




TCR Affinity for Self-Ligands Influences the Development and Function of Encephalitogenic T Cells

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Abstract

The specificity and affinity of self-reactive T cells is likely to impact the development of autoimmune-disease causing T cells in the thymus as well as their function in the periphery. We identified a naturally occurring, low affinity variant of an MBP Ac1-11/I-A^b specific TCR that is known to induce EAE. Thymocytes in mice carrying the transgenes for this low affinity TCR were poorly positively selected, as compared to their high affinity TCR expressing counterparts. Nonetheless, CD4 T cells bearing the low affinity TCR accumulated in the periphery of the mice. Unlike mice expressing the high affinity TCR, these mice very rarely developed disease. However, if endogenous TCR expression was eliminated by breeding to RAG1 deficient mice, 100% of the mice carrying either the high or the low affinity versions of the TCR developed EAE. Intriguingly, while the incidence of EAE increased, the age of onset of disease in both mice was identical. These data suggest disease onset occurs during a short window of mouse development.

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
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Introduction

Developing T cells require a functional interaction between the TCR and self-MHC: self-peptide complexes [1,2]. In general, it is thought that low affinity interactions lead to maturation, whereas high affinity interactions result in clonal deletion. These complementary selection processes lead to the development of a self-referential T cell repertoire that is both tolerant to self, yet sufficiently diverse to respond to pathogenic challenges [3,4,5,6,7]. Data suggests that in addition to defining positive versus negative selection, TCR affinity for selecting ligands may also play an instructive role in defining lineage commitment. For example, high affinity interactions have been suggested to direct developing thymocytes towards functionally distinct lineages such Treg, iNKT, CD8 $\alpha\alpha$ and Ly6C⁺ T cells [1,8,9]. Additionally, commitment to the conventional CD4 and CD8 lineages may also be influenced by TCR affinity [10].

EAE is an inflammatory, demyelinating disease manifested by acute, chronic or relapsing paralysis [11], similar to human multiple sclerosis [12]. The disease is mediated primarily by MHC class II-restricted CD4⁺ T cells that secrete inflammatory cytokines [11]. Stimulation of anti-inflammatory Th2 T cells that secrete IL-4 and IL-10, however, has been shown to inhibit EAE [13]. To determine if TCR affinity influences the onset of EAE, we identified and cloned a TCR that has considerably lower affinity for the Ac1-11 MBP peptide presented by I-A^b as compared to a

previously published TCR [14]. Transgenic mice expressing this new low affinity TCR were generated and compared to an existing high affinity TCR transgenic mouse.

On a wild type B10.PL background, the incidence of spontaneous disease initiated by T cells expressing the low affinity TCR was very low, as compared to the high affinity TCR transgenic mouse. When bred to a RAG1 deficient background, which is known to increase the frequency of EAE, at least in part due to the loss of residual CD4⁺CD25⁺ Tregs [15,16,17], both the high and low affinity TCRs induced the onset of EAE in 100% of mice. Intriguingly, the age of onset of disease in mice with and without Tregs was nearly identical.

Materials and Methods

Animals

All animal work was done in compliance with Memorial Sloan-Kettering Cancer Center's Internal Animal Care and Use Committee (IACUC) and the guidelines of the Federal Office of Laboratory Animal Welfare. The approved protocol number was 05-03-005. Mice were housed and bred in specific pathogen-free environments in microisolators in the Research Animal Resources Center (RARC) of Memorial Sloan-Kettering Cancer Center (MSKCC). Additional mice were purchased from The Jackson Laboratory. Animal housing rooms were under temperature and humidity control, the mice were not subjected to water

or food restrictions and bedding material was placed in each cage. Four full time veterinarians and six veterinarian technicians staff the facility. The veterinary staff is located on site and a clinical veterinarian is available at all times. Animal care staff carried out routine husbandry procedures including changing cages, feeding and watering. All mice were sacrificed prior to use. Euthanasia was conducted in accordance with the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. Briefly, mice were sacrificed by asphyxiation with CO₂. CO₂ is delivered into the cages at less than 5psi per second. Death of the animal is confirmed by lack of respiration and toe pinch. CO₂ euthanasia stations are inspected regularly by IACUC personnel. After confirmation of death, tissues were removed for experiments. In studies involving the incidence of spontaneous EAE, mice were examined at least three times per week for evidence of disease. Histological analysis confirmed pathological lesions associated with EAE in hindlimb-paralyzed mice (data not shown). Of note were mononuclear cell accumulations in the perivascular, meningeal and parenchymal regions of the brain.

