hand, for several alloreactive T cell clones the exact peptides recognized, which can be present at both high and low copy numbers, have been identified (9–13). On the other hand, recognition of MHC molecules in the absence of peptides has been clearly demonstrated (14, 15). There are also cases where the peptide changes structural characteristics of the MHC molecule and leads to recognition by certain T cell clones in a peptide-dependent rather than peptide-specific manner (16–18). Today, the two models of allorecognition may be seen as two extremes on a scale where MHC and peptide contribute in different degrees to the overall binding energy between the TCR and its ligand. Accordingly, Daniel et al. explained the decreased peptide specificity of the alloreactive T cell clone 2.102 with certain allelic α -helical residues of the foreign MHC, which supposedly supply more binding energy to the TCR than the respective self-MHC molecule (19).

The involvement of peptides in alloreactivity that are present on stimulator cells in low copy numbers (3, 13) raised the possibility of generating allorestricted CTLs against tumor-associated self-peptides (self as seen from the tumor's host) presented by MHC molecules (nonself for

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the T cells) for adoptive immunotherapy (20–22). For this purpose, referring to allogeneic repertoires is necessary because T cell tolerance is MHC restricted (20, 23–25). Stauss and colleagues and our group demonstrated high avidity allorestricted CTLs against tumor-associated peptides and libraries from allogeneic mouse and human repertoires (21, 26–28). However, a T cell repertoire, which contains T cells recognizing peptides bound to nonself-MHC molecules with high avidity contradicts the well-established bias of T cells to react against antigens preferentially in the context of self-MHC (29).

To analyze the peptide repertoire of alloreactive T cells and the potential influence of self-MHC molecules, we stimulated naive splenic T cells of MHC class I mutant or H2 recombinant mice against RMA-S cells (H2^b) loaded with defined K^b- or D^b-specific peptide libraries (K^bL, D^bL)¹ under limiting dilution conditions. The relative frequencies of peptide-specific versus peptide-nonspecific T cells were determined, and several T cells of interesting peptide specificity were expanded and further analyzed. The results allow an unexpected insight into the relations between self-MHC and the repertoire of alloreactive T cells.

Materials and Methods

Mice, Cell Lines, and Virus. C57BL/6 (abbreviated B6) and BALB/c mice were purchased from Charles River. B10.A(5R) (5R), B10.HTG (HTG), and B6.C-H2^{bm1} (bm1) animals were obtained from The Jackson Laboratory and maintained in our animal facility. B6.C-H2^{bm13} (bm13) and B6.C-H2^{bm14} (bm14) mice were received from Drs. R. Brandt and C.J.M. Melief (Leiden University Medical Center, The Netherlands). FVB/N transporter associated with antigen processing 1^{-/-} (TAP)-1^{-/-} [30] mice were obtained from Drs. H. ter Rile and A. Berns (Cancer Institute, Amsterdam, The Netherlands) and also bred in our animal facility. EL4, RMA (H2^b, TAP⁺), and RMA.S (H2^b, TAP⁻) cell lines were maintained in RPMI 1640 (Sigma Chemical Co.) supplemented with 2 mM l-glutamine (BioWhittaker), 2 μM 2-ME, and 10% FCS (Sigma Chemical Co.). For generating blasts, 0.5–2 × 10⁷ splenocytes (in 5–10 ml RPMI) were stimulated for 2 d with 2.5–5 μg/ml Con A (Boehringer Mannheim), replated in CTL medium (see below), and used on days 3–5 of culture. Vesicular stomatitis virus (VSV) was obtained from Dr. R. Zatzky (German Cancer Research Center, Heidelberg, Germany).

