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Negative Selection during the Peripheral Immune Response to Antigen

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Abstract

Thymic selection depends on positive and negative selective mechanisms based on the avidity of T cell interaction with antigen–major histocompatibility complex complexes. However, peripheral mechanisms for the recruitment and clonal expansion of the responding T cell repertoire remain obscure. Here we provide evidence for an avidity-based model of peripheral T cell clonal expansion in response to antigenic challenge. We have used the encephalitogenic, H-2 A^u-restricted, acetylated NH_2-terminal nonameric peptide (Ac1-9) epitope from myelin basic protein as our model antigen. Peptide analogues were generated that varied in antigenic strength (as assessed by in vitro assay) based on differences in their binding affinity for A^u. In vivo, these analogues elicited distinct repertoires of T cells that displayed marked differences in antigen sensitivity. Immunization with the weakest (wild-type) antigen expanded the high affinity T cells required to induce encephalomyelitis. In contrast, immunization with strongly antigenic analogues led to the elimination of T cells bearing high affinity T cell receptors by apoptosis, thereby preventing disease development. Moreover, the T cell repertoire was consistently tuned to respond to the immunizing antigen with the same activation threshold. This tuning mechanism provides a peripheral control against the expansion of autoreactive T cells and has implications for immunotherapy and vaccine design.

Key words: T cells • repertoire selection • autoimmunity • encephalomyelitis • apoptosis

Introduction

The repertoire of T lymphocytes responding to a given antigen is shaped by selection during thymic development coupled with homeostatic mechanisms active in the periphery (1). It is clear that the fate of a developing T cell (clonal deletion or maturation to become part of the peripheral T cell pool) is determined by its sensitivity for peptide antigens encountered in the thymus (1–4). However, peripheral homeostasis and selection are only now beginning to be explored (5–11). It has been suggested that a degree of T cell cross-reactivity is necessary to maintain an immune system with sufficient flexibility to adapt to a continuously changing antigenic environment (12). Therefore, it would follow that a single antigenic epitope will have the potential to stimulate a heterogeneous population of T cells that expresses diverse TCRs and responds with a range of antigen sensitivities. What is not known is the sensitivity that a T cell must display to form part of a productive immune response. To address this issue, we have investigated how clonal expansion during an immune response is influenced by the strength of antigenic challenge.

The acetylated NH_2-terminal nonameric peptide (Ac1-9) of myelin basic protein (MBP)[1] is an immunodominant T cell epitope in mice expressing the A^u MHC class II restriction element (13, 14). Remarkably, this peptide forms highly unstable complexes with A^u such that a binding affinity cannot be determined (15, 16). Nevertheless, immunization with either the Ac1-9 peptide or intact myelin can efficiently activate Ac1-9–reactive T cells and induce experimental autoimmune encephalomyelitis (EAE [13, 17]). We can assume, therefore, that the expanded T cells must

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[1]*Abbreviations used in this paper:* 7-AAD, 7-amino actinomycin D; CFSE, carboxyfluorescein diacetate succinimidyl ester; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PLNC, primed lymph node cell; TCL, T cell line.

be highly sensitive for the A^u–Ac1-9 complex to compensate for such poor affinity for MHC.

The Ac1-9 peptide binds A^u through the interaction of residues 4Lys and 5Arg with the peptide binding groove (17–19). Mutational analyses coupled with modeling studies indicate that 4Lys interacts unfavorably with a hydrophobic pocket within the A^u binding cleft resulting in an unstable interaction (20–22). Peptide analogues in which 4Lys is altered to a hydrophobic residue show greatly enhanced affinity for A^u (16, 17, 23).

We have used the Ac1-9 system to assess how the strength of the antigenic stimulus influences T cell clonal expansion in vivo. Peptide analogues displaying a hierarchy of affinities for A^u were thus created: Ac1-9(4Tyr) > Ac1-9(4Val) > Ac1-9(4Ala) >> Ac1-9(4Lys). These analogues were used to compare both antigenic and immunogenic properties of the peptide antigens. Previous studies had demonstrated the direct correlation between affinity for A^u and the capacity of the peptides to induce a secondary T cell response in vitro (antigenicity). Here we demonstrate conversely that the capacity of the peptides to induce EAE (pathogenicity) correlates inversely with their affinity for MHC. This is explained by the fact that strongly antigenic peptides induce cell death among highly sensitive Ac1-9–reactive T cells and simultaneously recruit cells with low affinity TCRs. Based on these findings, we propose an avidity-based model for tuning of the peripheral immune response that allows productive expansion of T cells with a defined sensitivity for the priming antigen.

Materials and Methods

Mice, Antigens, and Immunizations. B10.PL (H-2^u), B10.PL× SJL (H-2^{uxs}), and Tg4 transgenic mice were bred at the School of Medical Sciences, Bristol University. The Tg4 mouse is transgenic for a Vα4/Vβ8.2 TCR specific for MBP(Ac1-9; reference 2) and was backcrossed onto the B10.PL genetic background.

Synthetic peptides based on the sequence of murine MBP Ac1-9 (Ac-ASQKRPSQR) were prepared as described previously (24) using an ABIMED AMS 422 multiple peptide synthesizer. Analogue peptides with the wild-type Lys at position 4 being altered to Ala, Val, or Tyr were used. Two peptide panels containing Ala substitutions at individual residues were also prepared. One panel contained wild-type Lys at position 4, and the other panel also used the 4Ala substitution. Binding affinities of peptides for purified I-A^u were assessed using a competitive binding assay as described previously (24).