Hybridomas

TCR V α 2 depleted or total T cells (see results section) were activated and fused to BW5147 cells as previously described [18]. Fused cells were plated at a density likely to generate clonal populations. In addition, hybridomas used for assays were cloned by limiting dilution and analyzed by FACS for TCR and CD4 expression. Stimulation assays were carried out as previously described [18].

TCR α transgene

cDNA made from the hybridomas was screened with a panel of 24 V α specific primers that we generated based on published sequences [19]. The primers were tested by amplifying cDNA generated from mRNA from wild type thymocytes RNA. PCR products were cloned (TOPO TA, Invitrogen) and sequenced. Based on this information, PCR primers were designed to amplify genomic DNA from one of the V α 6 hybridomas from 5' of the ATG to 150 bp 3' of the end of the J α segment (within the J α -C α intron). This DNA fragment was cloned, sequenced and then transferred into the p α Tris vector [20]. The purified transgene was injected into fertilized F1 eggs obtained by mating B10.PL and C57Bl/6 mice. Transgene positive founders were backcrossed to B10.PL and typed to confirm homozygous expression of I-A^u. Two different founder lines were established. Data from the two founder mouse lines (FACS, onset of EAE, etc.) was nearly identical and, therefore, only one founder was fully analyzed.

T Cell Proliferation Assays

Lymph nodes and spleens from healthy 6-8 week-old MBP $\alpha\beta$ TCR and OV $\alpha\beta$ TCR transgenic mice were harvested. Magnetic depletion (BioMag beads; PerSeptive Biosystems, Framingham, MA) using antibodies against MHC class II, CD8, Mac1 and NK1.1 was used to enrich for CD4⁺ T cells. Cell purity (typically >90%) was checked by FACS with antibodies against CD4 and TCR V β 8. APCs were prepared by incubation of single-cell suspensions of B10.PL splenocytes with antibodies against CD4, CD8, and Thy 1 followed by incubation with rabbit complement and mitomycin C for 30 min at 37°C. Experiments were set up in triplicate in 96-well, round-bottom plates with 2.5 \times 10⁴ T cells (total cell number adjusted based upon purity) and 1 \times 10⁵ APCs. Assays were pulsed with 0.5 μ Ci per well of [³H]thymidine after 48 hours and were harvested after an additional 24 hours.

Blocking assays were carried out as previously published [21]. No T cell proliferation is induced by the F23.1 antibody at the doses that were used.

Tissue preparation and FACS Analysis

Single-cell suspensions were prepared by dissociation of tissues between glass slides. To block nonspecific antibody binding, cells were first incubated for 15 minutes with anti-mouse FC-RIII antibody and normal mouse serum (Jackson Labs). Cells were washed and then stained for 30 minutes with the appropriate cocktail of the following antibodies from BD Biosciences: CD4-PerCP-Cy5.5, CD8-APC, V β 8 FITC, V α 2-PE, CD69-PerCP-Cy5.5, CD44-FITC, CD25-PE, CD62L-APC and TCR α -APC. Cells were washed and analyzed on a Becton Dickinson LSR or DakoCytomation Cyan.

Cell sorting

Cell sorts were done in MSKCC's Flow Cytometry Core Facility on a DakoCytomation MoFlo. Sorted populations were reanalyzed and proved to be over 98% pure.