Peptides. Peptides and libraries were synthesized and analyzed as described (27). The K^bL has been described previously (27), and the D^bL consisted of the amino acids indicated in Table I (anchor positions in bold). Both libraries bind to the respective MHC allele as strongly as control peptides from OVA and influenza nucleoprotein in an RMA.S induction assay (data not shown). The new H2-K^b ligands were identified as described previously (31) by immunoprecipitation of peptide-MHC complexes, treatment with 0.1% trifluoroic acid, ultrafiltration, fractionation by HPLC, and subsequent analysis by automated Ed-

Table I. H2-D^b Binding Peptide Library D^bL (n = 2,000)

1	2	3	4	5	6	7	8	9
F	Q	N	D	N	G	Y	T	M
I	A		V		A	V		
N	M		Q		L	T		
S	S		K		M	Q		
A			E			E		

man degradation (sequencer model Procise 494A; Applied Biosystems).

Generation of Effector Cells and CTL Assays. Splenocytes were plated in limiting dilution in round-bottomed 96-well plates (Greiner), starting with 1–2 × 10⁵ cells/well and diluting 1:2. Each well received 5 × 10⁵ irradiated (33 Gy) stimulators in 200 μl of α-MEM (GIBCO BRL) with the above-listed supplements, including 5% Con A-induced rat splenocyte supernatant, 50 μM α-methylmannoside (CTL medium; Roth), and, where applicable, a peptide library (500 ng/ml) or a peptide mixture. After 7 d, the cultures were stimulated as before with peptides at 50 ng/ml. The cultures were tested 4–6 d later in a split-well ⁵¹Cr-release assay, as described previously (27). For expansion, CTLs were restimulated in 48- and 24-well plates with 250 or 5 × 10⁵ irradiated TAP-1^{-/-} splenocytes, respectively.

Results

Differential Requirement for Synthetic Peptides in Alloreactivity. For the generation of tumor-specific CTLs, the holes in the self-restricted T cell repertoire led us to raise CTLs against peptide libraries presented on MHC molecules foreign to the T cells (27, 28). In the course of such experiments, we noticed difficulties in raising H2-K^b- or H2-D^b-restricted CTLs from H2^d and H2^k animals against several individual peptides (data not shown) and investigated the influence of the responder's haplotype.

Splenocytes from HTG, bm1, and B6 mice were stimulated with TAP-1^{-/-} (H2^b) splenocytes in the presence of an H2 K^bL under limiting dilution conditions. HTG animals express the K^d allele with 35 polymorphic amino acids within the α1 and α2 domains, whereas the K^{bm1} allele carries merely 3 amino acid exchanges, both compared with K^b (Table II). After one restimulation, the cultures were tested against TAP⁻ RMA.S cells (H2^b) in the presence or absence of K^bL. H2 expression was not increased under our experimental conditions (data not shown). The library is a strong antigen for B6 responders, as it contains numerous nonself-peptides (Fig. 1, top right). Previously, we have shown that such CTLs can be of high avidity towards the particular peptide or peptides recognized (27). For the allogeneic responders, it turned out that the HTG splenocytes generated less allorestricted, i.e., K^b-restricted, and peptide-specific/dependent cultures (ratio of peptide-specific [P] to structure-specific [S] cultures 1:4) than bm1 splenocytes (P/S = 1:1; Fig. 1, top). These results suggest that the bm1 repertoire is more prone to react in a peptide-dependent

¹Abbreviations used in this paper: B6, C57BL/6; K^bL or D^bL, K^b or D^b binding peptide library, respectively; K^b_(self), mixture of 10 K^b binding self-peptides; TAP, transporter associated with antigen processing; VSV, vesicular stomatitis virus.

Table II.

