Molecular mimics can induce a nonautoaggressive repertoire that preempts induction of autoimmunity

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To determine the role that competition plays in a molecular mimic’s capacity to induce autoimmunity, we studied the ability of naïve encephalitogenic T cells to expand in response to agonist altered peptide ligands (APLS), some capable of stimulating both self-directed and exclusively APL-specific T cells. Our results show that although the APLs capable of stimulating exclusively APL-specific T cells are able to expand encephalitogenic T cells in vitro, the encephalitogenic repertoire is effectively outcompeted in vivo when the APL is used as the priming immunogen. Competition as a mechanism was supported by: (i) the demonstration of a population of exclusively APL-specific T cells, (ii) an experiment in which an encephalitogenic T cell population was successfully outcompeted by adoptively transferred naïve T cells, and (iii) demonstrating that the elimination of competing T cells bestowed an ability to expand naive encephalitogenic T cells in vivo. In total, these experiments support the existence of a reasonably broad T cell repertoire responsive to a molecular mimic (e.g., a microbial agent), of which the exclusively mimic-specific component tends to focus the immune response on the invading pathogen, whereas the rare cross-reactive, potentially autoreactive T cells are often preempted from becoming involved.

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The data are freely available online through the PNAS open access option.

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from EAE (Fig. L4). Thus, it was hypothesized that a molecular mimic of this encephalitogenic clone in vivo might correlate with its ability to induce EAE.

The Vβ8.2/Jβ2.7 Clonotype Responds In Vitro to LDVM1-9(Y4) and Ac1-6 (M4). To compare the ability of different APLs of Ac1-9 to stimulate the characteristic Vβ8.2/Jβ2.7 clonotype, a Vβ8.2/Jβ2.7 Ac1-9-specific DAGGGY T cell hybridoma (9), was incubated with different APLs in the presence of splenic APCs. Fig. 1B shows that LDVM1-9(Y4) was superior to Ac1-9 in its ability to stimulate the public clonotype in vitro. Thus, the natural ligand, Ac1-9, is a suboptimal agonist for the Vβ8.2/Jβ2.7 clonotype. In contrast, Ac1-6(M4) (open diamonds) was inferior to Ac1-9 in its ability to stimulate the Ac1-9-specific Vβ8.2/Jβ2.7 T cell clone.

Ac1-9(Y4) Is Able to Expand the Encephalitogenic Vβ8.2/Jβ2.7 Clonotype In Vivo. One might propose several possible mechanisms for failure of an APL agonist to expand a pathogenic self-directed repertoire from its naïve state within an animal. As mentioned earlier, in addition to stimulating self-directed T cells, an APL agonist might be able to stimulate a large number of exclusively APL-specific, non-self-directed T cells. These exclusively APL-specific T cells in turn may outcompete pathogenic self-directed clones for activation. Alternatively, an APL agonist may induce an antiinflammatory cytokine profile (12) or antagonize pathogenic T cells (13). Lastly, it may induce an active exhaustion of the pathogenic repertoire. In favor of the latter explanation, a published report demonstrated negative selection during the peripheral immune response to an APL (14). These results, however, are somewhat controversial. A more recent study conducted in the same experimental system reported enhanced antigen-specific T cell responses rather than negative selection. The authors of the latter study concluded that immunizing with a high concentration of an APL agonist resulted in an antiinflammatory feedback loop involving IFN-γ (15). To exclude immunologic exhaustion as a possible mechanism for our results, we immunized B10.PL animals with differing amounts of Ac1-9(Y4) and then characterized the intensity of the driver Vβ8.2/Jβ2.7 clonotypic expansion. Like Ac1-9 itself (Fig. L4), Ac1-9 (Y4) was an excellent inducer of the Vβ8.2/Jβ2.7 Ac1-9-specific public response (Fig. S1A and B). However, at a very high concentration of antigen (200 μg/mouse), the expansion of the Vβ8.2/Jβ2.7 clonotype decreased but was not entirely eliminated (Fig. S1C). Therefore, to avoid immunologic exhaustion and to focus on the competitive activities among T cell clones, an eightfold lower amount of 25 μg was used for all subsequent APL immunizations. These results correspond with the findings that Ac1-9(Y4) induces severe EAE at low but not high concentrations (15).