Results

Identification of a low affinity TCR

Mice carrying the transgenes for a MBP Ac1-11 peptide specific TCR (V α 2.3-J α 11; V β 8.2-J β 2.6) cloned from the 172.10 hybridoma have been described [14]. T cells from these TCR transgenic mice respond strongly to the Ac1-11 myelin basic protein (Ac1-11) peptide bound to I-A^u and these mice spontaneously develop EAE (1, 9). Previously, we have shown that mice carrying only the TCR β transgene (MBP β TCR) are highly enriched (approximately 0.01% of total CD4 SP thymocytes) for T cells specific for the Ac1-11:I-A^u complex [22]. As a result, in vitro culture of total lymph node cells taken from MBP β TCR transgenic mice with the Ac1-11 peptide results in a strong, dose-dependent T cell response. Other than the somewhat restricted TCR repertoire, MBP β TCR transgenic mice are indistinguishable from wild type mice since they have normal numbers of both CD4 and CD8 T cells that express a diverse TCR repertoire [22].

In an effort to identify T cells with a low affinity for the MBP Ac1-11:I-A^u ligand, we harvested lymph node cells from MBP β TCR transgenic mice and bulk cultured the cells with 10 μ M of the peptide. After seven days, cells were analyzed by FACS using mAbs against CD4, V β 8 and V α 2. As expected, nearly all of the surviving cells were CD4⁺ (Fig. 1A) and expressed that the transgene encoded V β 8⁺ TCR β chain (Fig. 1A). Interestingly, in six independent experiments, we consistently observed two different T cell populations when we stained with an anti-V α 2 mAb. Approximately 70% of the T cells were always found to be V α 2⁺, while approximately 30% of the cells were V α 2 negative (Fig. 1A). This ~2:1 ratio was found with several different doses of peptide and was stable if the T cells were activated several more times (data not shown).

Our finding that the non-V α 2 T cells were consistently a smaller percentage of the total cultured T cell population suggested that these cells might be expressing TCRs that were lower affinity as compared to the V α 2⁺ cells. To test this, we sorted the two populations from bulk cultured T cells that had gone through several rounds of activation in vitro. The sorted T cells were stimulated with titrated amounts of the Ac1-11 peptide presented by T cell depleted, irradiated B10.PL spleen cells. As shown, in Fig. 1B, the V α 2⁺ cells responded substantially better than their V α 2⁻ counterparts. While the magnitude of the T cell response

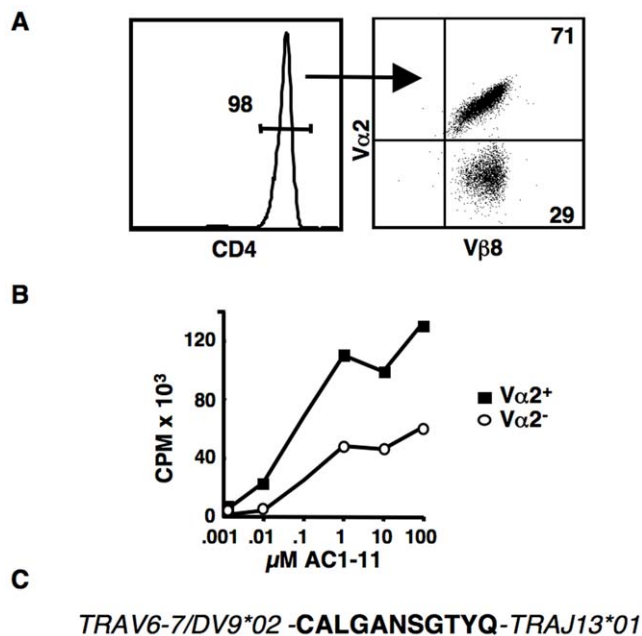


Figure 1. Analysis of lymphocytes from MBP β transgenic mice cultured with the AC1-11 peptide. Total lymphocytes from MBP β -only transgenic mice were bulk cultured with the MBP-derived AC1-11 peptide. Several additional rounds of activation were done by culturing with T cell depleted B10.PL splenocytes loaded with the AC1-11 peptide. FACS was carried out seven days after the final activation. (A) Nearly all remaining cells were CD4⁺ and expressed the transgene-encoded V β 8 TCR β chain. Across several experiments, approximately 30% of the T cells were not V α 2 positive. (B) The V α 2⁺ and V α 2⁻ T cells were sorted and then stimulated by the addition of irradiated, T cell depleted B10.PL splenocytes and titrated amounts of the AC1-11 peptide. Cells were pulsed with 3[H]thymidine after 48 hours and harvested after a total of 72 hours. Approximately 5 \times 10⁴ T cells and 1 \times 10⁵ APCs were used per well. Assays were carried out in triplicate and averaged. Results shown are representative of three independent experiments. (C) Predicted amino acid sequence of the CDR3 α region of the V α 6 (TRAV6-7/DV9*02) - J α 13 (TRAJ13*01) TCR cloned from the V α 2⁻ population. Gene nomenclature based on the IMTG Marie-Paule convention [46].

