Howard M. Grey,[1] Ulrich Zügel[2] and Rongfang Wang[1]

Molecular Mimicry: Breaking Tolerance with Heteroclitic Antigens

Introduction

The mechanism by which autoimmunity is triggered in susceptible individuals is not known. One popular concept is that of molecular mimicry. According to this idea, sequence similarity between a foreign pathogen and a self-antigen leads to the induction of an immune response to the foreign antigen that cross-reacts with the self-antigen, thereby initiating an autoimmune process. There are two possible explanations for how such a foreign antigen might induce an immune response to a self-antigen. First, the specific self-peptide that shares homology with the foreign antigen may never have induced a state of self-tolerance (1-3). Normally, no autoimmunity to this epitope is observed because the epitope is not expressed on a potent antigen processing cell (APC) or, because of its structure or the structure of flanking regions of the protein from which it is derived, it is inefficiently processed and displayed on the surface of an APC (i.e., it is a cryptic epitope) (4, 5). In the course of the immune response to the foreign antigen, the cross-reactive, non-self epitope becomes effectively processed by a professional APC and induces the latent immune response to the cross-reactive self-epitope.

The second possible mechanism by which a cross-reactive epitope on a foreign antigen may give rise to an anti-self response involves potentially immunodominant T cell epitopes that have actively induced a state of self-tolerance. In this case, central and/or peripheral tolerance to the epitope exists in the host. Due to the structural characteristics of the foreign cross-reactive epitope, a subset of untolerized T cells (perhaps with too low an affinity to have been tolerized by the self-epitope) or previously anergized T cells become activated by the foreign epitope and can now recognize the self-epitope and initiate the autoimmune process (3, 6, 7).

We have been investigating this second explanation for antigenic mimicry. In our first series of experiments, we have utilized the tolerant state induced to the dominant I-E^k restricted epitope of moth cytochrome c ($MCC_{88\text{-}103}$) as a model system. The immune response to this peptide has been well-characterized by several investigators (8-13). One of the advantages of this epitope is that in H-2^k animals it generates a rather restricted T cell response dominated by T cell receptors (TCRs) that utilize $V\alpha_{11}$ and $V\beta_3$ (14-16), and although different T cell clones vary somewhat in their fine specificity, the major T cell contact residues appear to be conserved. Thus, information on the relative immunogenicity of $MCC_{88\text{-}103}$ analogs gained at the clonal level may be pertinent for the polyclonal in vivo response as well.

The data obtained in this system indicate that tolerance to MCC can be terminated by certain cross-reactive antigens. Strikingly, those peptides capable of breaking tolerance were all characterized as being heteroclitic antigens, in that they were more potent stimulators of the $MCC_{88\text{-}103}$ specific T cell clone than the parental antigen.

Initial experiments were performed to characterize the state of tolerance induced in adult animals given $MCC_{88\text{-}103}$ in incomplete Freund's adjuvant (IFA). Ten days after intraperitoneal injection of 300 μg of $MCC_{88\text{-}103}$ in IFA, animals were immunized subcutaneously with the same peptide in complete Freund's adjuvant (CFA), and 10 days later, lymph node cells (LNC) were stimulated in vitro with $MCC_{88\text{-}103}$ and proliferative and cytokine responses were subsequently measured. The cytokine responses from the nontolerized animals indicated that interferon-γ (IFN-γ) and IL-2 were the predominant cytokines made to this antigen, with no detectable IL-4 or IL-10 being observed (Figure 1, and data not shown). The proliferative response of