Mutation	Position	Exchanges	Pocket (peptide res.)	TCR interaction
K ^{bm1}	152	Glu→Ala	D (3), C (6), E (7)	–
	155	Arg→Tyr	D (3), C (6), E (7)	CDR1 α , 2 α , 3 β
	156	Leu→Tyr	D (3), C (6), E (7)	–
D ^{bm13}	114	Leu→Gln	E (7), F (9/10) C (6), E (7),	–
	116	Phe→Tyr	F (9/10)	–
	119	Glu→Asp		
D ^{bm14}	70	Gln→His	B (2), D (3), C (6), F (9/10)	–

Mutated MHC class I molecules of mouse strains used in this study. Compiled from references 34, 56–58.

way towards K^b than the HTG repertoire. Out of the 35 amino acids polymorphic between K^d and K^b, 13 make contact with the bound peptide (32, 33), whereas 2 (residues 79 and 155) are bound by the 2C TCR directly (34). However, one of the three amino acids that are polymorphic between K^b and K^{bm1} contacts the 2C TCR directly (residue 155; Table II). It is tempting to speculate that this residue is responsible for the finding that about half of the cultures recognize RMA.S cells in the absence of K^bL. These data show that allogeneic MHC molecules, which carry amino acid exchanges at positions that are responsible for peptide binding, preferentially elicit an alloresponse against the bound peptides, as one would expect from considering the structure of the MHC–peptide complex.

None of the polymorphic amino acids of H2-D^{bm13} and H2-D^{bm14} (compared with D^b) are directly involved in TCR contacts (Table II). Consequently, after stimulation of splenocytes with TAP-1^{-/-} cells and a D^bL, the majority of cytotoxicity was directed towards D^bL-coated RMA.S cells (Fig. 1, bottom). Amino acid 70, which is exchanged in D^{bm14}, is positioned at the inner rim of the α 1 helix and seems to cause some structural change that is detected by several cultures reacting to both D^bL-coated and untreated RMA.S cells (P/S = 5:1). The contribution of cultures specific for structural elements to overall alloreactivity is even further reduced in the case of bm13 responders, which carry polymorphic residues primarily determining the chemical environment of the binding groove's F pocket (P/S = 10:1). Our data indicate that increasing similarity between α -helical residues of the responder's and the stimulator's MHC increases the contribution of peptide-specific or -dependent T cells to an alloresponse.

Differential Requirement for Natural Peptides in Alloreactivity. In the experiments described above, we made use of peptide libraries of limited complexity to replace the more diverse set of self-peptides on TAP⁺ cells. Under our loading conditions (1 h of peptide incubation at 37°C), no increase in the expression of H2 molecules on RMA.S cells could be detected. This was true both for incubation with the peptide library and with individual peptides (data not shown). We wondered whether the above observations also apply to alloreactivity against cells expressing TAP, the wild-type complement of peptides and, therefore, the natural density of class I molecules on the cell surface. BALB/c, bm1, bm13, and bm14 splenocytes were stimulated with B6 cells under limiting dilution conditions and tested against RMA versus RMA.S target cells. In general, a higher proportion of microcultures reacting with the foreign MHC was detected irrespective of TAP expression (Fig. 2). This result suggests that TAP⁺ cells could stimulate more T cells specific for structural elements of K^b present on both TAP⁺ and TAP⁻ cells, because they express a higher density of