Mice were immunized subcutaneously with 200 nmol of peptide emulsified in 100 μl CFA supplemented with 400 μg *Mycobacterium tuberculosis* H37RA (Difco). For induction of EAE, mice also received 200 ng pertussis toxin (Speywood Pharmaceuticals) intraperitoneally on the day of immunization and 2 d later. Clinical signs of EAE were assessed for 35 d after immunization.

Generation of Antigen-specific T Cell Lines and Hybridomas. Draining lymph nodes were removed 10 d after immunization and stimulated in vitro with 10 μM of the immunizing peptide for 3 d. To generate T cell hybridomas, blasts were fused with the TCR-deficient thymoma BW5147 as described previously (25) at this point. The generation of the Ac1-9–reactive T cell lines (TCLs) 4Kuxs.TCL and the Tg4.TCL has been described previously (24). TCLs were also generated from peptide-primed mice by expansion of blasts subsequent to maintenance through continuous cycles of restimulation with the immunizing peptide and expansion with T cell growth factors, as described previously (24). All TCLs were $CD4^+$ and A^u restricted. Activation of TCLs was assessed using 72-h proliferation assays in 96-well microtitre plates (2×10^4 T cells/well) using irradiated B10.PL splenocytes (3×10^5/well) as APCs. Results are expressed as ^{3}H-TdR incorporation (mean cpm of triplicate cultures). Activation of T cell hybridomas was assessed as IL-2 secretion in response to antigen presented by the A^u-expressing B cell hybridoma PL8 (26). Proliferation of the IL-2–dependent cell line CTLL-2 was used as a measure of IL-2 levels in hybridoma supernatants. TCLs and hybridomas were maintained and assayed in IMDM supplemented with 2 mM L-glutamine, 5×10^{-5}M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL/Life Technologies), and 5% FCS (Sigma-Aldrich). For analysis of primed lymph node cell (PLNC) proliferative responses (10 d after immunization), 3×10^5 cells/well were cultured for 72 h using X-VIVO 15 serum-free medium (BioWhittaker) supplemented with 2 mM L-glutamine (GIBCO BRL) and 10^{-5} M 2-ME.

Tg4 Transgenic T Cell Transfer Experiments. Tg4 transgenic splenic T cells were purified using a nylon wool adherence negative selection step. These T cell–enriched populations (>80% $CD4^+$) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) as described previously (27) and 5×10^6 cells injected intravenously into nontransgenic littermates. 1 d after transfer, mice were immunized with either Ac1-9, Ac1-9(4Tyr), or CFA alone as described above. 5 d later, draining lymph node cells were removed and $CFSE^+$ cells assessed for apoptosis by staining with 7-amino actinomycin D (7-AAD) and PE-conjugated annexin V (BD PharMingen) as per manufacturer's instructions.

Analysis of A^u–Ac1-11(4Tyr) Tetramer Staining of TCLs. The generation of a soluble construct of A^u linked to the Ac1-11 (4Tyr) peptide is described elsewhere (28). Tetrameric complexes were prepared by incubation with PE-conjugated streptavidin and used to stain TCLs by incubation at 4°C for 3 h. Analysis of staining was performed using a FACScan™ (Becton Dickinson) and the CELLQuest™ (Becton Dickinson) and WinMDI 2.8 analysis software (Scripps Research Institute; http://facs.scripps.edu). Kinetics of loss of tetramer staining were assessed using the previously described method (29). In brief, after incubation of TCLs with tetramer, cells were washed twice and suspended in PBS containing 100 μg/ml of the A^u-binding monoclonal antibody 10-2-16 to prevent tetramer rebinding and incubated for various time points at 4°C before analysis of tetramer staining. Results were expressed as total fluorescence within each population as a percentage of that found before incubation with 10-2-16. Receptor expression by TCLs was assessed using FITC-conjugated anti-TCR (H57-597; BD PharMingen) or anti-CD4 (H129.19; Sigma-Aldrich).

Results

Enhanced Antigenicity Correlates Inversely with Induction of Autoimmunity. To study the influence of the strength of antigenic stimulus on selection of the T cell repertoire, we required a well-defined model system in which precise alterations in peptide sequence confer marked differences in antigenic properties in vitro. The MBP(Ac1-9) model has been shown previously to fulfill these criteria (17, 23).

Table I. *A^u Binding Affinity Correlates Inversely with EAE Induction*

Peptide	Sequence	Relative binding affinity for A^u	Incidence of EAE
			%
4Lys	AcASQKRPSQR	<0.00001	60
4Ala	AcASQARPSQR	0.01	10
4Val	AcASQVRPSQR	0.2	0
4Tyr	AcASQYRPSQR	1	0

Binding affinities of peptides for A^u were assessed as described previously (reference 24). Incidence of EAE represents cumulative data from experiments shown in Fig. 1, D and E.

For this study, we selected Ac1-9 analogue peptides in which the wild-type 4Lys was substituted with either Ala, Val, or Tyr. Results from competitive binding assays confirmed previous reports that these analogues showed greatly enhanced binding to the A^u MHC class II molecule (Table I [16, 17, 23]). The 4Tyr peptide proved to be the strongest binder with an affinity at least 5 logs higher than wild-type Ac1-9. The 4Val peptide showed slightly weaker binding than 4Tyr, whereas the 4Ala peptide showed intermediate binding (>1,000-fold stronger than wild-type, but 100-fold weaker than 4Tyr).