The Driver Vβ8.2/Jβ2.7 Clonotype Is Highly Susceptible to T Cell Competition In Vivo. Previous reports have indicated that residues flanking a minimal core of amino acids can have profound effects on T cell activation, either enhancing or interfering with the activation of particular T cells (16, 17). Ac1-9's interaction with I-Ak is unique in that when bound within the MHC binding groove it is predicted that the first MHC pocket remains unoccupied. Thus, of our panel of agonistic ligands for the Vβ8.2/Jβ2.7 DAGGGY clonotype (Fig. 1B), we hypothesized that the APL LDVM1-9(Y4), which was composed of additional flanking residues, LDVM, might expand a unique population of APL-specific T cells focused on the N-terminal LDVM residues. In addition, a similar phenomenon was predicted to exist with the very short agonist Ac1-6(M4). To investigate the latter, B10.PL animals were immunized with 25 μg of Ac1-6(M4). Lymphocytes from these animals were then fused with BWS157 (α 8.2J) to make Ac1-6(M4)-specific T cell hybridomas. Twenty Ac1-6(M4)-responsive hybridomas were screened, none of which could recognize Ac1-9 (one representative hybridoma is shown in Fig. 2A). Thus, the majority of the Ac1-6(M4)-specific T cells were not cross-reactive to Ac1-9. In contrast, when B10.PL animals were initially immunized with Ac1-9, Ac1-6(M4) was very effective at expanding the public Vβ8.2/Jβ2.7 driver clonotype in vitro (Fig. 2B). To test whether Ac1-6(M4) could expand the Vβ8.2/Jβ2.7 clonotype in vivo, B10.PL animals were immunized with 25 μg of Ac1-6(M4) and T cell repertoire analysis was conducted 10 days later on draining lymph node cells. Although Ac1-6(M4) was effective at expanding the Vβ8.2/Jβ2.7 encephalitogenic repertoire in vitro (Figs. 1B and 2B), Fig. 2C reveals that active immunization with Ac1-6(M4) failed to expand the naive Vβ8.2/Jβ2.7 Ac1-9-specific repertoire. In addition, when B10.PL mice were primed with Ac1-6(M4) there was only a marginal in vitro recall response to Ac1-9 (data not shown). In agreement with these findings, TCR repertoire analysis of samples obtained from Ac1-6(M4)-immunized animals revealed Ac1-6(M4)-specific expansions, even within the Vβ8 family, which did not cross-recognize the longer peptide, Ac1-9 (Fig. 2D). The expansions seen in Fig. 2D are believed to be Ac1-6 (M4) specific because they arise from Ac1-6(M4)-immunized animals and are seen when lymphocytes are incubated with Ac1-6(M4) but not when lymphocytes are incubated with Ac1-9 or medium alone (Fig. 2D). Thus, one plausible explanation for the ability of Ac1-6(M4) to stimulate the Vβ8.2/Jβ2.7 Ac1-9-specific T cell repertoire in vitro (Figs. 1B and 2B) but not in vivo (Fig. 2C) is that upon priming a naïve B10.PL animal with Ac1-6(M4), the non-Ac1-9-specific Ac1-6(M4)-specific T cells (Fig. 2A and D) outcompete the encephalitogenic Vβ8.2/Jβ2.7 T cells. Although we have suggested competition as the mechanism accounting for these effects, several other potential mechanisms remain possible. For example, the K4M substitution could somehow interfere with the activation of naïve Vβ8.2/Jβ2.7 T cells. To address this possibility, we immunized B10.PL animals with 25 μg of the longer peptide, Ac1-9(M4), capable of strong stimulation of Ac1-9-specific T cells (18). Fig. 2E shows that lengthening Ac1-6