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varied in repeats of this experiment due to variability in the total cell numbers used, the difference in the response between the two T cell populations was consistent. Importantly, the anti-V α 2 mAb itself does not induce or block activation of T cells and, therefore, is not a factor in these T cell assays [23].

In an effort to clone the TCR α chain or chains used by the V α 2⁺ and V α 2⁻ populations, we generated T cell hybridomas [18]. For our first fusion, we used T cells that had been bulk cultured by feeding several times with irradiated B10.PL splenocytes loaded with 10 μ M Ac1-10 peptide. Prior to the final activation, the bulk culture was split and magnetic beads were used to deplete out the V α 2⁺ cells from one of the two cultures. Two days after activation, the T cells were fused to BW5147 and plated at a density likely to produce single cell clones [18]. The resulting hybridomas were screened by FACS with mAbs against the TCR β chain, V α 2 and CD4. Specificity of the clones was confirmed by carrying out T cells assays with Ac1-11 peptide loaded APCs. Activated T hybridomas produced IL-2, which was detected by the IL-2 dependent cell line, CTLL-2 (data not shown). Dose-response assays confirmed that, similar to the sorted T cells, the V α 2⁺ hybridomas responded much better to the Ac1-11 peptide (data not shown).

Twenty V α 2⁺, CD4⁺ clones and twenty V α 2⁻, CD4⁻ clones were selected and used for further studies. cDNA from the V α 2⁺ clones was PCR amplified with primers specific for V α 2 and the C α gene segments. The amino acid sequences deduced from the sequences of the PCR products were identical both to each other and to the original Ac1-11/I-A^u specific 172.10 hybridoma. This finding is consistent with our previous work in which we found that positive selection in the thymus resulted in a repertoire that was enriched for TCRs using the TCR α chain found in the original 172.10 T cell hybridoma [22].

The V α 2⁻ hybridomas were also negative for the three other available anti-V α gene segment mAbs. Therefore, we screened the clones by RT-PCR utilizing a panel of 24 different V α specific primers that we designed to amplify all known mouse V α gene segments. Other than the endogenous TCR α chain known to be expressed by the BW5147 cell line, only one primer set consistently resulted in a PCR product. We cloned and sequenced the PCR fragment and found that all twenty hybridomas expressed the identical TRAV6-7/DV9*02 - TRAJ13*01 (V α 6-J α 13) TCR α chain (Fig. 1C). Interestingly, other than a single amino acid change in the CDR3 α segment, the TCR α chain we cloned is identical to a TCR α chain cloned from an AC1-9/I-A^u specific T cell derived from a wild type B10.PL mouse [24]. This closely related TCR, however responds strongly to the AC1-9/peptide:MHC complex [24].

These data suggested that T cells from MBP β TCR transgenic mice bearing one of only two different TCRs could respond to the Ac1-11/I-A^u complex. We were concerned, however, that other T cells expressing different TCRs might have been lost during the extended culturing of these cells. Therefore, we generated a new set of hybridomas following a single stimulation of total lymph nodes cells harvested from the MBP β TCR mice. The hybridomas were screened with our panel of 24 V α -specific primers, as described above. cDNA was generated from approximately 20 V α 2⁺ and V α 2⁻ clones, PCR amplified and sequenced. Once again, we were only able to identify the same two TCRs. Therefore, under these conditions, only T cells expressing one of the two different TCRs responded to the Ac1-11 peptide presented by I-A^u APCs. Importantly, the V α 6-J α 13 TCR, which we named the OV α β TCR, responded less well to this antigen (Fig. 1B and data not shown) and, therefore, can be considered a functionally lower affinity TCR.