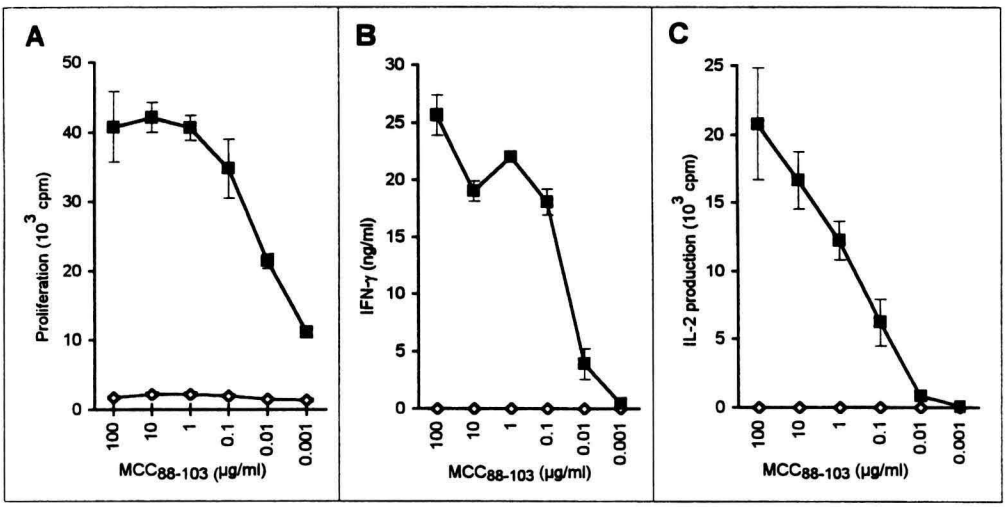

Fig. 1. Induction of tolerance to $MCC_{88\text{-}103}$. B10.A mice were tolerized with 300 μg of $MCC_{88\text{-}103}$ in IFA intraperitoneally. Ten days later tolerized and nontolerized mice were immunized subcutaneously with 2 μg of $MCC_{88\text{-}103}$ in CFA. Lymph node cells were collected 10 days after immunization and stimulated with the indicated concentrations of $MCC_{88\text{-}103}$. LNC from tolerized (*open diamonds*) and untolerized (*black squares*) mice were tested in a proliferation assay (A). For cytokine analysis, 50 μl of supernatants from the cultures established to measure proliferative response were removed and tested for IFN-γ (B), and IL-2 (C). Data are representative of three independent experiments.

cells from tolerized animals was drastically diminished to a level of 5-15% of the maximum response observed with cell cultures from untolerized animals. Also, almost complete loss of both IL-2 and IFN-γ production was observed, with responses in the range of 0-5% of the untolerized control group being obtained in the multiple experiments performed. Of particular note was the observation that there was no IL-4 or IL-10 detected in the cultures derived from tolerized animals as analyzed by ELISA or the more sensitive ELISPOT assay (not shown). Thus, immune deviation to a Th2-like response was not responsible for the decrease in the production of the Th1

cytokines or in the proliferative response (19-21). This is in keeping with the findings of Adorini's group, who observed immune deviation when an intact protein but not when a peptide epitope was used as the antigen (22).

Next, 15 single amino acid substituted MCC $_{88\text{-}103}$ analogs were selected to determine their capacity to break tolerance to $MCC_{88\text{-}103}$. The peptides were chosen on the basis of two criteria. First, they all possessed a relatively high binding capacity for I-E^k, having 50% inhibition (IC_{50}) values of less than 200 nM, a level that had been previously determined to be sufficient for a peptide to be potentially immunogenic (23). The second criterion by which they were selected was that they represented a broad range of antigenicity for a cytochrome-specific T cell clone, AD10. It has been previously demonstrated that this clone is fairly typical of the cytochrome c specific response generated in H-2^k mice; i.e., it contains a $V\beta_3/V\alpha_{11}$ TCR which recognizes K99 and T102 as dominant TCR contact residues on the MCC peptide (12, 13, 24). Thus, information on the relative immunogenicity of $MCC_{88\text{-}103}$ analogs gained at the clonal level may be pertinent for the polyclonal in vivo response as well. As shown in Table 1, the panel included nonantigenic peptides and TCR antagonistic peptides, as well as antigenic peptides, varying from weak to very strong.

Table 1. I-E^k binding and T cell stimulatory capacity of $MCC_{88\text{-}103}$ analogs

Peptide #	Substitution[a]	I-E^k Binding[b] IC_{50} (nM)	Stimulatory Capacity for AD10 Clone[c]
1	$MCC_{88\text{-}103}$	40	*1*
2	A96S	47	0.007
3	A96V	59	0.0001
4	Y97F	86	2
5	Y97V	86	0.001
6	L98F	51	2
7	L98A	79	9
8	K99Q	78	A[d]
9	K99R	25	0.0005
10	Q100T	49	0.5
11	Q100N	56	0.001
12	A101G	82	0.3
13	A101S	124	0.2
14	T102S	82	0.1
15	T102G	143	A[d]
16	K103R	89	1

[a] The substitution made in the $MCC_{88\text{-}103}$ sequence (ANERADLIAYLKQATK) is denoted by the wild type residue, the residue number, followed by the substitution.
[b] The concentration of peptide required to inhibit binding to I-E^k of the radiolabeled ligand by 50% (IC_{50}).
[c] Data are presented as the ratio of the concentration of $MCC_{88\text{-}103}$ required to stimulate 40% of the maximal response compared to the concentration of the analog peptides needed to achieve the same degree of stimulation.
[d] A = TCR antagonist. The ability of nonantigenic peptide analogs to act as TCR antagonists for the AD10 clone was determined by the 'pre-pulse' assay previously described (32).