Fig. 1. Characterization of the Ac1-9-specific Vβ8.2/Jβ2.7 clonotype. (A) Mice were immunized with CFA alone or Ac1-9-CFA and draining lymph nodes were removed for analysis. Vβ8.2/Jβ2.7 spectra are shown. Arrows indicate a TCR CDR3 length of 9 aa. Significant expansions within the Vβ8.2/Jβ2.7 spectra were not seen when B10.PL animals were immunized with CFA alone. Likewise, when lymph node cells isolated from Ac1-9-CFA primed animals were incubated with medium alone, no significant expansions were found. In contrast, when lymph node cells from Ac1-9-CFA primed animals were cultured with Ac1-9, an expansion correlating to a CDR3 length of 9 aa was seen. This 9 aa expansion was also seen without the need of an in vitro culturing step when mononuclear cells were obtained from spinal cords isolated from mice suffering from EAE. This figure shows one of several similar experiments. (B) The MBP Ac1-9-specific Vβ8.2/Jβ2.7 T cell hybridoma 172.10 can be stimulated by Ac1-6(M4), Ac1-9, and LDVM1-9(Y4). Stimulatory responses are measured in units of IL-2 production. LDVM1-9(Y4) (△) is superior to Ac1-9 (□) in its ability to stimulate the Ac1-9-specific hybridoma, 172.10. In contrast Ac1-6(M4) (○) is less effective when compared to Ac1-9. This figure shows one of several similar experiments.
class II I-Au molecule (19). Fig. 3 showed strong expansions of the public VβAc1-9, (hybridomas isolated, none were able to cross-recognize Ac1-9. One representative example is shown here. B10.PL animals were then pricked with (B) Ac1-9, (C and D) Ac1-6(M4), or (E) Ac1-9(M4) emulsified in CFA. Ten days later, these mice were killed and draining lymph nodes were removed for immunoscope analysis. (B) Lymphocytes isolated from Ac1-9-primed animals showed strong expansions of the public Vβ8.2Jβ2.7 clonotype when cultured in vitro with either Ac1-9 or Ac1-6(M4). (C) Although Ac1-6(M4) is capable of stimulating the Vβ8.2Jβ2.7 clonotype in vitro, priming with Ac1-6(M4) failed to expand the public Vβ8.2Jβ2.7 Ac1-9-specific clonotype. (D) Priming with Ac1-6(M4) expanded a population of Ac1-6(M4)-specific T cells, which did not cross-recognize Ac1-9. (E) In contrast to Ac1-6(M4), Ac1-9(M4) was effective at expanding the naïve Vβ8.2Jβ2.7 clonotype when used as an immunogen. This represents one of several similar experiments.

The Results Obtained with Ac1-6(M4) Hold True for Another APL of Ac1-9. Because the Vβ8.2Jβ2.7 response to Ac1-9 arises after priming with Ac1-9, Ac1-20, MBP, or whole spinal cord homogenate, where the amino terminus is relatively available, we synthesized LDVM1-9(Y4), which includes the adjacent 5′ Golli (genes of the oligodendrocyte lineage) residues, LDVM. In this peptide, the 4K-to-4Y substitution is required for induction of 104 cells) incubated with medium or MBP:Ac1-9 in the presence of splenic APCs resulted in no IL-2 production (as measured in this HT-2 cell assay). The ability to expand non-cross-reactive, nonpathogenic T cells correlates well with an antigen’s inability to expand a pathogenic population. Other factors such as antigen dose and the MHC binding affinity of the mimic agonist may also play a role in the ability/ inability to expand self-reactive pathogenic T cells.