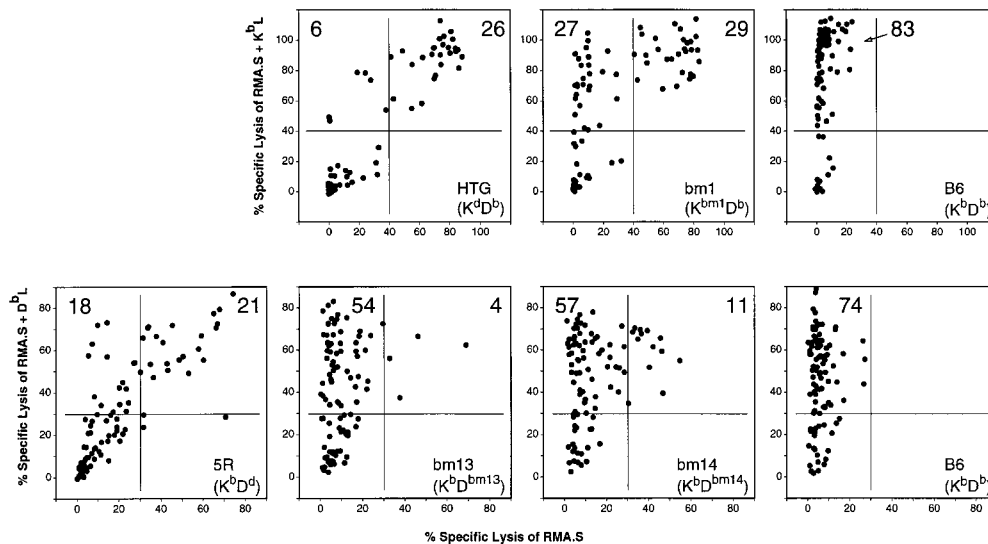


Figure 1. The responder H2 haplotype influences the role of peptide libraries in CTL alloreactivity. Splenocytes of the given strains were plated in limiting dilution and stimulated with irradiated TAP-1^{-/-} (H2^b) splenocytes in the presence of K^bL (top) or D^bL (bottom). The 96 cultures were assayed for CTL activity against RMA.S cells ⁵¹Cr labeled in the absence or presence of the respective library. Numbers of cultures within the arbitrarily chosen regions are indicated to allow for better comparison. Spontaneous release was <10%. The experiments were repeated with similar results.

H2 molecules on the cell surface. But again, bm1 responds with more peptide-specific cultures ($P/S = 1:3$) than the completely allogeneic responder BALB/c ($P/S = 1:5$), and bm13 and bm14 respond with even more ($P/S = 3:1$) than bm1 (Fig. 2). These results extend our conclusion that the particular responder–stimulator combination determines the role of peptides to alloresponses among wild-type stimulators and targets.

Allorestricted CTLs against Defined Self-Peptides. To further characterize alloreactive CTLs raised from a responder expressing an H2 molecule closely related to the stimulating one, we cultured bm1 splenocytes with irradiated TAP-1^{-/-} cells in the presence of 10 self-peptides eluted from K^b (K^b_{self}) by us and other laboratories (Fig. 3, a and b). Peptide-specific microcultures were expanded, and the specific peptide recognized was determined. We detected reactivities against determinants from the transcription factor Erk2, the cytosolic protein β -catenin, and the endothelial pas domain protein (EPAS-1; Fig. 3 c) as well as from RNA helicase p54 and the mouse mammary tumor virus envelope protein (data not shown). The specificity of the “aberrant” CTL lines recognizing RMA-S in the absence but not presence of added peptides (see Fig. 3 b, right) could not be elucidated. Importantly, all peptide-specific CTL lines recognized RMA tumor cells as well as B6 Con A blasts, but not bm1 Con A blasts, not even in the presence of peptides (Fig. 3 d). These results demonstrate that allorestricted and peptide-specific CTLs can be generated easily without depletion or extensive cloning from a related mouse strain. The lines that were raised against synthetic self-peptides can efficiently lyse target cells expressing physiological levels of the self-antigen and thereby circumvent peptide-specific self-tolerance.

