

# Identification of High Potency Microbial and Self Ligands for a Human Autoreactive Class II-restricted T Cell Clone

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## Summary

CD4<sup>+</sup> class II-restricted T cells specific for self antigens are thought to be involved in the pathogenesis of most human autoimmune diseases and molecular mimicry between foreign and self ligands has been implicated as a possible mechanism for their activation. In this report we introduce combinatorial peptide libraries as a powerful tool to identify cross-reactive ligands for these T cells. The antigen recognition of a CD4<sup>+</sup> T cell clone (TCC) specific for myelin basic protein peptide (MBP) (86-96) was dissected by the response to a set of 220 11-mer peptide sublibraries. Based on the results obtained with the libraries for each position of the antigen, artificial peptides were found that induced proliferative responses at much lower concentrations than MBP(86-96). In addition stimulatory ligands derived from protein sequences of self and microbial proteins were identified, some of them even more potent agonists than MBP(86-96). These results indicate that: (a) for at least some autoreactive CD4<sup>+</sup> T cells antigen recognition is highly degenerate; (b) the autoantigen used to establish the TCC represents only a suboptimal ligand for the TCC; (c) a completely random and unbiased approach such as combinatorial peptide libraries can decrypt the spectrum of stimulatory ligands for a T cell receptor (TCR).

CD4<sup>+</sup> T cells recognize via their TCR 11-14-mer peptides derived from exogenous proteins after processing and presentation by a MHC class II-expressing APC (1). CD4<sup>+</sup> MHC class II-restricted T cells are not only essential for the specific immune response to foreign pathogens but also are thought to be involved in the pathogenesis of many autoimmune diseases. Based on sequence homologies, cross-recognition by autoreactive T cells of foreign antigenic peptides has been demonstrated, and it was hypothesized that self-reactive T cells expanded by foreign antigens may be involved in the pathogenesis of autoimmune diseases (2-4).

In recent years, peptide binding motifs of class II molecules have been identified by several experimental approaches. These motifs allow predictions about the binding of peptides to the MHC molecule and have helped to understand which ligands are available for recognition by CD4<sup>+</sup> T cells. In parallel, determinants derived from foreign and self antigens that are important for T cell recognition have been mapped in detail using modified (truncated, single

amino acid [aa]<sup>1</sup>-substituted) peptides. Based on the concept of conserved TCR contact positions (5) it is well established that CD4<sup>+</sup> T cells can recognize different peptides, but the number of possible ligands for a single T cell receptor is thought to be limited. However, it was also demonstrated that peptides sharing only a few amino acids are able to stimulate the same T cell clone (6) as long as they have conserved TCR contact residues (7). Using this information, a novel strategy to identify molecular mimicry peptides has been described, and stimulatory ligands derived from microbial proteins have been identified for autoreactive T cells, which do not share sequence homologies with the autoantigen in positions other than TCR contacts (7).

However, approaches using single amino acid exchanges of the peptide antigen in order to identify the anatomy of antigen recognition and to search for cross-reactive ligands are impaired by the large number of individual peptides

<sup>1</sup>Abbreviations used in this paper: aa, amino acid; CM, complete medium; MBP, myelin basic protein; TCC, T cell clone.

necessary to define a single T cell epitope. Furthermore, little is known about how multiple alterations of a ligand influence T cell recognition. Therefore, it has been impossible to define by formerly used approaches, how many ligands are recognized by a single T cell receptor and the full extent to which molecular mimicry can occur.

In contrast to the experiments mentioned above, combinatorial peptide libraries were introduced recently as an approach to allow epitope identification that is unbiased by sequence homologies or binding motifs. Peptide libraries have been successfully applied to define unknown antibody specificities (8), peptide recognition by SH2-domains (9) or enzyme substrate specificity (10). Peptide libraries have also allowed a more detailed dissection of MHC class I and II binding motifs (11, 12). Finally, 9-mer peptide libraries were used to identify stimulatory ligands for tumor-specific (13) or alloreactive CD8<sup>+</sup> T cell clones (14–16).

In this report, we used combinatorial peptide libraries to study antigen recognition of a CD4<sup>+</sup> T cell clone and to search for cross-reactive high potency ligands. The response of a MBP(86–96)-specific DR2b-restricted TCC to an undecapeptide library in the positional scan format (17, 18) was used to dissect its recognition pattern. Artificial peptide ligands based on the results derived from the library were shown to be more potent ligands than MBP(86–96). Furthermore, screening of protein databases with the library information revealed not only MBP as potential ligand for the TCC but also several peptides derived from self and foreign antigens. These ligands induced proliferation of the TCC, some of them even at lower concentration than MBP(86–96).

These data indicate: (a) for at least some class II-restricted T cells antigen recognition is highly degenerate; (b) for these TCC a high number of ligands with different stimulatory potency exist; (c) the autoantigen that was used to establish the TCC represents a suboptimal ligand; (d) peptide ligands from self and microbial antigens can be identified which are more potent than MBP(86–96).

These results not only have important implications for the understanding of how cross-reactivity is related to autoimmunity, but may also provide a model as to how degeneracy of antigen recognition might contribute to thymic selection.