LDVM1-9(Y4) is effective at expanding the Vβ8.2Jβ2.7 clonotype from Ac1-9-primed animals but not when used as an immunogen. (A) B10.PL mice were primed with Ac1-9 and in vitro recall responses to medium, Ac1-9, and LDVM1-9(Y4) were analyzed. Cultures incubated with Ac1-9 or LDVM1-9(Y4) showed significant expansion of the characteristic Vβ8.2Jβ2.7 clonotype. (B) Mice were then primed with LDVM1-9(Y4). After a preliminary survey of all Vβ-Jβ combinations, a “public” Vβ7Jβ2.5 LDVM1-9(Y4)-specific expansion was detected. This expansion was seen in all B10.PL animals primed with LDVM1-9(Y4), one of six replicates shown here. (C) B10.PL animals were then immunized with Ac1-9 and the Vβ7Jβ2.5 spectrum was inspected. No significant expansions were seen. (D) An LDVM1-9(Y4)-specific hybridoma (1 x 10⁶ cells) incubated with medium or MBP:Ac1-9 in the presence of splenic APCs resulted in no IL-2 production (as measured in this HT-2 cell assay). The hybridoma was, however, able to strongly respond to LDVM1-9(Y4). This clone is an example of an LDVM1-9(Y4)-specific, non-Ac1-9-specific T cell clone. (E) Immunization with LDVM1-9(Y4) failed to strongly expand the public Vβ8.2Jβ2.7 clonotype. Six individual mice are shown. Very small expansions were seen in two animals.

Fig. 2. Ac1-9-specific or APL-specific T cell expansions following immunization with Ac1-9 vs. Ac1-6(M4). (A) B10.PL mice were primed with 25 μg of Ac1-6(M4). Draining lymph nodes were removed 10 days later and cells were cultured with Ac1-6(M4) for 3 days and then fused with the 8.2Jβ2.7 hybridoma, 172.10 (Fig. 1B). B10.PL mice were primed with 25 μg of Ac1-9 and draining lymph nodes were removed 10 days later and cells were cultured with Ac1-9 for 3 days and then fused with the 8.2Jβ2.7 hybridoma, 172.10 (Fig. 1B). T cell repertoire analysis revealed a strong expansion within the Vβ8.2Jβ2.7 clonotype. (B) Ac1-9(M4) was an inferior in vitro activator of the characteristic Vβ8.2Jβ2.7 response from Ac1-9-primed animals. Similarly, it was an excellent stimulator of the Vβ8.2Jβ2.7 hybridoma, 172.10 (Fig. 1B). Characterize the LDVM1-9(Y4)-specific T cell repertoire, B10.PL animals were immunized with LDVM1-9(Y4) and draining lymph nodes were harvested on day 10. T cell repertoire analysis revealed a strong “public” LDVM1-9(Y4)-specific expansion within the Vβ7Jβ2.5 spectrum that was non-cross-reactive with Ac1-9 (Fig. 3B). As additional evidence that this Vβ7Jβ2.5 expansion was non-cross-reactive with Ac1-9, B10.PL mice were immunized with Ac1-9 and draining lymph nodes were analyzed for expansions within the Vβ7Jβ2.5 spectrum. The Gaussian distributions seen in Fig. 3C are evidence in support of the non-cross-reactive nature of the Vβ7Jβ2.5 LDVM1-9(Y4)-specific response. Fig. 3D shows the response of an LDVM1-9(Y4)-specific T cell hybridoma that is unable to recognize Ac1-9. We then asked whether the ability to expand a population of non-cross-reactive T cells correlated with an inability to expand the Ac1-9-specific Vβ8.2Jβ2.7 DAGGYY clonotype in vivo. Fig. 3E shows that, when used as the initial immunogen, LDVM1-9(Y4) was only able to minimally expand the public encephalitogenic repertoire in two out of six animals. In summary, there is no direct relationship between the in vitro stimulatory potency of a peptide agonist and its ability to expand a self-reactive clone from its naïve state within the animal. In addition, the ability to expand non-cross-reactive, nonpathogenic T cells correlates well with an antigen’s inability to expand a pathogenic population. Other factors such as antigen dose and the MHC binding affinity of the mimic agonist may also play a role in the ability/ inability to expand self-reactive pathogenic T cells.
The Ability of a Peptide to Induce EAE Correlates Well with Its Ability to Expand the Encephalitogenic Clonotype. Because Ac1-6(M4) was capable of expanding a population of Ac1-9-specific T cells in vitro (Fig. 2) but was incapable of expanding the naïve Vβ8.2Jβ2.7 clonotype in vivo, it was of interest to determine the encephalitogenic potential of this peptide. Table 1 shows that Ac1-6(M4) was incapable of inducing EAE in naïve wild-type animals (0/6). Similarly, when EAE induction was attempted with the longer 13-mer peptide, LDVM1-9(Y4), only a fraction (4/10) of the animals developed EAE and disease severity was limited to minimal tail paralysis (EAE scores = 1). On the other hand, correlating well with their ability to expand the naïve encephalitogenic Vβ8.2Jβ2.7 repertoire (Figs. 1D and 2E), both Ac1-9 and Ac1-9(M4) were able to induce severe EAE (Table 1). Lastly, like Ac1-9(M4), we have previously shown that 25–75 μg of Ac1-9(Y4) is also an effective inducer of EAE (19). Thus, the ability of an APL to expand autoreactive T cells in vitro does not correlate with its ability to induce autoimmunity in vivo. This experiment also highlights the role of the driver response in autoimmunity.