When tolerized mice were immunized with the antigen analogs listed in Table 1, in most instances no greater response to $MCC_{88\text{-}103}$ was observed than that obtained following immunization with the tolerogen. Although tolerance was not broken, in all instances a significant response against the immunizing analog was obtained (stimulation indices between 2 and 10 in tolerized animals, and 3 and 14 in non-tolerant animals). A representative example of the failure to respond to $MCC_{88\text{-}103}$ is shown in Figure 2A. Proliferative responses of LNC were in the range of 10% or less of that of the untolerized control animals, with little or no cytokine production being detected.

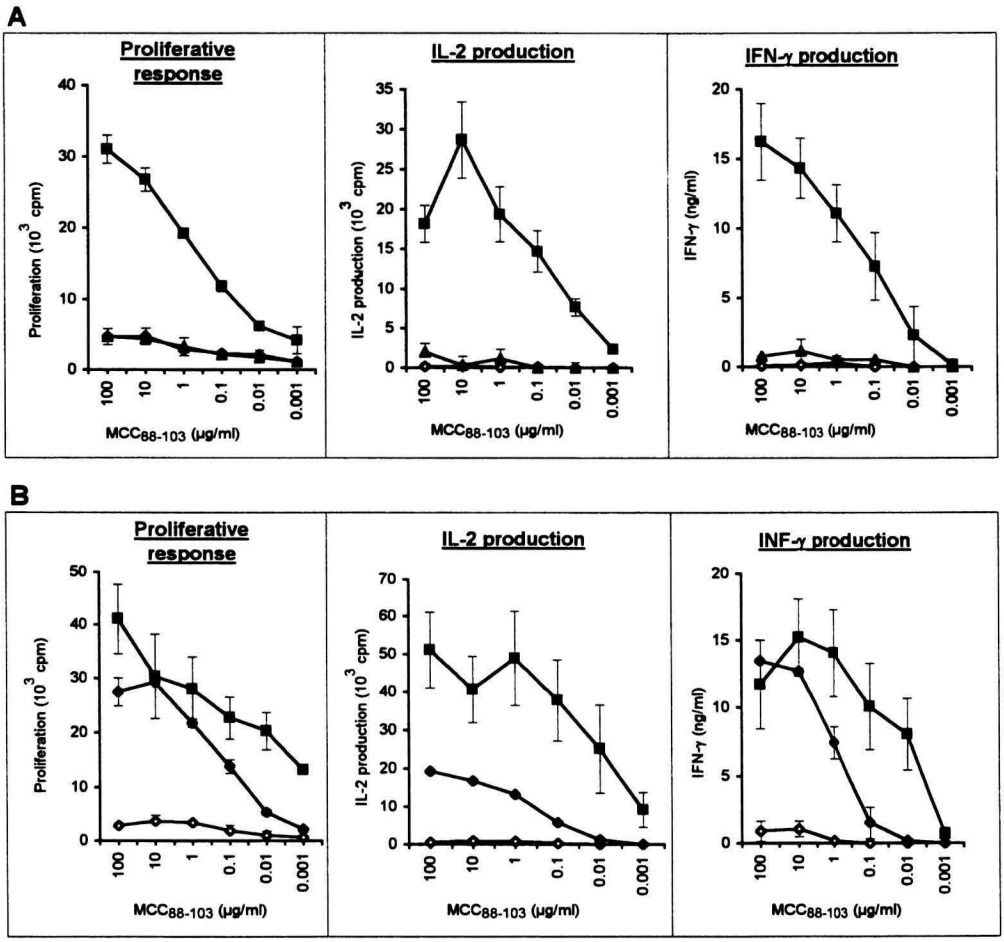

Fig. 2. Capacity of antigen analogs to terminate tolerance. Analog peptides T102S (A) and L98A (B) were used to immunize mice that had been previously tolerized to $MCC_{88\text{-}103}$. LNC from nontolerized mice (*black squares*), tolerized and $MCC_{88\text{-}103}$ immunized mice (*open diamonds*), and tolerized and MCC analog immunized mice [*black triangles* (T102S); *black diamonds* (L98A)] were analyzed for their proliferative activity and secretion of IL-2 and IFN-γ. Data are representative of three independent experiments. No IL-4 or IL-10 production was detected (data not shown).