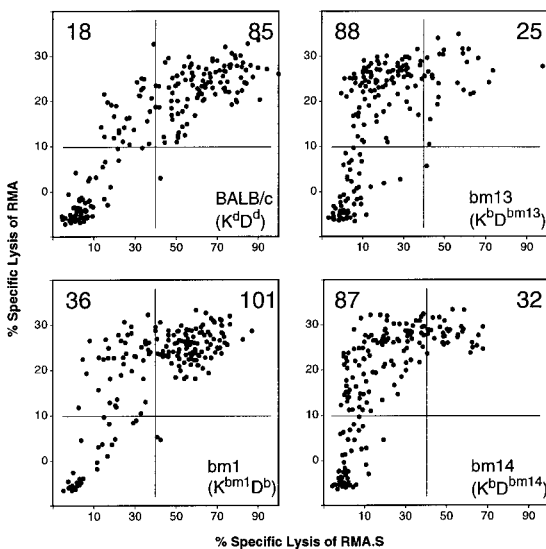


Figure 2. The responder H2 haplotype influences the role of endogenous peptides in CTL alloreactivity. Splenocytes of the given strains were stimulated under limiting dilution conditions with irradiated B6 splenocytes. 192 cultures were assayed against RMA and RMA.S. Numbers of cultures within the arbitrarily chosen areas are indicated to allow for better comparison. Spontaneous release was <21%.

Allorestricted CTLs against Defined Viral Peptides. To demonstrate more clearly the exclusive specificity of allorestricted CTLs from a related strain, a line was generated from bm1 against K^b and the immunodominant peptide from VSV (pVSV). The CTL line B6E10 was specific for pVSV, since it did not lyse RMA.S cells coated with the OVA257–264 peptide (Fig. 4 a) or any other of 38 K^b binding peptides tested (data not shown). The line also does not cross-react with self-peptides presented by K^b, since the TAP⁺ cell line EL4 was not recognized. In contrast, however, EL4 cells infected with VSV were lysed efficiently (Fig. 4 b). Thus we show, to our knowledge for the first time, that allorestricted and highly specific CTLs can be generated in vitro with synthetic peptides to lyse virus-infected targets.

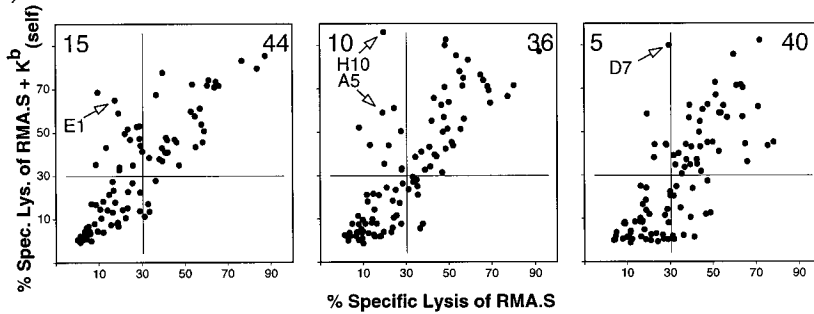
Discussion

We have previously shown that K^b-restricted and peptide-specific CTLs can be detected among BALB/c splenocytes. Such cells were very rare and were obscured by many alloreactive cells recognizing the target cells in a peptide-independent manner. We had to deplete such cells in order to generate several peptide-specific CTL lines (27). Similar protocols have been successfully used to deplete graft-versus-host activity without compromising the graft-versus-leukemia effect of allogeneic PBLs in bone marrow transplantation (35–37). However, since we (data not shown) and others (38) had difficulty generating allorestricted CTLs against individual peptides when CTLs and target cells were completely allogeneic to each other, we wished to analyze the spectrum of CTL precursors specific for allogeneic MHC–peptide complexes in closely related versus unrelated MHC combinations. Our results show that an alloresponse against a related MHC molecule contains more peptide-specific T cells than a response against an unrelated one: alloactions against an MHC molecule carrying groove mutations only (bm13 anti-B6, bm14 anti-B6) were clearly dominated by peptide-specific cells compared with a response against a molecule with both groove and α -helical replacements (bm1 anti-B6). Among responses against molecules with several groove and α -helical exchanges, peptide-specific cultures were further reduced (HTG anti-B6, 5R anti-B6, BALB/c anti-B6; Figs. 1 and 2). In addition, K^b-restricted CTLs from bm1 against self- as well as viral peptides showed high specificity and efficiently recognized naturally presented peptides (Figs. 3 and 4). We take these findings as evidence for an increased contribution of molecular mimicry due to partial identity to the alloresponse against similar MHC molecules, as first suggested by Lechler et al. (39): the self haplotype determines whether an alloresponse is dominated by cells recognizing nonself via multiple binary interactions or rather by cells seeing the allelic MHC molecules regardless of bound peptides. The mode of allorecognition, however, seems unrelated to the “antigenic strength,” as skin grafts done in the mutant strain combinations used in our study are rejected in a similar time frame (40, 41).