These differences in MHC binding translated directly into antigenic activity in vitro. We generated TCL specific for the wild-type Ac1-9(4Lys) peptide from three different sources. Two TCLs were derived from H-2^{uxs} mice immunized with either intact myelin (4Kuxs.TCL) or the 4Lys peptide (4Lys.TCL). The third TCL was derived using splenocytes taken from an unprimed Tg4 transgenic mouse (this mouse expresses an Ac1-9–specific TCR derived from PL/J mice immunized with rat MBP [2]). Each of these three TCLs was maintained by in vitro restimulation with the wild-type 4Lys peptide. These Ac1-9–specific TCLs were tested for proliferative responses to 4Lys and the three analogue peptides (Fig. 1, A–C). Each TCL showed the same response profile, with responses to wild-type 4Lys evident at a concentration of 10 nM. The three analogue peptides all showed "superagonist" activity, with 4Tyr and 4Val capable of inducing responses at femtomolar (fM) concentrations. The response to 4Ala was lost below 100 fM, consistent with the intermediate A^u binding displayed by this peptide.

Given the increased antigenic activity in vitro of the superagonist peptides, one might predict that these peptides would be powerful inducers of EAE in vivo. However, this was not the case. Whereas immunization with wild-type 4Lys induced EAE in 60% of mice, disease was observed rarely using the intermediate 4Ala analogue and not at all with the strongest superagonists 4Val and 4Tyr (Table I, and Fig. 1, D and E). These findings indicated that the ef-

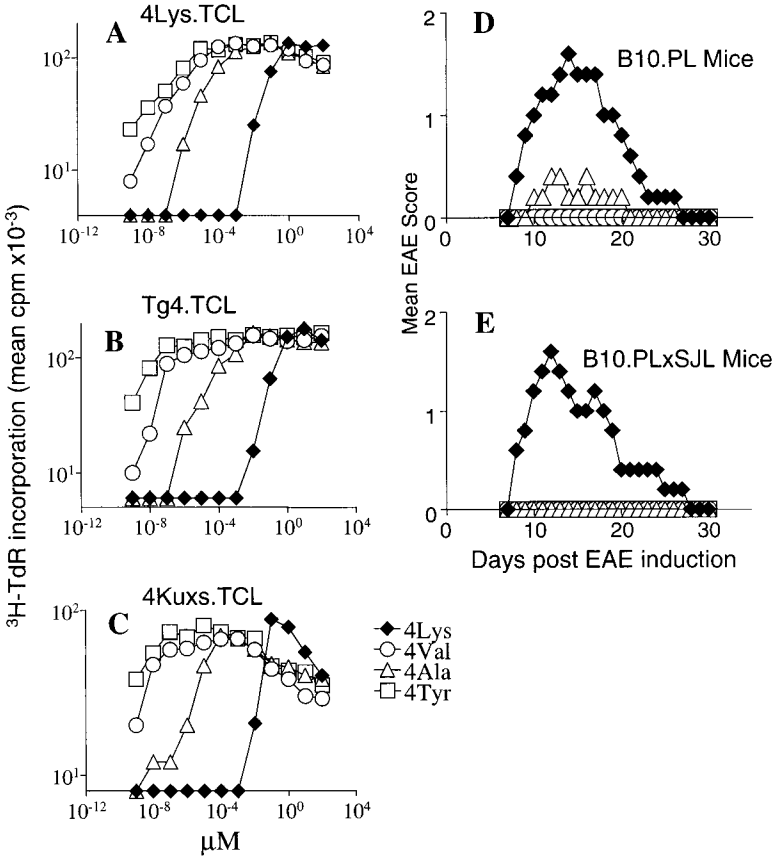

Figure 1. Ac1-9 position 4 analogues behave as superagonists in vitro, but show markedly reduced encephalitogenic activity in vivo. (A–C) TCLs generated against wild-type Ac1-9 were tested for proliferative responses to Ac1-9 and position 4 analogue peptides. Mice were immunized to induce EAE as described in Materials and Methods. Results represent two identical experiments, one using B10.PL mice (D) and the other using B10.PL×SJL mice (E). In each experiment five mice were used per group. The incidence of EAE induced with 4Lys was 3/5 in each experiment.

fect of increasing antigenic strength in vivo was to reduce the immunogenicity of the peptides, correlating with a reduction in the productive activation of pathogenic T cells. The markedly reduced encephalitogenic activity of the 4Ala peptide has been reported previously (17). These studies also reported deficits in T cell activation at the primed lymph node level, and it was therefore assumed that the 4Ala peptide was non (or weakly) immunogenic (17, 30).

The Immune Response Tunes T Cell Reactivity to a Predetermined Threshold of Sensitivity. To clarify the immunogenic potential of our analogue peptides, mice were immunized with each of the four peptides individually and the draining lymph node cells were restimulated in vitro with the same peptide used for priming. Using this approach, we were able to derive TCLs specific for each peptide (4Lys.TCL, 4Ala.TCL, 4Val.TCL, and 4Tyr.TCL). Analysis of each of these TCLs showed striking differences in antigen sensitivity (Fig. 2). First, stimulation with the wild-type 4Lys peptide revealed that the 4Lys.TCL responded normally and was activated by a dose of 10 nM. However, the TCLs primed against the superagonists were far less sensitive to stimulation with the wild-type peptide (Fig. 2 A). The antigenic hierarchy described above was reversed such that priming with the 4Ala peptide induced T cells that responded to 4Lys at 1 μM, whereas 100 μM was required to stimulate the 4Tyr.TCL. Therefore, it seems likely that the superagonist peptides failed to induce EAE in vivo because they were unable to expand T cells of sufficient sensitivity to respond to wild-type Ac1-9 in the central nervous system.