In striking contrast to these negative results, a few peptides were very efficient in terminating tolerance. Data from one such peptide, L98A, are shown in Figure 2B. Cells from animals that were first tolerized to MCC_{88-103} and then immunized with L98A gave a peak proliferative response to MCC_{88-103} that was about 75% of that obtained from the untolerized controls. Also, a virtual complete reconstitution of the IFN-γ response and a partial reconstitution of IL-2 production was achieved. Cells from animals tolerized and immunized with MCC_{88-103} were included and served as tolerized control cultures.

Table 2. Summary of the capacity of MCC_{88-103} analogs to terminate tolerance to MCC_{88-103}

		Restoration of MCC_{88-193} Response (%)[a]		
Peptide #	Substitution	Pro-liferation	IFN-γ Secretion	IL-2 Secretion
2	A96S	-	-	-
3	A96V	5 (2)	-	-
4	Y97F	**25** (13)[b]	-	1 (1)
5	Y97V	-	-	-
6	L98F	**65** (14)[c]	**62** (20)[c]	**30** (13)[c]
7	L98A	**76** (12)[c]	**81** (23)[c]	**34** (13)[c]
8	K99Q	-	-	-
9	K99R	2 (1)	1 (1)	-
10	Q100T	-	1 (1)	-
11	Q100N	-	-	-
12	A101G	3 (2)	6 (4)	-
13	A101S	1 (0.3)	-	-
14	T102S	1 (0.2)	5 (4)	3 (3)
15	T102G	-	1 (2)	-
16	K103R	7 (2)	1 (2)	-

[a] Percent restoration of the MCC_{88-103} response of tolerant animals following immunization with analog peptides. Numbers in parentheses represent standard deviation from the mean.
[b] Significant response over that of tolerant controls ($P<0.05$).
[c] Significant response over that of tolerant controls ($P<0.01$).

A summary of the data with all 15 antigen analogs is shown in Table 2. Three peptides were consistently successful in inducing a significant immune response to the MCC_{88-103} peptide following the induction of tolerance to that peptide. Peptide 4 (Y97F) was the least efficient, restoring the proliferative response to 25% of normal but was unable to induce significant cytokine production. The two most potent analogs in terminating tolerance had a substitution at position 98, L to A (peptide 7) and L to F (peptide 6). None of the other 12 peptides studied had any significant effect on the tolerant state to MCC_{88-103}. In comparing the data obtained in Tables 1 and 2, it is striking that the peptides capable of terminating tolerance to MCC_{88-103} were better antigens than the wild type MCC_{88-103} for the AD10 clone; i.e., they were heteroclitic (25-28). The heteroclitic nature of the two L98 analogs was not restricted to the AD10 clone, but was also observed with several other MCC_{88-103} specific T cell

clones, and could also be detected with a bulk response using T cells from MCC_{88-103} immunized mice when limiting antigen concentrations were used.

With respect to the heteroclitic reactivity of these analogs, there would appear to be two potential explanations of how an antigen analog with the same affinity for major histocompatibility complex (MHC) as the antigen may be a more effective antigen than the immunogen that induced the original response. First, the heteroclitic antigen may bind with higher affinity to the TCR due to replacement of a TCR contact residue with a closely related amino acid capable of even stronger interactions with the TCR. This is probably the mechanism for the heteroclicity of the Y97F analog, since Y97 has been characterized as a subdominant TCR contact residue by ourselves and others (12, 13).