Inefficient positive selection of thymocytes expressing the low affinity OV α β TCR

To analyze the in vivo function of T cells expressing the low affinity OV α β TCR, we generated TCR transgenic mice. The original MBP Ac1-11 specific transgenic mice (MBP α β) were generated as single-chain transgenics (TCR α and TCR β were not coinjected). Therefore, it was only necessary for us to construct the OV α TCR transgene. A 650 bp fragment containing the V α 6-J α 13 gene segment was generated by PCR of DNA from one of the hybridomas described above. The PCR product was cloned, sequenced and then transferred into the \sim 20 kb pT α cassette vector [20]. This vector contains a TCR V α promoter and the complete TCR C α gene, including the C α enhancer and LCR. Overall, this transgene vector is very similar to the cosmid vector used for generating the MBP α transgene [14] and, therefore, it is expected that the two transgenic constructs should behave similarly in vivo. The purified transgene was microinjected into F1 blastocysts obtained from B10.PL \times C57Bl/6 female mice.

Two founder transgenic mice were identified and backcrossed to B10.PL mice that carried the transgene for the MBP β TCR chain. The phenotype of the two founder lines was nearly identical

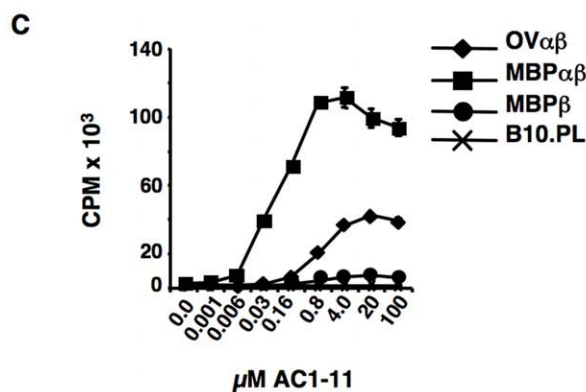
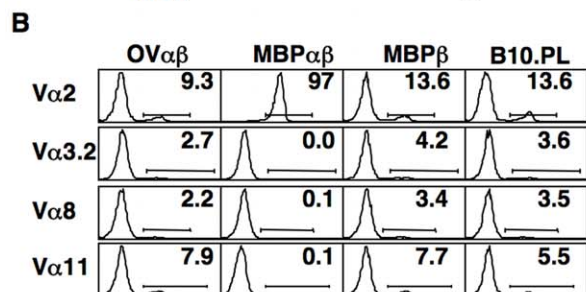
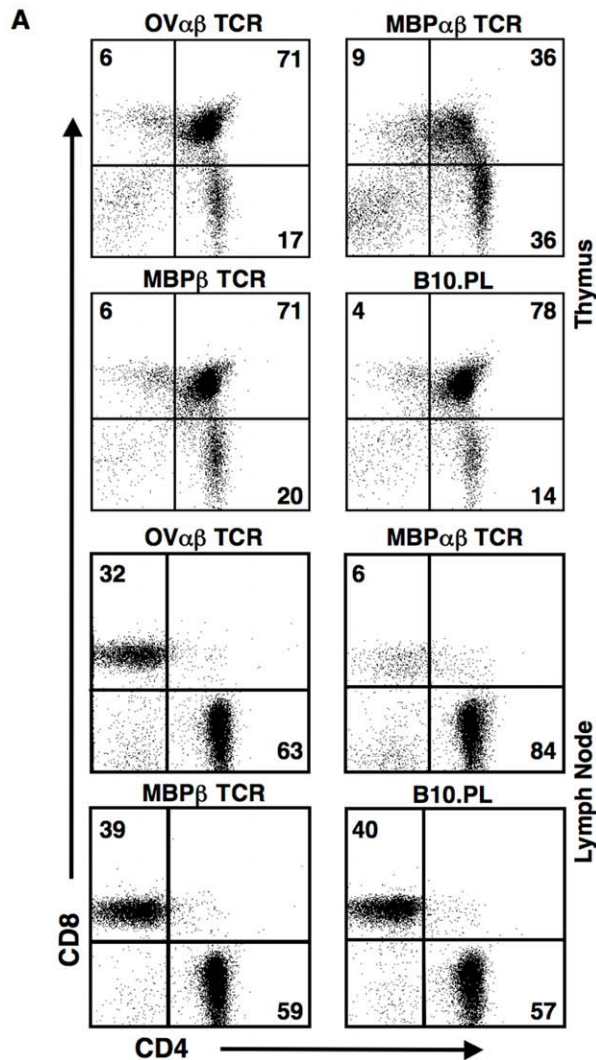