## Materials and Methods

**Synthesis and Analysis of Undecapeptide Amides and Undecapeptide Amidelibraries.** The synthetic randomized peptide libraries varying in length from 6 to 15 amino acids, the undecapeptide amide sublibraries as well as individual undecapeptide amides were prepared by solid phase peptide synthesis using Fmoc/tBu chemistry and Rink amide MBHA polystyrene resins as described recently (12). In brief, in the case of peptide libraries, the introduction of randomized sequence positions was performed in a double coupling step with equimolar mixtures of Fmoc-L-amino acids that were used in an equimolar ratio with respect to the coupling sites of the resins. For coupling of defined sequence positions a five-fold molar excess of single Fmoc-L-amino acids was used. An optimized diisopropylcarbodiimide/1-hydroxybenzotriazole method

was used for the synthesis of the libraries to yield equimolar mixtures (19). Defined peptides were synthesized by multiple peptide synthesis (20). The identity of the defined undecapeptides was confirmed by electrospray mass spectrometry and the purity of the peptides was determined by HPLC. The amino acid composition in the randomized sequence positions of the sublibraries was determined by pool sequencing, electrospray mass spectrometry, and amino acid analysis. Deviations from equimolar representation of the amino acids in randomized sequence positions were found to be within the error limits of the analytical methods.

**T Cell Clone TL 5G7.** The T cell clone TL 5G7 was established from peripheral blood lymphocytes of an MS patient by a limiting dilution split well technique with MBP protein as described before (21). The TCC is DR2b (DR $\alpha$  + DRB1\*1501)-restricted and specific for MBP(86–96/83–99).

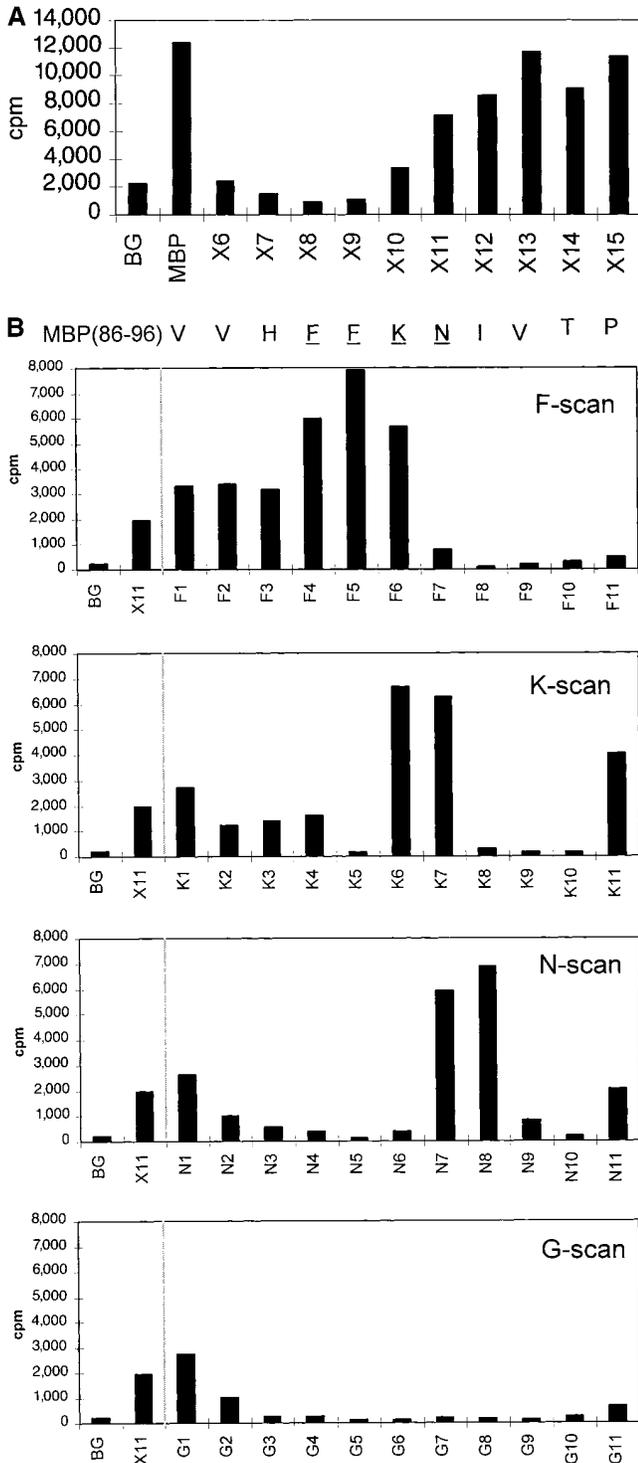
**T Cell Proliferation.** Cell proliferation was measured by standard [<sup>3</sup>H]thymidine-incorporation as described (22). TCC were rested for 8–12 d, washed and resuspended at  $1 \times 10^5$  cells/ml in complete medium (CM, IMDM containing 5% human serum, 1% penicillin/streptomycin, 0.2% Gentamycin). 100  $\mu$ l of this cell suspension were added to each well of 96-well U-bottom plates containing  $5 \times 10^4$  irradiated (3,000 R) PBL and varying concentrations of peptide or peptide libraries. Cells were cultured for 48 h at 37°C. During the last 6 h of culture, 1  $\mu$ Ci [<sup>3</sup>H]thymidine was added to each well. Cells were then harvested and incorporated radioactivity was measured by scintillation counting.