Most strikingly, analysis of the response of the individual TCL to peptides against which they were raised revealed identical dose response patterns. In each case, the TCL would respond at a dose of 1–10 nM (Fig. 2 B). These findings indicate that, as an immune response develops in vivo, an active mechanism tunes the T cell population that is expanded to have a predetermined sensitivity for antigen.

One possible explanation for the insensitivity of superagonist-primed T cells to the wild-type 4Lys peptide would be that they recognize distinct amino acids as TCR contact residues. Previous reports had identified MHC and TCR contact residues within the Ac1-9 sequence (17–19). In addition to position 4, position 5 Arg also contacts A^u, with the 5Ala analogue showing reduced affinity for A^u and failing to stimulate Ac1-9–specific T cells. In contrast, Ala substitutions at positions 3 and 6 were shown to abrogate T cell activation without influencing A^u binding.

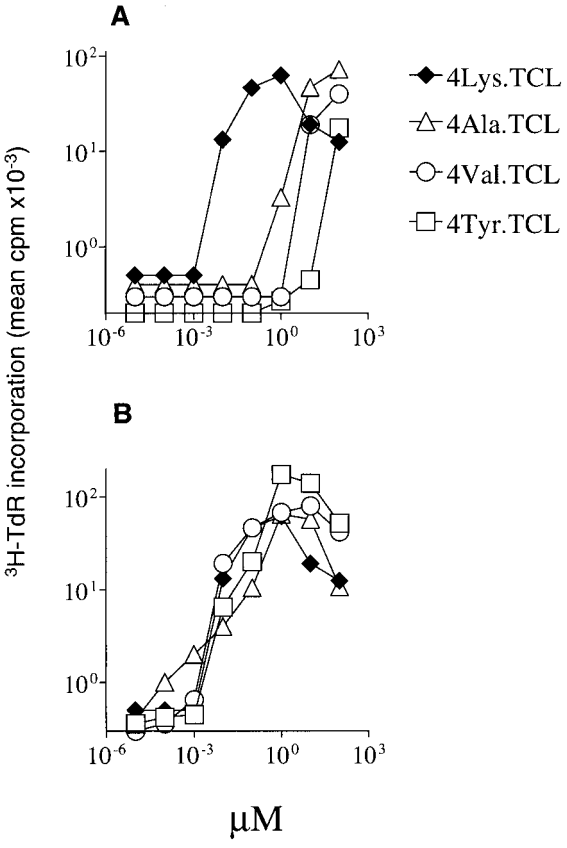

Figure 2. T cell activation thresholds are tuned to the strength of antigenic challenge. The response to Ac1-9 (A) and the response to immunizing antigen (B) are shown. TCLs were generated from mice immunized with either wild-type Ac1-9 (4Lys) or position 4 analogue peptides by in vitro stimulation of PLNCs with the immunizing peptide. TCLs were cultured with varying doses of 4Lys (A) or the immunizing peptide relevant to each TCL (B). Symbols refer to the peptide against which the TCL was generated: 4Lys (◆), 4Ala (△), 4Val (○), or 4Tyr (□). These priming and in vitro restimulation protocols were used in three independent experiments that yielded consistent results.

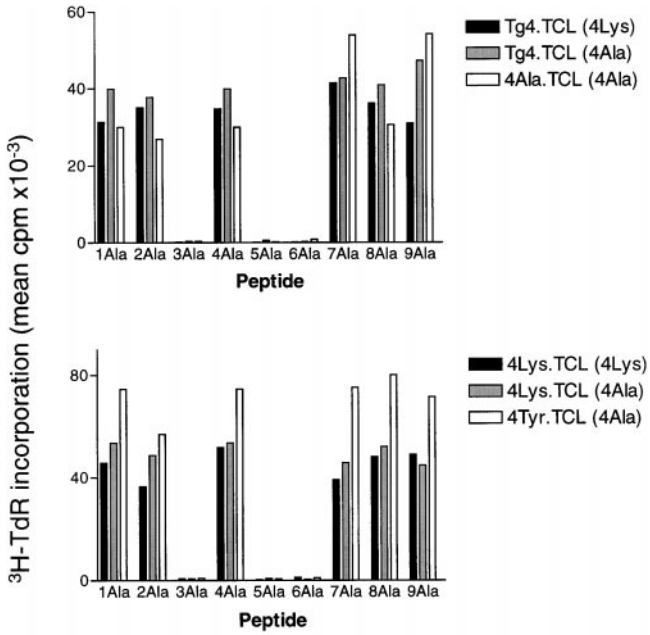

Figure 3. TCLs specific for either wild-type Ac1-9 or superagonist recognize the same TCR contact residues. TCLs were stimulated with two panels of Ac1-9 analogues with Ala substitutions at individual residues. One panel was based on wild-type (4Lys) Ac1-9, the other panel also incorporated a 4Ala substitution. Parentheses in legends refer to these panels (i.e., the amino acid used at position 4). The 1Ala peptides represent the wild-type sequence (Ala occurs naturally at this residue). Responses were tested over a dose range of peptide (10 μM shown).