Figure 2. Development of OV $\alpha\beta$ TCR expressing thymocytes.

The strong skewing of thymocytes expressing the high affinity MBP $\alpha\beta$ TCR towards the CD4 lineage is not seen in OV $\alpha\beta$ TCR expressing mice. Expression of only the MBP β chain does not markedly alter T cell development. (A) Single cell suspensions of thymocytes (top) and lymph node cells (bottom) from OV $\alpha\beta$, MBP $\alpha\beta$, MBP β transgenic and transgene negative mice were stained with antibodies against CD4, CD8 and TCR C β . Only TCR C β ⁺ cells are shown in the lymph node cell plots. The number indicates the percent of cells in the quadrant. Only subtle variations in the percent of cells within each quadrant were noted during repeat experiments. (B) CD4 SP thymocytes from OV $\alpha\beta$ TCR transgenic mice express a high percentage of endogenous TCR α chains, whereas MBP $\alpha\beta$ TCR transgenic mice predominantly express the transgene encoded V α 2 chain. Single cell suspensions of thymocytes from the indicated mouse strain were stained with antibodies against CD4, CD8 and individually with each of the four available TCR V α specific antibodies. The percent of cells within the CD4 single positive population expressing each of the V α s is shown. More than 1×10^6 events were collected per sample. One of two experiments is shown. Greater than 98% of T cells in the OV $\alpha\beta$, MBP $\alpha\beta$ and MBP β mice expressed the transgene encoded V β 8.2 TCR. (C) Increased levels of proliferation in response to the Ac1-11 peptide indicate a substantial number of antigen specific T cells develop in the OV $\alpha\beta$ mice. CD4 T cells were enriched by negative selection. Double cultures were set up with 2.5×10^4 CD4⁺ T cells and 1×10^5 T cell depleted, irradiated B10.PL splenocytes plus the indicated amount of the MBP Ac1-11 peptide. Assays were pulsed with ³[H]thymidine after 48 hours and harvested after an additional 24 hours. Each condition was set up in triplicate and data was averaged. One example of three experiments is shown. doi:10.1371/journal.pone.0017702.g002

and, therefore, data from only one line is presented. Mice carrying the transgenes for both the OV α TCR chain and the MBP β TCR chain as well as homozygous for the H-2u MHC haplotype were selected for analysis. Negative littermates, MBP β TCR only littermates and age matched MBP $\alpha\beta$ TCR transgenic mice were used for controls. In addition to the direct injection into B10.PL x C57Bl/6 F1 blastocysts, mice were backcrossed to B10.PL for at least five generations.

The increased percentage (consistently over 30%) of CD4 single positive (CD4SP) thymocytes expressing the MBP $\alpha\beta$ TCR as compared to nontransgenic littermates or littermates carrying only the MBP β TCR chain indicated strong positive selection of this TCR (Fig. 2A). In sharp contrast, thymocytes from mice expressing the OV $\alpha\beta$ TCR had little obvious skewing towards the CD4 lineage (Fig. 2A). Indeed, the percentage of cells in the various CD4 versus CD8 quadrants identified by FACS was very similar to what was seen for MBP β TCR only and wildtype mice (Fig. 2A). Overall cellularity of the thymuses from the OV $\alpha\beta$ TCR and MBP $\alpha\beta$ TCR mice was similar, although, as with all TCR $\alpha\beta$ transgenic mice, the cellularity was reduced as compared to TCR β only and wild type mice. Finally, in the periphery, in sharp contrast with the $\sim 15:1$ CD4:CD8 ratio of MBP T cells (Fig. 2), the CD4:CD8 ratio of T cells from the OV $\alpha\beta$ TCR mice was only $\sim 2:1$, similar to the MBP β only TCR transgenic mice.