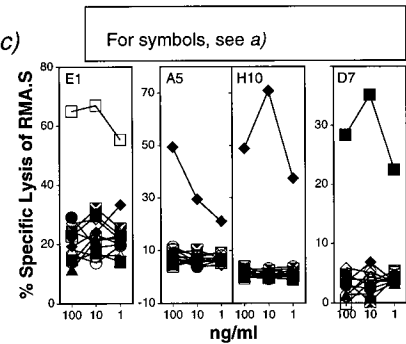
a)

Symbol in c)	#	Sequence	Source	Residues	Reference
■	8180	VGPRVTNL	Erk2	19-26	this paper
□	8181	RTYTYEKL	β-catenin	329-336	this paper
	8182	INFDFPKL	RNA helicase p54	407-414	this paper
	8183	GAYEFTTL	40kDa, unknown		(59)
	8184	EQYKFYSV	mMLRQ	61-68	(43)
	8185	VNVDYSKL	mMLRQ	68-75	(55)
	8186	ASYEFTQL	CDC, unknown		(59)
	7015	ANYDFICV	MMTV env	544-551	(12)
◆	4078	SNYLFTKL	lung, EPAS-1	386-393	(60)
	6172	HIYEFPLQ	P815p, unknown		(60)

b)



c)



d)

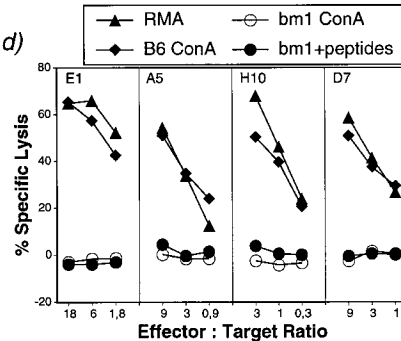


Figure 3. Allorestricted CTL lines from bm1 mice against K^b binding self-peptides. (a) Self-peptides eluted from K^b and used in this study as mixture K^b_(self) (references 12, 43, 55, 59, 60; and this study). (b) Splenocytes from bm1 animals were plated in limiting dilution and stimulated with TAP-1^{-/-} cells in the presence of a mixture of self-peptides at 500 (left), 100 (middle), and 50 ng/ml (right). After 10 d, the cultures were assayed against RMA.S in the absence or presence of the mixture. Spontaneous release was <10%. (c) Peptide specificity of expanded CTL lines. All peptides listed in a were titrated on ⁵¹Cr-labeled RMA.S cells. The indicated effector cells were added at effector to target ratios between 2 and 5. (d) The expanded CTL lines recognize tumor cells and Con A-induced blasts from B6, but not from bm1 mice. Spontaneous release was <10% for tumor cells and <18% for blasts.

The importance of the peptide-specific mode of alloreaction was not obvious from structural information published to date. Studies on molecules recognized by the 2C allo-TCR (4, 34, 42, 43) and the xenoreactive clone AHIII12.2 (5, 44) do not explain the clones' cross-reactivities by struc-

tural molecular mimicry. For 2C, only a critical negative charge on the self-MHC-peptide complex has been mimicked by the allogeneic ligand, as suggested in reference 4. We speculate that T cells responding to foreign MHC molecules carrying groove mutations interact with them in the same way as T cells interacting with their respective self-molecules. Such alloreactive T cells respond to the bound peptides, as these peptides had not been present during negative selection.