**CTL Assay.** CTL assays were performed as described (23). Briefly, target cells ( $5 \times 10^5$ ) were labeled overnight at 37°C in 500  $\mu$ l of CTL medium (RPMI + 5% fetal calf serum) with 50  $\mu$ Ci <sup>51</sup>Cr (Du Pont-New England Nuclear, Boston, MA). <sup>51</sup>Cr-labeled cells were incubated with different concentrations of peptide for 2 h, washed twice (200 g, 20°C, 10 min), and adjusted at  $2 \times 10^4$  cells/ml.  $2 \times 10^3$  targets were plated into 96-well U-bottom microtiter plates containing sufficient numbers of T cells to reach desired E/T ratios. After a 4-h incubation (37°C), supernatants were counted in a gamma counter (ME Plus; ICN Micro-medec, Huntsville, AL). Specific lysis was calculated according to the following formula:  $([\text{test release } \{\text{cpm}\}] - [\text{spontaneous release } \{\text{cpm}\}]) / ([\text{total incorporation } \{\text{cpm}\}] - [\text{spontaneous release } \{\text{cpm}\}]) \times 100$ .

**Database Searches.** The tolerated amino acids for each position of the 11-mer peptide were used to search human proteins in Swissprot using protein motif search engine (Virtual Genome Center, <http://alces.med.umn.edu/dbmotif.html>). In addition, sequences based on the peptide predictions were used to search Swissprot using BLITZ search engine ([http://www.ebi.ac.uk/searches/blitz\\_input.html](http://www.ebi.ac.uk/searches/blitz_input.html)). Peptide sequences from self or foreign peptides that matched at least 6 aa of the predicted aa in core positions were chosen.

## Results

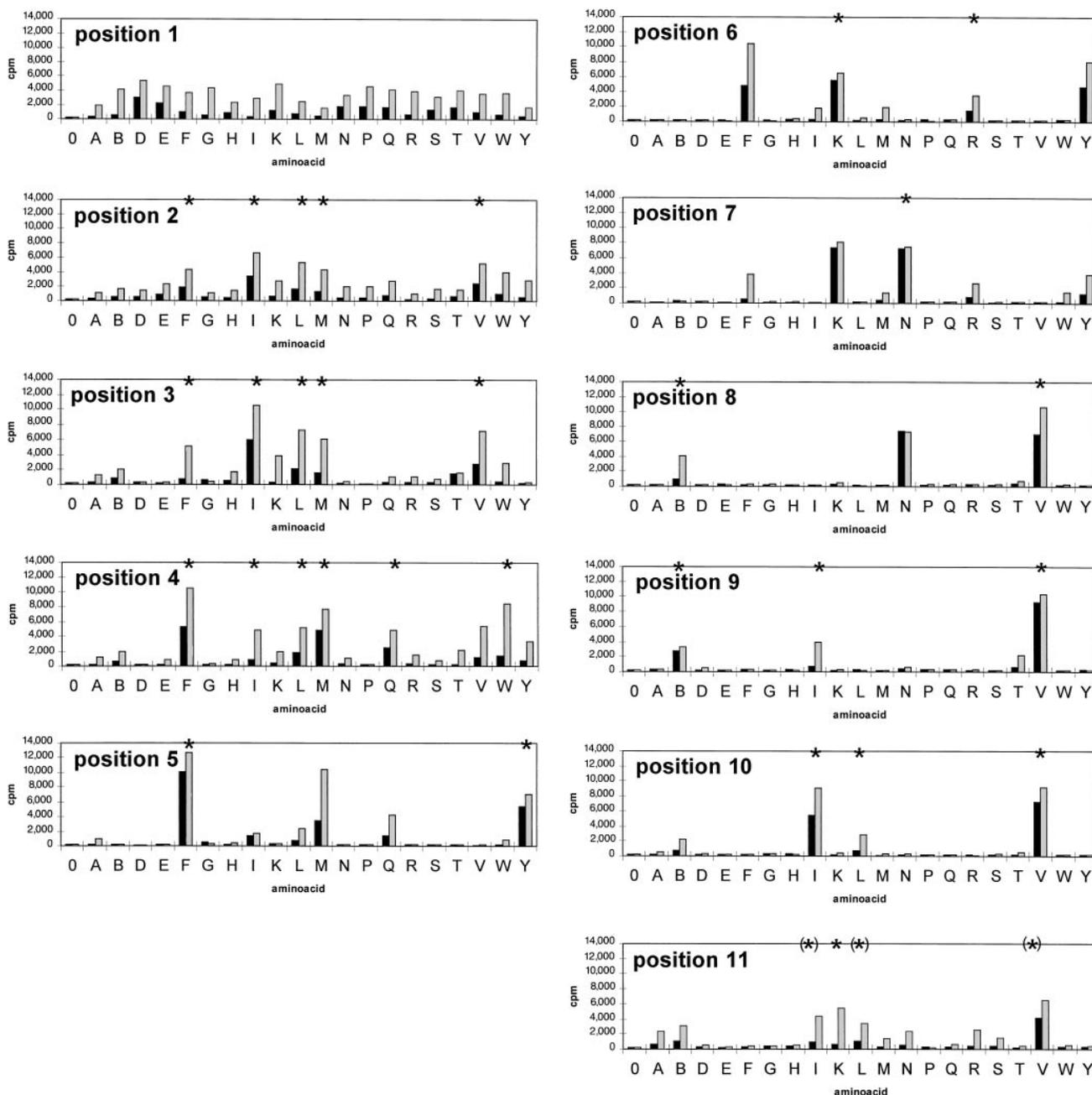
**The Response to Completely Randomized Combinatorial Peptide Libraries Suggests Degeneracy in Antigen Recognition.** TL 5G7 is a DR2b (DR $\alpha$  + DRB1\*1501)-restricted Th0-like TCC generated and grown using MBP as described (21, 22). The TCC is specific for the immunodominant peptide MBP(83–99) and MBP(86–96). The response to MBP(86–96) is two orders of magnitude weaker than to MBP(83–99) due to the lower MHC binding affinity of the shorter 11-mer peptide (24, 25). The TCC was first tested for its response to completely randomized peptide libraries. The libraries