Most likely the failure of the MHC class II restricted OV $\alpha\beta$ TCR to increase overall percentages of CD4SP thymocytes and CD4⁺ T cells was due to poor positive selection. Inefficient positive selection has been shown to result in the replacement of transgenic TCR α chains with α chains from the endogenous locus [25,26]. It has also been shown that a rearranged V α J α gene segment that was introduced into the genomic J α region was removed by secondary TCR α rearrangements when the selecting MHC allele was not present [27,28]. We were not able to directly measure the loss of cell surface expression of the transgene encoded TCR α chain since a mAb specific for this V α gene segment does not exist and a published I-A^u:Ac1-11 tetramer was no longer available. Therefore, to estimate the loss of the transgene-encoded TCR α ,

we stained thymocytes from OV $\alpha\beta$ TCR, MBP $\alpha\beta$ TCR, MBP β TCR and wild type B10.PL mice with anti-TCR V α 2, V α 3.2, V α 8 and V α 11 mAbs (Fig. 2B). In the MBP $\alpha\beta$ TCR mice, over 97% of the CD4SP thymocytes expressed the transgene encoded V α 2 TCR (Fig. 2B), while the other V α s were hardly detectable. In contrast, CD4SP thymocytes from the OV $\alpha\beta$ TCR mice (Fig. 2B) expressed high levels of all four TCR α chains in CD4 single positive thymocytes (9.3% V α 2, 2.7% V α 3.2, 2.2% V α 8 and 7.9% V α 11) (Fig. 2B). Indeed, the percentage of CD4SP thymocytes expressing each endogenous V α was similar to what was seen in MBP β TCR only and wild type B10.PL mice (Fig. 2B). These data strongly suggest that thymocytes carrying the transgene expressing the low-affinity OV $\alpha\beta$ TCR commonly revised their TCR and, as a consequence, expressed endogenously rearranged TCR α chains.

To directly test for the presence of AC1-11 specific T cells in the OV $\alpha\beta$ TCR mice, we purified CD4 T cells from the spleen and lymph nodes of OV $\alpha\beta$ TCR, MBP $\alpha\beta$ TCR, MBP β TCR only transgenic mice as well as from control transgene negative mice. T cell proliferation assays were carried out by incubating CD4⁺ T cells with irradiated B10.PL splenocytes plus titrated amounts of the AC1-11 peptide (Fig. 2C). As anticipated, purified MBP $\alpha\beta$ TCR T cells proliferated strongly, while wild type CD4 T cells did not respond at all. MBP β TCR CD4 T cells also responded due to a relatively high frequency of antigen specific T cells, as previously published [22]. The CD4⁺ OV $\alpha\beta$ T cells clearly responded much more vigorously than the MBP β TCR only T cells. Therefore, although we did not observe strong skewing towards the CD4 lineage in mice carrying the OV $\alpha\beta$ TCR transgenes, we were able to detect a relatively strong antigen-specific T cell response.

Selection of MBP $\alpha\beta$ TCR and OV $\alpha\beta$ TCR expressing thymocytes in the absence of endogenous TCRs

To exclude the T cells bearing endogenous TCRs, we generated B10.PL, RAG1 deficient mice carrying the transgenes for either the OV $\alpha\beta$ TCR or the MBP $\alpha\beta$ TCR. Similar to the data from the RAG sufficient mice, most MBP $\alpha\beta$ TCR expressing thymocytes (Fig. 3A) were found to be at the CD4SP positive stage or had downregulated CD4 and CD8 and were likely on their way towards becoming CD4SP [22,29]. In contrast, over 85% of the OV $\alpha\beta$ TCR expressing thymocytes were found to be at the DP stage, with only 7% progressing to the mature CD4SP stage. Although the TCR is MHCII restricted, a small number of CD8⁺ cells are seen in both mice. Such “mismatched” cells are commonly found in the TCR transgenic mice.