The T cell repertoire is shaped by selection events in the thymus and the periphery (45-48). It is currently assumed that a certain MHC haplotype selects 15-20% of CD4⁺ CD8⁺ thymocytes (49), of which 50% or more undergo clonal deletion by bone marrow-derived APCs (50, 51). Despite this profound imprint of the self-MHC on the T cell repertoire, an influence of selection events on peptide requirements of alloreactivity has not been shown. Our detailed analysis of alloreactive cells on a population level now

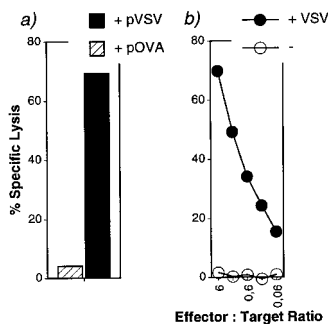


Figure 4. Allorestricted CTLs can recognize VSV-infected cells. (a) The bm1-derived CTL line B6E10 raised against TAP-1^{-/-} and the VSV-N52-59 peptide (pVSV) lyses RMA.S cells after incubation with the stimulating peptide, but not with the OVA257-264 peptide (pOVA). Effector to target ratio was 2. (b) The same line recognizes EL4 cells after infection with 2 × 10⁷ PFU VSV only. Spontaneous release was <20%.

shows that allorecognition is readily influenced by the selecting MHC molecule—rather than “hard-wired into the TCR genes” (45, 52)—and mirrors the resemblance between self and foreign.

It is not clear whether negative or positive selection, peripheral survival, or all three are skewing the repertoire towards peptide-specific/dependent recognition of related MHC molecules. Favoring negative selection, one could argue that, for example, the bm13 repertoire, due to its similarity to B6, is simply purged of T cells reactive against structural determinants of D^b and contains peptide-specific cells only. However, negative selection may not completely explain the skewing, since the antigenic strength of the pocket mutants bm13 and bm14 is almost as high as that of bm1, which carries a polymorphic amino acid at a position in direct contact with the TCR (40, 41). It is tempting to speculate that positive selection and peripheral survival enrich for cells able to recognize similar MHC molecules in a peptide-specific way and thereby modify the alloresponse as well.

The fact that tolerance is MHC restricted (20, 23–25) permits the generation of high avidity CTLs against MHC–self-peptide complexes for adoptive tumor therapy in three ways. First, a responder’s T cell can react towards a peptide from its own MHC groove if it is presented in a new context (same groove, new α helices; references 3, 53, 54). Second, CTLs have been raised against peptides presented by allogeneic MHC molecules carrying several α -helical and groove mutations (new groove, new helices; references 21, 27). Third, alloreactive CTLs can recognize an MHC molecule with groove mutations only because of the many new peptides it carries, as in a self-restricted response (new groove, same helices [reference 55, and this study]). We show here that the latter possibility allows for the easy generation of allorestricted CTLs against defined self- and viral peptides. We therefore suggest that this approach is more likely to yield high avidity human CTLs against self- and other peptides, including tumor-associated antigens. For example, to raise HLA-A*0201-restricted CTLs, it may be useful to start with T cells from a donor expressing A*0206 (1 mutation in pocket B) rather than from a donor expressing, e.g., A*0301 (3 pocket mutations and 11 exchanges outside the groove). In fact, 9 out of 22 HLA-A2 subtypes carry exclusively pocket mutations relative to A*0201 (56). Which of these subtypes is best suited to select T cells that respond to A*0201-bound self-peptides remains to be determined.

Taken together, we have shown that self-MHC shapes the repertoire not only of self-restricted, but also of alloreactive T cells. A practical consequence of our data bears on the generation of allorestricted, peptide-specific T cells, especially for those directed against tumor-associated peptides: the success rate for getting such T cells should be higher if T cell donor and stimulating APCs express MHC molecules with similar α helices but different peptide binding grooves.

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