**Figure 1.** Proliferative response of TL 5G7 to MBP(86-96) and different combinatorial peptide libraries. **A** displays the response to a sizing scan with completely randomized libraries ranging in length from 6 (X6) to 15 aa (X15). **B** shows the response to peptide sublibraries with fixed amino acid phenylalanine (F), lysine (K), asparagine (N), or glycine (G) in position 1 to 11. The proliferation is compared either to MBP(86-96) (**A**) or randomized library X11 (**B**). Note that each selected amino acid of the MBP sequence is recognized by two adjacent sublibraries, whereas the double F of the MBP sequence is recognized by three adjacent sublibraries. The peptide library concentration is 100  $\mu\text{g/ml}$ . BG = background. One representative out of three separate experiments yielding similar results is shown.

with defined peptide length were generated by random synthesis for each position with all 20 naturally occurring aa (only C is replaced by B[ $\alpha$ -aminobutyric acid]) (12). They contain a homogeneous mixture of all possible combinations of the 20 aa within the given peptide length. The equimolar distribution of all 20 aa in each randomized position of the defined undecapeptides was confirmed by electrospray mass spectrometry and the purity of the peptides was determined by HPLC. The aa composition in the randomized sequence positions of the sublibraries was determined by pool sequencing, electrospray mass spectrometry, and amino acid analysis (12). The peptide libraries contain large numbers of different peptides; the number is related to the length of peptides in the library (i.e., for a 11-mer peptide library  $20^{11} = 2 \times 10^{14}$  different sequences). Peptide libraries with different peptide length ranging from 6-mer (X6) to 15-mer peptides (X15) were used in the assay (Fig. 1 **A**). X6 to X9 induced no response whereas X10 to X15 induced significant proliferation of the TCC. The observation that the TCC responds to a mixture of  $2 \times 10^{14}$  different peptides (X11) almost as strongly as it does to 11-mer MBP peptide(86-96), suggests a high degree of degeneracy in antigen recognition by this TCC.

*Positional Scanning Detects Important Amino Acids in the Core of the Peptide.* The X11 library induced a nearly maximal response and is less diverse than libraries of longer peptides. Therefore, further studies were based on a 11-mer peptide library in the positional scanning format consisting of 220 sublibraries each containing 10 degenerated (X) and one defined sequence position (0). According to peptide binding data of MBP(83-99/87-99) to DR2b (the restriction element of TL 5G7) and the crystal structure of peptides bound to HLA-DR molecules (26, 27), V87 is bound in pocket I, F90 in pocket IV and T95 in pocket IX of the MHC molecule. Amino acids H88, F89, K91, N92, I93, and V94 are exposed to the TCR. To evaluate whether the TCR determinants can be detected with a library approach, we tested TCC TL 5G7 with positional scanning sublibraries, based on three aa that represent potential TCR determinants located in the middle of the epitope (F89 [F1 {XXXXXXXXXX} to F11 {XXXXXXXXXXF}], K91 [K1 to K11], N92 [N1 to N11]) and on G (G1 to G11) that is not present in the MBP sequence. The sublibraries with F in position 4, 5, and 6, K in position 6, 7, and 11, and N in position 7 and 8 elicited an increased proliferative response compared to the X11 library (Fig. 1 **B**). The response to the sublibraries with the fixed G in core positions 4 to 8 was reduced. The results matched the sequence of MBP(86-96) (VVHFFKNIVTP) with F89 (position 4), F90 (position 5), K91 (position 6), and N92 (position 7) or MBP(85-95) (PVVHFFKNIVT) with F89 (position 5), F90 (position 6), K91 (position 7), and N92 (position 8) (Fig. 1 **B**). Single aa N and K in the core of the epitope were detected by a positive response for two adjacent sublibraries, whereas double F in the MBP epitope (F89, F90) was detected by a positive response to three adjacent sublibraries. Based on these results, we can expect, that each matched aa of the epitope will appear as a double peak in the response to a