TCR contact residues for the various cell lines described above were assessed by measuring response profiles of TCLs using two panels of peptide analogues containing Ala substitutions at individual peptide residues. One peptide panel contained wild-type Lys at position 4, and the second panel also possessed the 4Ala substitution. This was necessary, as superagonist-primed TCLs were insensitive to the wild-type 4Lys peptide but all responded to the 4Ala analogue. We used the Tg4.TCL and 4Lys.TCL to represent the "normal" T cell repertoire. We found that both of these TCLs failed to respond to the 3Ala, 5Ala, and 6Ala peptides, and that this was also the case whether position 4 was Lys or Ala (Fig. 3). This confirms previous observations (17–19) and indicates that the 4Ala analogue must form a similar conformation to that of wild-type 4Lys within the groove of A^u. We also tested the 4Ala.TCL and 4Tyr.TCL for responses to the Ala-substituted analogues that also contained 4Ala, and observed an identical response pattern with the 3Ala, 5Ala, and 6Ala analogues being nonstimulatory (Fig. 3). The 4Tyr.TCL also failed to respond to 3Ala and 6Ala analogues that contained the 4Tyr substitution (data not shown). Taken together, these data indicate that there was no significant qualitative deviation of TCR recognition caused by alterations at position 4, with all TCLs tested focusing on positions 3 and 6 as TCR contacts.

We then addressed the question of whether tuning of the cell lines had occurred in vivo or was a result of repeated in vitro stimulation with antigen. T cell hybridomas generated directly from PLNC populations were tested for response patterns to the priming antigen and wild-type peptide (Table II). Again we observed that hybridomas from mice primed with superagonists were increasingly insensitive to 4Lys and responded to the priming antigen at concentrations generally within the 1–100 nM range. Furthermore, analysis of proliferative responses of PLNC from mice immunized with either 4Lys or 4Tyr showed evidence of tuning (Fig. 4). Although the 4Tyr peptide behaved as a superagonist after priming with 4Lys, the response after priming with 4Tyr was equivalent to that seen to 4Lys after priming with 4Lys. From these data we conclude that the tuning event occurred during the development of immunity in vivo and was not the result of continued exposure to antigen in vitro.

Two possible mechanisms could account for the tuning of T cell sensitivity: (a) biochemical detuning of the signaling machinery within a homogeneous Ac1-9–specific repertoire, or (b) the selective expansion of distinct repertoires of T cells in response to the different peptides. No differences in antigen receptor expression levels between the different panels of hybridomas were evident (serological analysis of TCR-α/β and CD4 expression; data not shown). Thus, lack of sensitivity could not be attributed to reduced TCR availability. The response to wild-type Ac1-9 has been reported to be dominated by T cells expressing TCR using Vβ8 variable region gene products (31). Serological analysis confirmed this for the 4Lys-induced hybridomas (11 of 12 V$\beta8^+$) whereas the superagonist-primed hybrid-

Table II. *Superagonist Peptides Select T Cells with Reduced Antigen Sensitivity In Vivo*

Priming peptide	4Lys		4Ala		4Val		4Tyr	
In vitro peptide	4Lys	4Tyr	4Lys	4Ala	4Lys	4Val	4Lys	4Tyr
μM								
0.00001		6						
0.0001		6				1		
0.001						1		2
0.01	3			4		5		6
0.1	8			2	1	7		8
1	1		1		1		2	3
10			3		4		3	
100			2		5		7	
No response					3		7	
Total	12		6		14		19	

Peptide-specific T cell hybridomas were generated from PLNCs as described in Materials and Methods and tested for responsiveness to peptide analogs over a range of concentrations. The minimal stimulatory dose of each peptide is shown in terms of the number of hybridomas beginning to respond at each concentration. Note that 0.00001 μM was the lowest concentration used in these experiments. Therefore, the six 4Lys hybridomas responding at this concentration of 4Tyr may have been more sensitive than these results reveal.

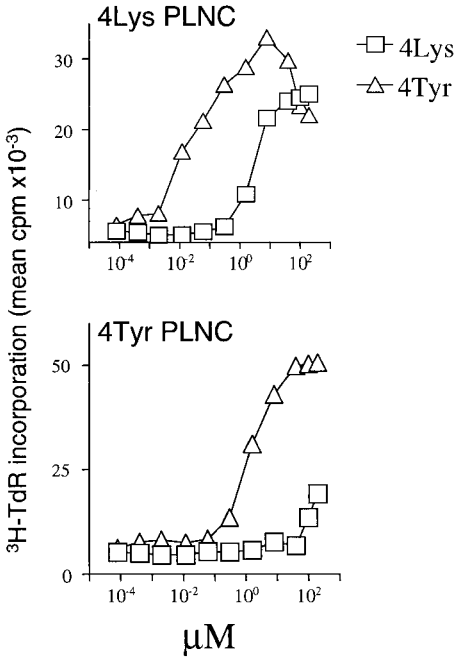

Figure 4. Tuning of T cell responsiveness is evident at the primed lymph node level. PLNCs were isolated from B10.PL×SJL mice immunized with either the 4Lys or 4Tyr peptides. PLNCs were tested for proliferative responses to dose ranges of each peptide. Data are from one of three experiments which gave consistent results.

omas tended not to use Vβ8 (4Ala, 1 of 6; 4Val, 0 of 14; 4Tyr, 0 of 16 tested). These findings rule out the possibility that superagonist-primed T cells were simply Ac1-9–specific T cells that had been desensitized. Rather, they imply that tuning of the immune response depends on expansion of distinct T cell repertoires.