An alternative explanation of these findings might simply be that the APL, Vβ8.2Jβ2.7, and LDVM1-9(Y4) are both naïve and the APL is more effective at expanding a population of Ac1-9(Y4) in vivo. To investigate this possibility, draining lymph node cells from both Ac1-9-immunized and LDVM1-9(Y4)-immunized B10.PL animals were analyzed for IFN-γ production. Fig. 4 reveals that LDVM1-9(Y4) was excellent at generating an IFN-γ cytokine response regardless of the priming antigen.

T Cell Adoptive Transfer Experiments Confirm the High Sensitivity of the Driver Response to T Cell Competition. To seek conclusive evidence for T cell competition as a mechanism preventing the expansion of naïve pathogenic T cells, T cell transfer experiments were performed in which naïve Ac1-9-specific T cells were transferred into wild-type B10.PL recipients before priming with Ac1-9. These naïve T cells were isolated from the Ac1-9-immunized and LDVM1-9(Y4)-immunized B10.PL mice as described in Materials and Methods. On day 8, the draining lymph node cells were cultured with Ac1-9. As expected, the naïve Ac1-9-specific repertoire developed EAE and disease severity was limited to minimal tail paralysis (EAE scores = 1). On the other hand, correlating well with their ability to expand autoreactive T cells in vitro does not correlate with its ability to induce autoimmunity in vivo. This experiment also highlights the role of the driver response in autoimmunity.

Table 1. Ac1-6(M4) cannot induce EAE

<table>
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<th>Exp. no.</th>
<th>Antigen</th>
<th>Dose, μg</th>
<th>Sequence</th>
<th>Incidence, %</th>
<th>Mean day of onset</th>
<th>Mean severity</th>
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<tbody>
<tr>
<td>1</td>
<td>Ac1-11</td>
<td>50</td>
<td>Ac-ASQKRPSQRK</td>
<td>3/3 (100)</td>
<td>13.0</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>(1-9) Y4</td>
<td>100</td>
<td>ASQYRPSQR</td>
<td>4/4 (100)</td>
<td>14.0</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>LDVM1-9(Y4)</td>
<td>100</td>
<td>LDVMASQYRPSQR</td>
<td>2/4 (50)</td>
<td>13.0</td>
<td>1.0</td>
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<tr>
<td></td>
<td>Ac1-9</td>
<td>75</td>
<td>Ac-ASQKRPSQR</td>
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<tr>
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<td>6/6 (100)</td>
<td>13.0</td>
<td>2.5</td>
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<tr>
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<td>12.0</td>
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The clinical severity of EAE was scored daily as follows: 1, loss of tail tonus; 2, hind limb weakness or forelimb involvement alone; 3, total hind limb paralysis; 4, hind and forelimb paralysis; and 5, moribund/death. Mean severity was calculated using scores from only those mice exhibiting clinical signs of EAE. Bold indicates altered amino acid from the native sequence.
absence of competing T cells, the naïve Vβ8.2Jβ2.7 clonotype expanded robustly to priming with LDVM1-9(Y4) in vivo (Fig. 5G). This experiment provides conclusive evidence that T cell competition can prevent expansion of autoreactive T cells in vivo.