The second explanation for heteroclicity is the substitution of a residue which is not itself a TCR contact residue, but nevertheless has the ability to influence the interaction between peptide and TCR. On the basis that multiple substitutions at position L98 were tolerated with respect to MHC binding and had relatively minor effects on T cell recognition (Table 1, and H. M. Grey, unpublished results), this residue was thought to be either a 'spacer' residue not directly involved in MHC or TCR contact, or a minor TCR contact residue. However, recent crystallographic analysis of peptide/I-E^k complex indicates that L98 should be an MHC contact residue (29). Thus, the most likely explanation for the heteroclicity of MCC_{88-103} analogs with L98 substitutions is that changes in the way residues at this position engage the MHC alter the orientation of the TCR contact residues, resulting in an enhancement of their binding to the TCR. Another example of a change in an MHC contact residue that had an effect on TCR interaction has been documented with another I-E^k restricted response (30, 31). This type of heteroclicity may be ideally suited to terminate the tolerant state to an antigen, since the TCR contact residues are identical to the tolerized antigen, and therefore most of the T cell repertoire induced to the analog should be capable of reacting with the tolerized antigen as well.

The possible mechanisms by which tolerance can be terminated with heteroclitic antigens include: immune deviation, reversal of anergy, and the activation of nontolerized cross-reactive clones of T cells. There was no evidence that tolerance induction led to a switch from a Th1 to a Th2 response, either before or following tolerance termination, thus eliminating immune deviation as a factor in this system. With respect to activation of previously anergized clones, there are no data for or against this possibility, and we are currently attempting to evaluate this possibility by inducing tolerance in a TCR transgenic population of T cells to determine whether tolerance can be reversed at the clonal level.

There are data, however, that support the hypothesis that breaking of tolerance involves stimulation of low affinity, non-tolerized T cell clones. As illustrated in Figure 2, cells from previously tolerized animals required 10- to 100-fold more antigen than cells from nontolerized animals in order to be comparably stimulated by MCC_{88-103}. These findings are compatible with the concept that T cell clones with too low an affinity for MCC_{88-103} to be tolerized were stimulated by the heteroclitic analogs. In further support of this concept is the finding that there appears to be a somewhat different repertoire of T cells stimulated by the L98A analog compared with MCC_{88-103}. As shown in Figure 3, analysis of short-term lines for $V\beta_3$ and $V\alpha_{11}$

TCR utilization of MCC and MCC-A immune T cell lines

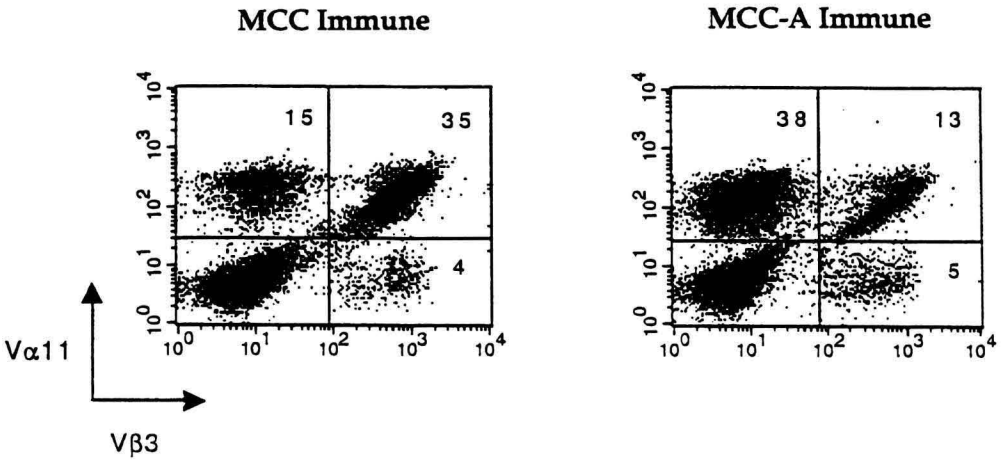

Fig. 3. FACS analysis of $V\beta_3$ and $V\alpha_{11}$ expression by short-term T cell lines derived from $MCC_{88\text{-}103}$ and L98A immunized animals. The L98A T cell line (MCC-A) has fewer $V\beta_3^+$, $V\alpha11^+$ cells and more $V\beta_3^-$, $V\alpha11^+$ cells than the $MCC_{88\text{-}103}$ cell line.

usage indicated that L98A elicited fewer $V\beta_3^+/V\alpha_{11}^+$ cells than $MCC_{88\text{-}103}$ (13 vs. 35%), and more $V\beta_3^-/V\alpha_{11}^+$ cells (38 vs. 15%). It is proposed that following activation by the heteroclitic antigen, these T cells with low affinity for $MCC_{88\text{-}103}$ (perhaps $V\beta_3^-/V\alpha_{11}^+$) become responsive to secondary stimulation with the tolerogen. Preliminary data with T cell hybridomas derived from animals whose tolerance to $MCC_{88\text{-}103}$ was broken by immunization with L98 analogs confirm that there is a high incidence of clones that are $V\alpha_{11}^+/V\beta_3^-$ and that these clones require high concentrations of $MCC_{88\text{-}103}$ to be stimulated.