**Figure 2.** Proliferative responses of TL 5G7 to 220 undecapeptide sublibraries with defined amino acid (20 for each of the 11 positions). Sublibraries starting with the defined amino acids in position P2 elicit very different responses in the TCC. Positional scan sublibraries, that induce responses stronger than X11 are considered positive for the first position they appear (\* marked). If one amino acid induced an optimal response in three or more consecutive positions, it was considered positive in more than one position. Proliferation is shown by CPM induced by 100 (black bars) or 250  $\mu\text{g}/\text{ml}$  (gray bars) of the libraries. Lane B represents  $\alpha$ -aminobutyric acid.

large panel of sublibraries, whereas two identical aa in adjacent positions of the peptide will show a triple peak in the response to the panel of sublibraries. This doubling effect can be attributed to different mechanisms. The minimal epitope for the TCC is less than 10 aa, and therefore, the sublibraries allow sliding by one amino acid in the binding groove, which influences the way they are recognized by the TCR. For example, if K is fixed in position 6 the amino acid side chains of positions 2, 5, and 10 could bind

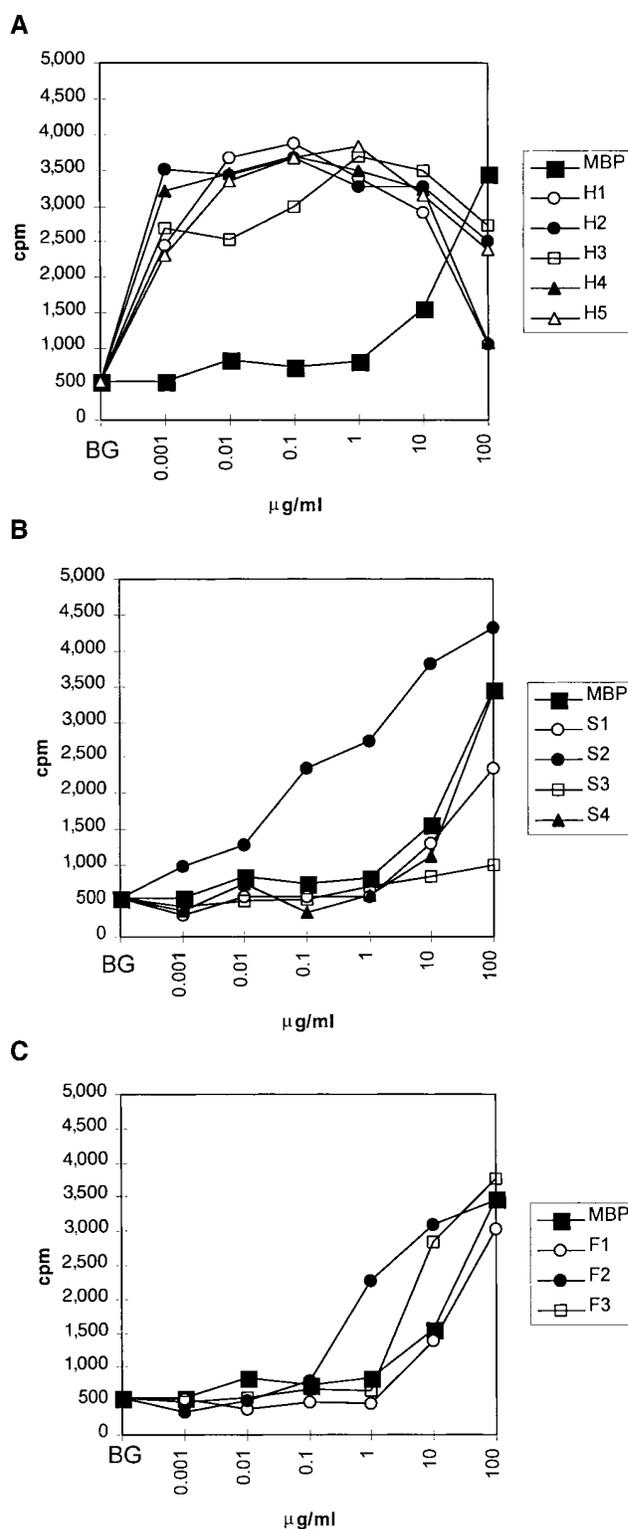
to the MHC molecule, whereas if K is fixed in position 7, position 3, 6, and 11 would contact the MHC. The second possibility, would indicate a sliding of the TCR when interacting with the MHC peptide complex. However, given the recent crystal structure of the TCR-MHC peptide complex (28), this seems highly unlikely.