Tuning Results from TCR Affinity–based Selection of T Cells with Permitted Sensitivity for Antigen. Based on our results, we propose an avidity model of T cell clonal expansion in which those cells with sensitivity to antigenic challenge above a certain threshold are purged from the productive immune response. Irrespective of the signal given by the priming antigen, the resulting immune response will be tuned to fall within a window of sensitivity by selecting distinct repertoires of T cells for expansion. To test the avidity model, we addressed the fate of highly sensitive Ac1-9–reactive T cells in mice primed with the high affinity 4Tyr analogue. T cells from the Tg4 mouse are highly sensitive to 4Tyr stimulation in vitro (2; Fig. 1 B). T cells were isolated from naive Tg4 spleen, labeled with CFSE, and transferred into nontransgenic littermates. In preliminary experiments to assess the incorporation of the transferred cells in lymphoid organs, spleens isolated from recipient mice 2 d after cell transfer contained ~1% $CFSE^+$ cells (Fig. 5 B).

In further experiments, recipient mice were immunized with either 4Lys, 4Tyr, or adjuvant alone 1 d after cell transfer. Significant numbers of $CFSE^+$ cells could not be detected in popliteal lymph nodes taken 5 d after priming with CFA alone (Fig. 5 C). Therefore, in this system the presence of antigen was required for the accumulation of antigen-specific T cells in the nodes. In the absence of antigen (CFA alone), antigen-specific cells presumably either do not home to these lymph nodes or their frequency is diminished to a level below detection, due to the accumulation and expansion of cells specific for antigens in CFA. In contrast, discrete but heterogeneous populations of $CFSE^+$ cells were identified in the draining popliteal and inguinal lymph nodes of mice primed with antigen. Fig. 5 C shows the $CFSE^+$ population present after immunizaton with 4Lys. Populations staining with this low level of CFSE were consistently found whether priming with either 4Lys or 4Tyr. This weak level of CFSE label compared with the levels shown in Fig. 5 B corresponds with the gated cells having undergone three to five rounds of division in response to immunization. As this gate was used for both the 4Lys- and 4Tyr-primed populations, the superagonist did not cause a noticeably more rapid proliferation of the transferred cells. These $CFSE^+$ cells ranged from 1.5 to 3.4% of 4Lys-PLNCs and 1.2 to 3.1% of 4Tyr-PLNCs. Counterstaining with annexin V and 7-AAD revealed that after priming with 4Tyr, >70% of $CFSE^+$ cells were showing signs of early apoptosis (annexin V^+, 7-AAD^-) compared with <10% after priming with 4Lys (Fig. 5 D). Therefore, Ac1-9–sensitive T cells were deleted after exposure to the 4Tyr superagonist in vivo.

The next outstanding question was how cells that were selected after immunization with strongly antigenic ligands avoided deletion. The relative insensitivity of T cells expanded after challenge with superagonist was not due to expression of reduced levels of TCR or CD4 (Fig. 6, A and B). An alternative explanation was that cells escape deletion through expression of low affinity TCRs. Measurement of the relative affinity of TCR expressed by individual T cells has been made possible through the development of soluble MHC class II–peptide complexes (29, 32, 33). Tetrameric complexes of A^u–Ac1-11(4Tyr) (28) were used to stain TCLs generated against 4Lys or 4Tyr and to assess the affinity of their TCRs. These complexes reacted specifically with Ac1-9–reactive T cells, as no staining was observed when using splenic T cells from unimmunized B10.PL mice or short-term T cell populations expanded against purified protein derivative of *Mycobacterium tuberculosis* (28). Analysis of tetramer staining patterns (Fig. 6 C) revealed a homogeneous population of Tg4.TCL (expressing a single TCR). However, the 4Lys.TCL displayed a broader pattern of staining, indicating a heterogeneous population using a range of TCRs with varying affinities for the tetrameric complex. This pattern is similar to those described in other systems using tetramers to stain TCRs specific for foreign antigens (29,

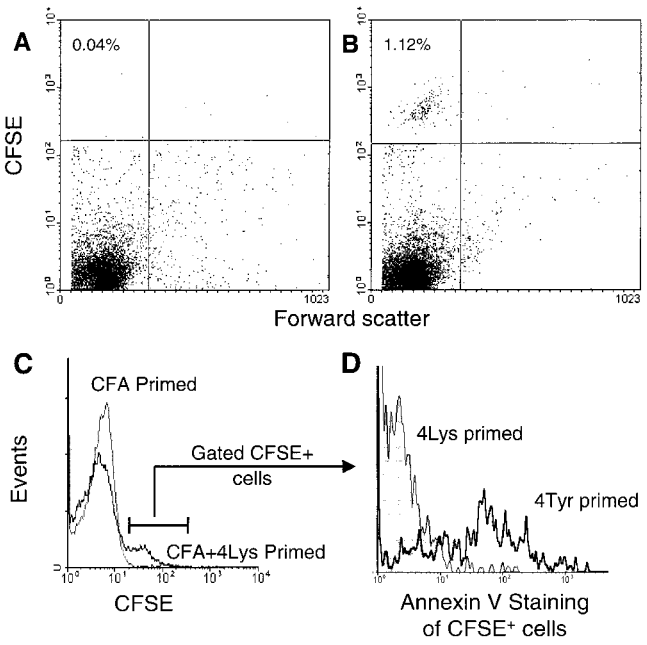

Figure 5. In vivo activation with superagonist results in apoptotic removal of highly sensitive Ac1-9–reactive T cells. CFSE-labeled Tg4 transgenic T cells were transferred to nontransgenic littermates. Presence of $CFSE^+$ cells was assessed in spleens of nontransferred mice (A) versus recipient mice 2 d after cell transfer (B). (C) Presence of CFSE-labeled T cells in primed lymph nodes 5 d after immunization with Ac1-9(4Lys) or CFA alone. Similar $CFSE^+$ populations to those after 4Lys priming were also evident after priming with Ac1-9(4Tyr). The percentage of $CFSE^+$ cells ranged between 1.5 and 3.4% for 4Lys-PLNCs and 1.2 and 3.1% for 4Tyr-PLNCs. (D) Annexin V–PE staining of 4Lys versus 4Tyr PLNC populations after gating on $CFSE^+$ cells. 8% of the 4Tyr-primed, CFSE/annexin V double-positive population also stained with 7-AAD. Data represent results from individual mice. Consistent results were obtained with a total of eight mice per group over three separate experiments.