In conclusion, our experiments show that one mechanism by which molecular mimicry can lead to the induction of autoimmunity is by raising an immune response to a cross-reactive heteroclitic antigen capable of activating low affinity T cells specific for the self-antigen. Once stimulated, these T cells have a lower threshold for activation and will recognize and be further activated by the self-antigen, leading to an autoimmune inflammatory disease.

References

1. Cibotti, R., J. M. Kanellopoulos, J. P. Cabaniols, O. Halle-Panenko, K. Kosmatopoulos, E. Sercarz, and P. Kourilsky, 1992. Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants. Proc. Natl. Acad. Sci. USA **89**:416.

2. Mamula, J., 1993. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. J. Exp. Med. **177**:567.
3. Liu, G. Y., P. J. Fairchild, R. M. Smith, J. R. Prowle, D. Kioussis, and D. C. Wraith, 1995. Low avidity recognition of self-antigen by T cells permit escape from central tolerance. Immunity **3**:407.
4. Brett, S. J., K. B. Cease, and J. A. Berzofsky, 1988. Influences of antigen processing on the expression of the T cell repertoire. Evidence for MHC-specific hindering structures on the products of processing. J. Exp. Med. **168**:357.
5. Mougdil, K. D., I. S. Grewal, P. E. Jensen, and E. E. Sercarz, 1996. Unresponsiveness to self-peptide of mouse lysozyme owing to hindrance of T cell receptor-major histocompatibility complex/peptide interaction caused by flanking epitopic residues. J. Exp. Med. **183**:535.
6. Tam, C., E. V. Fedoseyeva, M. Moskalenko, M. R. Garovoy, and G. Benichou, 1996. T cell tolerance is influenced by concomitant T cell recognition of cross-reactive self-peptides. J. Immunol. **156**:3765.
7. Cerasoli, D. M., J. McGrath, S. R. Carding, F. F. Shih, B. B. Knowles, and A. J. Caton, 1995. Low avidity recognition of a class II-restricted neo-self peptide by virus-specific T cells. Int. Immunol. **7**:935.
8. Oehen, S., L. Feng, Y. Xia, C. D. Surh, and S. M. Hedrick, 1996. Antigen compartmentation and T helper cell tolerance induction. J. Exp. Med. **183**:2617.
9. Fox, B. S., C. Chen, E. Fraga, E. C. A. French, B. Singh, and R. H. Schwartz, 1987. Functionally distinct agretopic and epitope sites: analysis of the dominant T cell determinant of moth and pigeon cytochromes c with the use of synthetic peptide antigens. J. Immunol. **139**:1578.
10. Bhayani, H., and Y. Paterson, 1989. Analysis of peptide binding patterns in different major histocompatibility complex T cell receptor complexes using cytochrome c-specific T cell hybridomas. J. Exp. Med. **170**:1609.
11. Sorger, S. B., Y. Paterson, P. J. Fink, and S. M. Hedrick, 1990. T cell receptor junctional regions and the MHC molecule affect the recognition of antigenic peptides by T cell clones. J. Immunol. **144**:1127.
12. Reay, P. A., R. M. Kanor, and M. M. Davis, 1994. Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of moth cytochrome c (93-103). J. Immunol. **152**:3946.
13. Page, D. M., J. Alexander, K. Snoke, E. Appella, A. Sette, S. M. Hedrick, and H. M. Grey, 1994. Negative selection of $CD4^+$ $CD8^-$ thymocytes by T-cell receptor peptide antagonists. Proc. Natl. Acad. Sci. USA. **91**:4057.
14. Fink, P. J., L. A. Mathis, D. L. McElligot, M. Bookman, and S. M. Hedrick, 1986. Correlations between T-cell specificity and the structure of the antigen receptor. Nature **321**:219.
15. Winoto, A., J. L. Urban, N. C. Lan, J. Goverman, L. Hood, and D. Hansburg, 1986. Permanent use of Vα gene segment in mouse T-cell receptors for cytochrome c. Nature **324**:679.
16. Nakano, N., R. Rooke, C. Benoist, and D. Mathis, 1997. Positive selection of T cells induced by viral delivery of neopeptides to the thymus. *Science* **275**:*678*.
17. La Face, D. M., C. Couture, K. Anderson, G. Shih, J. Alexander, A. Sette, T. Mustelin, A. Altman, and H. M. Grey, 1997. Differential T cell signaling induced by antagonist peptide-MHC complexes and the associated phenotypic responses. J. Immunol. **158**:2057.
18. Sette, A., S. Southwood, D. O'Sullivan, F. C. A. Gaeta, J. Sidney, and H. M. Grey, 1992. Effect of pH on class II-peptide interactions. J. Immunol. **148**:844.
19. Nicholson, L. B., J. M. Greer, R. A. Sobel, M. B. Lees, and V. J. Kuchroo, 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. Immunity **3**:397.
20. Constant, S., C. Pfeiffer, A. Woodward, T. Pasqualini, and K. Bottomly, 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. J. Exp. Med. **182**:1591.
21. Degermann, S., E. Pria, and L. Adorini, 1996. Soluble protein but not peptide administration diverts the immune response of a clonal $CD4^+$ T cell population to the T helper 2 cell pathway. J. Immunol. **157**:3260.
22. Degermann, S., E. Pria, and L. Adorini, 1996. Soluble protein but not peptide administration diverts the immune response of a clonal $CD4^+$ T cell population to the T helper 2 cell pathway. J. Immunol. **157**:3260.
23. Schaeffer, E. B., A. Sette, D. L. Johnson, M. C. Bekoff, J. A. Smith, H. M. Grey, and S. Buus, 1989. Relative contribution of 'determinant selection' and 'holes in the T cell repertoire' to T cell responses. Proc. Natl. Acad. Sci. U.S.A. **86**:4649.