*Complete Dissection of the Epitope for TCC TL 5G7.* The proliferative response of the TCC to different concentrations of the 220-peptide sublibraries in the positional

scan format was tested. Each sublibrary carries one defined amino acid in one of the positions P1–P11, and the entire panel covers all 20 amino acids in the 11 different positions (only cysteine was replaced by the structurally related synthetic analogue B [ $\alpha$ -aminobutyric acid]). Differences in the proliferative response were found at 100 and 250  $\mu\text{g}/\text{ml}$  (Fig. 2). The results were analyzed taking into account the rules established with the F-, K-, and N-scan sublibraries; (a) each matched aa of the MBP sequence resulted in a response with two adjacent sublibraries and (b) two adjacent tolerated aa resulted in a response in at least three adjacent sublibraries (Fig. 1 B). Therefore, when a sublibrary induced a stronger response than the X11 library (positive response), the fixed corresponding aa of the sublibraries was evaluated as being optimal for this position of a putative antigenic peptide (first peak). If the same aa was positive in two more adjacent sublibraries, the aa was also considered optimal for the following position of the antigenic peptide. If one specific aa gave a positive response in only two position, the second was considered an artefact due to the sliding effect.

No significant differences were observed in position (P1) (Fig. 2). Sublibraries with defined residues I, L, M, V, and F in position 2 induced an increased response in comparison to X11. The same aa were found for P3. In P4 aa F, M, Q, I, L, and W and in P5 F and Y were identified. In P6 aa K and R and in P7 aa N elicited increased responses. aa V and B in P8 and aa V, I, and B in P9 were positive. Finally, sublibraries with aa I, L, and V in P10 and aa K in P11 induced a positive response. Whether the positive response of aa I, L, and V in P11 is real or just the double peak from P10 can not be defined. Table 1 shows the results for each of the 11 positions. To summarize, aa in positions 2, 5, and 10 are thought to interact with the HLA-DR2b molecule. In accordance with binding studies, only hydrophobic aromatic and aliphatic aa were tolerated in position 2 (pocket I) and only the aromatic, uncharged aa F and Y in position 5 (pocket IV). For P10 (pocket IX) only aliphatic aa were found with the library. In predicted TCR contact positions only the aliphatic aa were detected in position 3 (P3) and Q and the hydrophobic aromatic and aliphatic aa in P4. In contrast, P6 tolerated only the positively charged aa K and R and P7 only the aa N. P8 and P9 tolerate only aliphatic aa and the aa B. As described above, no clear conclusions can be drawn for P1 and P11. Interestingly, only 6 aa of the MBP(86–99) sequence matched with optimal library predictions for P2–P10.

*Deduced Synthetic Peptides Selected From Peptide Sublibraries are Better Ligands than MBP(86–96).* Based on the results of the 220 peptide sublibraries individual (defined) 11-mer peptides with optimal aa in position P2 to P10 were deduced and synthesized (Table 1). In P1 a D residue and in P11 a K residue was introduced to increase solubility. Testing these peptides in proliferation assays, it became evident that TCC TL 5G7 responded to all peptides at much lower antigen concentration than MBP(86–96). The ligands induced maximal stimulation at a concentration as low as 1  $\mu\text{g}/\text{ml}$ , whereas 100  $\mu\text{g}/\text{ml}$  were needed for MBP(86–96)



**Figure 3.** Responses to individual peptides based on the prediction of the peptide library for optimal aa combination (H1–H5, A) or peptides derived from self (S1–S4, B) or microbial proteins (F1–F3, C) in comparison to MBP(86–96). Ligands H1 through H5 as well as S2, F2, and F3 induce stronger responses in TCC TL 5G7 than MBP(86–96). Even ligands that share only 2 aa with the MBP sequence are well recognized by the TCC.

**Table 1.** Library Predictions and Deduced Peptide Sequences

Position		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
	Library predictions	No	I,V,L,M,F	I,V,L,M,F	F,M,W,Q,L,I	F,Y	K,R	N	V,B	V,I,B	I,V,L	K (I,V,L)
MBP	Myelin basic protein (86-96)	V	<u>V</u>	H	<u>F</u>	<u>F</u>	<u>K</u>	<u>N</u>	I	<u>V</u>	T	P
H1	Hypothetical peptide	D	<u>L</u>	<u>I</u>	<u>F</u>	<u>Y</u>	<u>R</u>	<u>N</u>	<u>V</u>	<u>V</u>	<u>I</u>	K
H2	Hypothetical peptide	D	<u>L</u>	<u>I</u>	<u>F</u>	<u>Y</u>	<u>K</u>	<u>N</u>	<u>V</u>	<u>V</u>	<u>I</u>	K
H3	Hypothetical peptide	D	<u>L</u>	<u>I</u>	<u>M</u>	<u>Y</u>	<u>K</u>	<u>N</u>	<u>V</u>	<u>V</u>	<u>I</u>	K
H4	Hypothetical peptide	D	<u>L</u>	<u>I</u>	<u>M</u>	<u>Y</u>	<u>R</u>	<u>N</u>	<u>V</u>	<u>V</u>	<u>I</u>	K
H5	Hypothetical peptide	D	<u>L</u>	<u>I</u>	<u>M</u>	<u>Y</u>	<u>R</u>	<u>N</u>	<u>V</u>	<u>V</u>	<u>I</u>	A
S1	Ras GTPase-activating-like protein IQGAP1(1581-1591)	Q	<u>V</u>	N	<u>Q</u>	<u>F</u>	<u>K</u>	<u>N</u>	<u>V</u>	<u>I</u>	F	E
S2	Protein-glutamine gamma- glutamyltransferase(675-685)	A	<u>V</u>	K	G	<u>F</u>	<u>R</u>	<u>N</u>	<u>V</u>	<u>I</u>	<u>I</u>	G
S3	NAD-dependent methylenetetrahy- drofolate dehydrogenase(311-321)	V	A	<u>M</u>	<u>L</u>	M	<u>K</u>	<u>N</u>	T	<u>I</u>	<u>I</u>	A
S4	Tyrosine-protein kinase BTK (124-134)	W	<u>I</u>	H	<u>Q</u>	L	<u>K</u>	<u>N</u>	<u>V</u>	<u>I</u>	R	Y
F1	Protein kinase CHK1[schizosaccharo- myces pombe](473-483)	W	R	K	<u>F</u>	<u>F</u>	<u>K</u>	<u>N</u>	<u>V</u>	<u>V</u>	S	S
F2	Hypothetical protein UL71[human cytomegalovirus](166-176)	D	<u>I</u>	<u>L</u>	<u>I</u>	L	<u>K</u>	L	<u>V</u>	<u>V</u>	G	E
F3	UDP-N-acetylenolpyruvoyl- glucosamine reductase [ <i>Salmonella typhimurium</i> ](227-237)	A	G	S	<u>F</u>	<u>F</u>	<u>K</u>	<u>N</u>	P	<u>V</u>	<u>V</u>	A