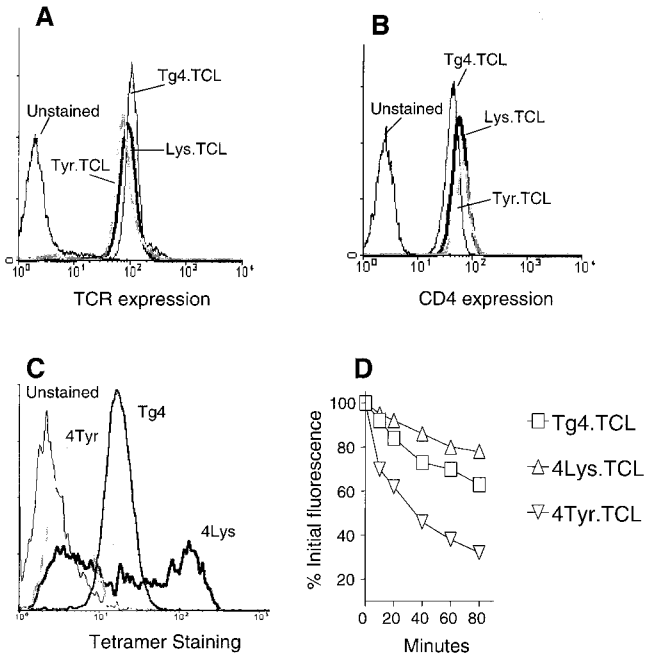

Figure 6. Immunization with the 4Tyr superagonist leads to preferential expansion of T cells expressing TCRs with low affinity for Ac1-9. The 4Lys.TCL, 4Tyr.TCL, and Tg4.TCL were stained with TCR-α/β (A), CD4 (B), or A^u–Ac1-11(4Tyr) tetrameric complexes (C). Each plot shows unstained 4Lys.TCL cells. (D) After tetramer staining, cells were washed and incubated for varying times. Results are expressed as the levels of total tetramer staining in each population as a percentage of the level of staining before incubation.

32). In contrast, the 4Tyr.TCL stained weakly with tetramer, indicating a more homogeneous population expressing only TCRs of low affinity. In further experiments we assessed the rate of dissociation of the tetramers once loaded to each TCL (Fig. 6 D). The less sensitive 4Tyr.TCL showed more rapid loss of tetramer staining than either 4Lys.TCL or Tg4.TCL. Immunization with the superagonist therefore led to the expansion of T cells expressing TCRs with lower affinity for peptide–MHC than those raised against the wild-type antigen.

Discussion

The notion of avidity-based tuning of T cell reactivity as a mechanism for central tolerance is well rehearsed (3, 34–36). Indeed, the absence of endogenous MBP in the shiverer mouse has been reported to allow T cells with specificity for high affinity MHC binding peptides to escape thymic negative selection (37, 38). This is the first study to address the influence of the strength of the antigenic stimulus on peripheral T cell repertoire selection during the initiation of an immune response. The results reveal an inherent tuning event involving selective expansion of T cells to maintain antigen reactivity within a consistent threshold of sensitivity. This threshold, between 1 and 10 nM, is not restricted to the MBP(Ac1-9) system, as other TCLs generated in our laboratory (reactive against self- or foreign antigens) also display this characteristic. Furthermore, a remarkable number of reports describe similar thresholds for $CD4^+$ T cell activation (39–44). In this study, we have altered the strength of the antigenic signal by manipulation of the peptide sequence so as to vary the affinity for MHC class II and hence antigenicity. Quantitative variations in the antigenic signal may also be achieved by manipulation of other parameters, such as duration of exposure and antigen dose (33).

Although our study addresses how the strength of signal affects repertoire selection against a single immunodominant epitope, other factors will also influence repertoire selection against complex antigens. First, efficiency of antigen processing and MHC–peptide complex formation has an important bearing on immunodominance (45). Determinant capture (45) can influence epitope selection involving either single or distinct MHC loci (46, 47). Once formed, the relative abundance of MHC–peptide complexes is not the sole factor governing immunodominance (48). Indeed, studies of immune recognition of virus have reported highly abundant MHC–peptide complexes against which T cell reactivity could not be found (49, 50). This may be due to deletion via clonal exhaustion (51) or a tuning mechanism due to excessive antigenic stimulus, as described here. Clearly, such deletional effects will influence subsequent responses on encounter of the same or a cross-reactive antigen. There is also evidence that the presence of T cells recognizing one epitope can inhibit T cell expansion against other epitopes during an immune response (48). The tuning mechanism will therefore serve as a check on T cell sensitivity as part of a multiparameter selection process.

Our findings indicate that, at least in our system, tuning is a deletional event based on the affinity of TCR for the peptide–MHC complex. When T cells bearing high affinity TCRs receive a powerful antigenic stimulus in vivo (i.e., "normal" Ac1-9–reactive T cells primed with 4Tyr) they rapidly progress to the contraction phase of the response and are removed by apoptosis. The T cells expanded after priming with superagonist express lower affinity TCRs, and this compensates for the strong antigenic stimulus to maintain antigen reactivity within the permitted sensitivity range. This mechanism is consistent with a recent report that T cells with low affinity TCRs are selected by prolonged exposure to high doses of foreign antigen (33).