**Discussion**

The theory of molecular mimicry is based upon the idea that T cells specific to a foreign antigen can induce autoimmunity by cross-recognition of a self-determinant. However, there are clear constraints on the relationships that have to exist among the foreign and self-determinants if a state of autoimmunity is to be induced via this pathway. Most importantly, the foreign mimic-determinant must be readily processed from the foreign antigen to ensure that it can be presented to autoreactive T cells. Because only a fraction of the potential determinants are ever effectively processed and presented, a large proportion of mimicking peptides will remain poorly displayed, unable to engage a potentially autoreactive repertoire. Most studies characterizing molecular mimicry have not identified naturally processed, dominant molecular mimics. Instead, these studies have focused on identifying any microbial peptide capable of stimulating autoreactive T cells.

In addition, when a self-antigen is used to prime an animal, T cells with unique TCRs can potentially expand. Each of these autoreactive T cells will fall along a spectrum of inherent pathogenic potential. This is evidenced by the fact that different self-directed TCR transgenic animals, with specificities for the same determinant, will display a characteristic incidence of autoimmunity (10, 11, 20, 21). Although such animals may also differ in their regulatory T cell repertoires, it is likely that the difference in their TCR affinity is also responsible for the varied disease incidences. In addition, different autoreactive T cell clones with specificities for the same self-determinant will often induce unique disease courses when passively transferred into naïve recipients (22). In fact, several autoreactive clones induce disease only when transferred in high numbers. It is likely that among the diverse sets of T cells potentially expandable to a self-antigen, only a small proportion are able to induce and drive states of autoimmunity, a group we have called driver clones (7, 8, 19). Thus, even if a mimic can activate self-directed T cells, it may not stimulate the pathogenic subset; individual T cells may respond to unique sets of molecular mimics. Lastly, as shown here, being able to stimulate the driver subset in vitro, is still not sufficient to induce autoimmunity. The mimic also needs to be able to activate and expand this subset from its naïve state, which exists among a group of highly competitive memory and naïve T cells (23–25). Studies linking molecular mimicry to autoimmunity have relied heavily upon deductive reasoning; in most cases, ligands have been characterized that are capable of stimulating already primed autoreactive T cells in vitro. The reasoning behind such studies is as follows: if molecular mimic X can stimulate autoreactive T cells specific to self-determinant Y, then a microbially induced immune response to mimic X should result in autoimmunity directed to self-determinant Y. There are several caveats to such reasoning. For example, due to a wide array of TCR specificities, it is likely that many (possibly most) T cells raised to mimic X will be specific to X and non-cross-reactive to self-determinant Y. Thus, immunizing with mimic X will expand a large population of nonautoreactive clones. Owing to competitive forces among T cells for activation, this potentially large population of nonautoreactive T cells may interfere with the activation of the self-reactive pathogenic driver T cell repertoire; in fact, we have just described such a case. Ligands capable of stimulating primed encephalitogenic T cells in vitro can be ineffective at expanding these same T cells as they reside in a naïve state within the animal. We find a strong correlation between an APL’s ability to expand APL-specific, non-self-directed T cells with its inability to expand the encephalitogenic self-directed repertoire in vivo. In
our study, we used APLs that differed only slightly from the native sequence, Ac1-9. We predict that differences between a microbial molecular mimic and the corresponding self-determinant will allow for a mimic-specific non-cross-reactive repertoire to be expanded in the setting of infection. Our experiments argue that this mimic-specific, non-self-directed repertoire, through competitive means, will focus the immune response on the pathogen, thereby precluding the induction of autoimmunity. These relationships help to explain why, given such a largely degenerate T cell repertoire, that there are relatively few cases of molecular mimicry. They also have direct implications on the design of APLs for the treatment of cancer and autoimmunity and for the use of APLs in vaccines.