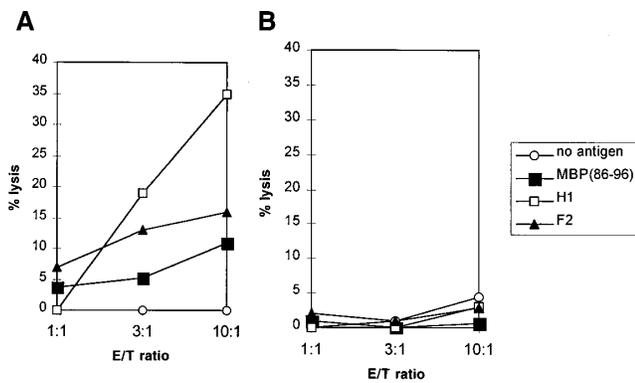
(Fig. 3 A). These data confirm the library predictions and identify MBP(86-96) as a suboptimal ligand for TCC TL 5G7.

*Library Predictions Identify Stimulatory Peptides Derived from Microbial and Self Peptides.* To identify peptides derived from natural proteins we searched the Swissprot database for peptide sequences using the library predictions for P2 to P10. The protein motif search for human entries in Swissprot identified several candidate self peptides. Interestingly, the MBP sequence was among them. Another search with sequences based on optimal aa combinations for P2 to P10 of the 11-mer peptide using BLITZ-search for the entire Swissprot database revealed several sequences of microbial antigens. Candidate peptides from both database searches, which matched at least 6 aa with the library prediction for P2-P10 were synthesized and tested in proliferation assays with TCC TL 5G7 (Table 1). Several peptides derived from human (Fig. 3 B) and microbial proteins (Fig. 3 C) were stimulatory for the TCC. In addition, self and foreign peptides (S2, F2, F3, Fig. 3) were identified that were more potent agonist for TCC TL 5G7 than MBP(86-96). To exclude any non-TCR-mediated activation of the TCC by these new ligands, four other MBP(86-96 or 83-99)- and two influenza hemagglutinin HA(306-318)-specific TCC were tested with the same concentration range and showed no re-

sponse to any of the synthetic ligands listed in Table 1 (data not shown). In addition, we tested the weakly cytotoxic TCC TL 5G7 for its ability to lyse HLA-matched targets pulsed with these peptides. B cells expressing HLA-DRB1\*1501 pulsed with MBP(86-96), H1 or F2 were lysed (Fig. 4 A), but not B cells expressing other HLA-DR molecules (Fig. 4 B). Again the peptides that were stronger agonists than MBP(86-96) in the proliferation assay induced stronger cytotoxicity. When we compared the sequences of the stimulatory ligands to the aa predicted by the library, we found a correlation between agonist activity and number of aa matched with library predictions. Peptides that matched 9 of 9 (H1-H5, not considering P1 and P11) induced proliferation at a concentration below 1 ng/ml, whereas peptides that matched 6 or 7 of 9 aa induced a proliferative response at 0.1–10  $\mu$ g/ml.