Our use of tetrameric 4Tyr–A^u complexes to assess TCR affinity revealed a continuous range of affinities from low to high after immunization with the wild-type 4Lys peptide, (Fig. 6 C). This was in contrast with the homogeneous populations found with the Tg4.TCL (expressing a single transgenic TCR and therefore having a fixed affinity) and the 4Tyr.TCL, which expressed only low affinity TCRs. The survival of low affinity T cells in the 4Lys.TCL population indicates that these cells were fully capable of sustained expansion in response to antigen in vitro. This may reflect survival due to the in vitro conditions such as excess production of IL-2 by the high affinity T cells also

present in these cultures. An earlier study has indicated that such low affinity cells become increasingly rare as the dose of antigen used to stimulate polyclonal populations in vitro is reduced (33). Therefore, it is difficult to extrapolate to the possible survival of such low affinity T cells in vivo. However, the presence of low affinity cells does suggest that in this system the dominant factor influencing the repertoire during in vivo expansion is the loss of high affinity cells through deletion (when immunizing with the 4Tyr superagonist) rather than the loss of low affinity cells through insufficient strength of antigenic signal (when immunizing with wild-type 4Lys).

Recent reports of selective tolerance to a foreign antigen also provide evidence of a tuning mechanism. A wild-type peptide from moth cytochrome c was able to induce effective tolerance to itself, but subsequent immunization with a superagonist analogue led to expansion of a distinct T cell population bearing lower affinity TCRs (43, 44). Preliminary experiments indicate that T cells primed to 4Lys in vivo, and transgenic Tg4 T cells, initially proliferate and then undergo apoptosis after in vitro exposure to superagonist peptides (data not shown). Removal by apoptosis of T cells that have been overstimulated by antigen has been proposed as a major homeostatic mechanism in maintaining the immune repertoire (52). Our studies support this notion of "propriocidal" control and suggest that strength of antigenic signal may be as important as duration of exposure to antigen. Although our findings suggest that T cell sensitivity is largely controlled by TCR affinity, other factors such as level of expression of TCR (40) or CD4 (53), or rewiring of T cell signaling machinery (42, 54, 55), might also play a role in other situations.

Superagonist-induced apoptosis in mice transgenic for Ac1-9–specific TCRs has been reported previously by ourselves and others (41, 56, 57). A deletional mechanism is consistent with previous reports that the 4Ala superagonist inhibits EAE in nontransgenic mice but does not induce a suppressive T cell population (protection cannot be transferred with T cells [17, 30]). Similar deletional effects have also been used to inhibit EAE by use of a superagonist variant peptide based on a different MBP T cell epitope (58). Several studies have reported induction of T cell apoptosis after administration of high doses of antigen in tolerogenic form (39, 58, 59). The results of this study underline the notion that deletion can occur during an active peripheral immune response and that this is dependent on the sensitivity of the T cell for antigen such that only relatively insensitive T cells are allowed to expand. Furthermore, this study is the first to show that the net result of the balance between apoptosis and expansion is that the resulting T cell repertoire will consistently respond to the initiating antigen within a narrow dose–response window.

What would be the immunological benefit of restricting productive $CD4^+$ T cell immunity to a predetermined threshold of sensitivity? The 4Tyr superagonist was found to induce clonal deletion of Tg4 T cells both during thymic development when given in soluble form (2) and during an active peripheral immune response (this study). Therefore, both immature and mature T cells are sensitive to apoptosis induced with the same antigenic signal. A peripheral fail-safe mechanism of clonal deletion would remove any potentially autoaggressive T cells that escape central tolerance. This would serve to limit the risk of autoimmune disorders developing via molecular mimicry (60, 61). We found that immunization with superagonist (leading to hyporesponsiveness to Ac1-9) fails to effectively induce EAE. Therefore, priming for a pathogenic autoreactive response requires the stimulus derived from the eliciting antigen to fall within a limited range. Thus, only T cells capable of responding to both foreign and self-antigens with the permitted sensitivity would be pathogenic. Highly self-reactive T cells would be removed by thymic selection, while those that escape would be further purged through activation by superagonist foreign ligands during peripheral immune responses. A recent description that immunization with a peptide derived from human papillomavirus 7 led to EAE included findings relevant to this point (62). Low doses of viral peptide were found to activate T cells cross-reactive with an epitope within the 87–99 sequence of MBP and to elicit EAE. However, high doses of the viral peptide led to expansion of viral-specific T cells that failed either to cross-react with MBP or induce EAE. Loss of self-reactivity through the tuning of the T cell response to the stronger foreign antigenic challenge would account for these findings.

Activation-induced cell death is believed to serve as a counterbalance designed to avoid excessive T cell expansion (52) and prevent autoimmunity (63, 64). Keeping the threshold for $CD4^+$ T cell activation above a defined point ensures that the response is focused on sites of high ligand density, for example antigen-specific B cells requiring T cell help. The immune system therefore concentrates limited resources at sites of greatest impact.

The proposed avidity model of clonal expansion has important implications. First, it argues that susceptibility to avidity-based elimination is intrinsic to the T cell throughout its lifespan and is not restricted to thymocyte development. Second, superagonists should evidently be more effective treatments for silencing autoimmunity (56, 58, 65). Finally, these findings argue against the use of superagonists for vaccine design. Such an approach would inevitably lead to the removal of the highly antigen-sensitive T cells that would be most effective in combating infection.

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