Materials and Methods

Mice. B10.PL/J mice were purchased from The Jackson Laboratory. Clone 19 and clone 172.10 Ac1-9-specific Tg B10.PL animals were obtained from Juan Lafaille and Joan Goverman, respectively (10, 20). Experiments were approved by the UIAI Animal Care and Use Committee.

Hybridomas. The Ac1-9 specific hybridoma, 172.10, has been maintained in this laboratory and was generated originally at California Institute of Technology. Other T cell hybridomas were created as described in the text.

Peptides. Peptides were purchased from Macromolecular Resources. Purity was >95% as determined by mass spectrometry and capillary electrophoresis. The following peptides were used in this study: Ac1-9 (Ac-ASQKRPSQR), Ac1-9(4Y) (Ac-ASQYPQRPSQR), Ac1-9(M4) (Ac-ASQMPSQRPSQR), Ac1-9(M4) (Ac-ASQMMPRP), and LDVM1-9(Y4) (LDVMASQYPQRPSQR). LDVM1-9(Y4) was also synthesized at University of California, Davis.

Induction of EAE. For induction of EAE, mice were immunized s.c. with 100 μL of a CFA emulsion containing 200 μg of Mycobacterium tuberculosis H37RA (Difco) and the indicated amount and type of antigen.s.c. One and three days later, mice were injected i.p. with 150 ng of purified PTX (List Biological) as described (8).

T Cell Repertoire Analysis (ImmunoScope). Repertoire analyses were performed using a modified protocol similar to that described by Pannetier et al. Total RNA was isolated from cell suspensions of individual mice using the RNeasy kit (Qiagen). CDNA syntheses were then performed using an oligo-dT primer according to the manufacturer’s instructions (Life Technologies). From each cDNA, PCR reactions were then performed using a Vβ8.2 primer (cattatcatatgtgtcctgg) and a common Cβ primer (cactggtctggtttgac). Run-off reactions were performed with a single internal fluorescent primer for each Vβ tested. These products were then denatured in formamide and analyzed on an ABI PRISM 310 genetic analyzer using GeneScan 2.0 software (Perkin-Elmer). The relative intensity of signal (RIS) values were calculated as the area under the experimental peak divided by the area under the control peak found within a Gaussian distribution. Peaks were normalized before division. RIS values >4 are considered significant.

Adaptive Transfer of Naive TCR Transgenic T Cells. Spleen cells were isolated from Ac1-9-specific Vβ2Jij2.4 TCR transgenic mice. CD4 T cells were then purified using MACS beads (Miltenyi Biotec). T cells were then diluted in PBS, warmed to room temperature, and injected i.v. into 6-week-old wild-type B10.PL animals. Twenty-four hours later, animals were injected s.c. with 75 μg of Ac1-9 emulsified in CFA. Ten days later, draining lymph node cells were analyzed by TCR repertoire analysis.

Flow Cytometry. Splenocytes were isolated from transgenic mice. Cells were incubated with Fc-block (BD Biosciences) before staining. Briefly, cells were incubated at 12 °C with biotinylated, recombinant MBP-1(9-41)=A- tetramers that were generated as described in Radu et al. (27). Following a wash, tetramer-stained cells were incubated with PerCP labeled-anti-CD4 (BD Biosciences) on ice and then washed and analyzed; cells were incubated with PE-labeled H57-597 (BD Biosciences) on ice and then washed and analyzed.

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