## Discussion

Combinatorial peptide libraries are useful tools to define peptide protein interactions, such as MHC binding motifs or antibody antigen interactions (8, 10–12). Recently, peptide libraries have been used to define the peptide repertoire of CD8<sup>+</sup> TCC (13–16). CD8<sup>+</sup> TCC recognize 9-mer



**Figure 4.** TCC TL 5G7 lyses HLA-DRB1\*1501-positive B cells (A) but not HLA mismatched target cells (shown for DRB1\*0404/1\*1302-positive target cells, (B) pulsed with either MBP(86-96), H1 or F2. Similar to the results of the proliferation assay F2 and H1 induced a stronger killing of target cells than MBP(86-96).

peptides and therefore can be studied by a 9-mer combinatorial library. In contrast, for CD4<sup>+</sup> class II-restricted T cells the minimal epitope is usually 11 aa, and longer libraries (such as an X11) are required for studying antigen recognition of these cells. Allowing 20 different aa in a X position, extending the length by 2 aa results in an increase of complexity by a factor of 400. Despite this increase in complexity, we were able to apply an 11-mer peptide library to dissect the antigen recognition pattern of an MBP-specific human class II-restricted TCC and identified several tolerated aa for most positions of the epitope. The data obtained by 220 undecapeptide sublibraries were confirmed by the positive response to synthetic individual 11-mer peptides based on optimal amino acids for each position. Cross-reactive ligands derived from human and microbial proteins, some of them even more potent than MBP(86-96), were easily identified by database searches.

These results allow several important conclusions. Functional peptide recognition by at least some class II-restricted T cells is extremely degenerate. The fact that a TCC responds to a randomized mixture of 11-mer peptides suggests, that many ligands are recognized by this TCR. The X11 library consists of  $2.05 \times 10^{14}$  ( $20^{11}$ ) different peptides resulting in assay concentrations of  $4.88 \times 10^{-19}$  g/ml for each single peptide (at 0.1 mg/ml total peptide concentration). Given the minimal concentration of one of the best hypothetical ligands, H1, to induce proliferation ( $1 \times 10^{-9}$  g/ml, respectively) (Fig. 3), we can hypothesize that a high number of different ligands are stimulatory for the TCC. Although only a few of these amino acid sequences will be generated from natural proteins and even fewer will be presented to T cells after processing, a very high number of ligands that can positively engage the TCR of TL 5G7 will still occur naturally. This observation is consistent with our previous observations using MBP-specific TCC (29). Based on single aa substituted peptides we established that many amino acid exchanges in the MBP peptides are tolerated and some even induce a stronger agonist response than the MBP pep-

ptide itself without increasing the MHC binding (superagonist modifications). In addition, we demonstrated that alterations of primary and secondary TCR contact positions, which usually abrogate T cell responses, can be restored by superagonist modifications at other positions. This suggested that the recognition of these TCC is degenerate and ligands exist that are much stronger agonists than the autoantigen itself.

Peptide libraries provide a tool to test the hypothesis of degeneracy in antigen recognition. For each position of the peptide antigen the optimal amino acid can be defined by the response to the sublibraries. The agonist activity of ligands correlates with the number of optimal amino acids for the positions of the antigenic peptide. The fact that TCR recognition can be predicted based on peptide libraries with only one amino acid fixed suggests that each amino acid independently contributes to TCR recognition. Accordingly, the strength of interaction given by the integration of the effect of each aa defines the agonist activity of a ligand. Even if some aa do not fit with the criteria of being optimal for a certain position, the ligand may still be stimulatory if enough suitable aa can counterbalance with positive effects in other positions. This observation does not fit models that argue that specific conformations of the peptide are the major determinant of recognition by the TCR.

A large number of ligands exists for each T cell that can positively engage the TCR. Among these ligands, a hierarchy exists with respect to agonist activity. The potency of the ligand determines at which antigen concentration the T cell becomes activated. Two different routes of activation for T cell with degenerate antigen recognition can be hypothesized; a single strong agonist ligand can reach the threshold for activation when applied at sufficient concentration (i.e., during microbial infection). Alternatively, a large number of different stimulatory ligands, i.e., the pool of self peptides or the X11 library can activate the T cell. These observations may be relevant to several immunological processes. Thymic selection and peripheral tolerance take place in an environment that is characterized by high numbers of different ligands rather than high concentrations of one ligand. Therefore, degeneracy of antigen recognition combined with the possibility that each ligand can interact with several different TCRs (30) might determine selection in the thymus by a pool of self peptides (31). T cells carrying TCR with a very low level of degeneracy may not reach the threshold for activation to undergo positive selection (few ligands from the pool are recognized = low affinity interaction). In contrast, T cells with highly degenerate TCR recognition may undergo negative selection due to a strong activation imposed by the large amount of stimulatory ligands from the pool of self peptides (high-affinity interaction). Similar mechanisms may also regulate T cell responses in the periphery. The pool of self peptides that selected the T cells in the thymus will constantly impose a low level of activation on the T cells that escaped selection in the thymus. Once the immune system is exposed to a microbial organism, its antigens will replace locally part of the pool of self peptides. This will result in a stimulation

and expansion of cells expressing a high-affinity receptor for these antigens. The responding T cells will clear the microbe from the body and the pool of self peptides will then replace the foreign antigens, turning the microbial-specific T cells back to a state of low activation. However, the T cells that encountered the foreign antigen, will develop into memory cells that become more efficient in recognition of antigenic ligands during recall responses due to their different repertoire of costimulatory ligands. If such a differentia-

tion to the memory stage occurs with T cells that cross-react with self peptides, these cells will be more prone to activation by subsequent stimuli, if, for example, the respective autoantigen is released during local tissue damage. The release of these and other self peptides from the damaged target organ may activate other T cells with different specificities and eventually lead to acute or chronic organ destruction.

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