24. Hedrick, S. M., I. Engel, P. J. Fink, M.-L. Hsu, D. Hansburg, and L. A. Mathis, 1988. Selection of amino acid sequences in the β chain of T cell antigen receptor. Science **239**:1541.
25. England, R. D., M. C. Kullberg, J. L. Cornette, and J. A. Berzofsky, 1995. Molecular analysis of a heteroclitic T cell response to the immunodominant epitope of sperm whale myoglobin. J. Immunol. **155**:4295.
26. Michaëlsson, E., M. Andersson, A. Engström, and R. Holmdahl, 1992. Identification of an immunodominant type-II collagen peptide recognized by T cells in H-2^q mice: self tolerance at the level of determinant selection. Eur. J. Immunol. **22**:1819.
27. Baumhüter, S., C. J. A. Wallace, A. E. I. Proudfoot, C. Bron, and G. Corradin, 1987. Multiple T cell antigenic determinants identified within a limited region of the horse cytochrome c molecule. Eur. J. Immunol. **17**:651.
28. Wraith, D., D. E. Smilek, D. J. Mitchell, L. Steinmann, and H. O. McDevitt, 1989. Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. Cell **59**:247.
29. Fremont, D. H., W. A. Hendrickson, P. Marrack, and J. Kappler, 1996. Structures of an MHC class II molecule with covalently bound single peptides. Science **272**:1001.
30. Kersh, G. J., and P. M. Allen, 1996. Structural basis for T cell recognition of altered peptide ligands: Single T cell receptor can productively recognize a large continuum of related ligands. J. Exp. Med. **184**:1259.
31. Evavold, B. D., S. G. Williams, B. L. Hsu, S. Buus, and P. M. Allen, 1992. Complete dissection of the Hb(64-76) determinant using T helper 1, T helper 2 clones, and T cell hybridomas. J. Immunol. **148**:347.
32. De Magistris, M. T., J. Alexander, M. Coggeshall, A. Altman, F. C. A. Gaeta, H. M. Grey, and A. Sette, 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. Cell **68**:625.

[1] La Jolla Institute for Allergy and Immunology, Division of Immunochemistry, 10355 Science Center Drive, San Diego, CA 92121, USA
[2] Current address: Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, 89080 Ulm, Germany