Productive interactions of TCRs expressed by developing thymocytes with self-MHC:self-peptide complexes can be measured by staining for the upregulation of the early activation marker CD69 [29]. The minimal upregulation of CD69 on double positive (DP) thymocytes expressing the OV $\alpha\beta$ TCR, as compared to the MBP $\alpha\beta$ TCR, confirms that OV $\alpha\beta$ TCR expressing thymocytes are very poorly positively selected (Fig. 3B). These data also ruled out increased negative selection of the OV $\alpha\beta$ TCR expressing thymocytes since negative selection would also result in increased CD69 levels.

Despite poor positive selection, OV $\alpha\beta$ TCR transgenic T cells accumulate in the periphery (Fig. 3C) in RAG1 deficient mice. Similar to the MBP $\alpha\beta$ TCR T cells, nearly all of the OV $\alpha\beta$ TCR expressing T cells are found within the CD4⁺ compartment. Unlike the CD4:CD8 ratio of the peripheral T cells from OV $\alpha\beta$ TCR B10.PL mice, which was about 2:1, the OV $\alpha\beta$ TCR Rag1^{-/-} had a CD4:CD8 ratio greater than 10:1, similar to what was seen in MBP $\alpha\beta$ TCR Rag1^{-/-} mice (Fig. 3C). Spleen and

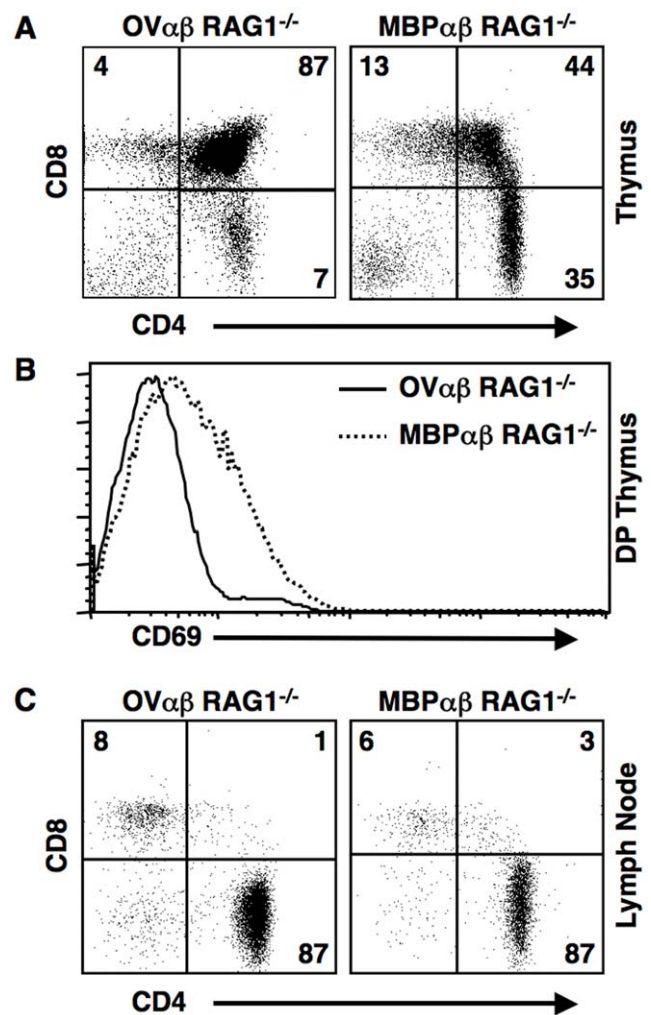


Figure 3. Thymocytes expressing the OV $\alpha\beta$ TCR are poorly positively selected even in the absence of endogenous TCRs. (A) As compared to thymocytes expressing the high affinity MBP $\alpha\beta$ TCR, the OV $\alpha\beta$ TCR is very inefficiently positively selected, which is reflected in a much lower percentage of CD4SP thymocytes. (B) OV $\alpha\beta$ TCR transgenic double positive (DP) thymocytes have a minimal upregulation of CD69, which is an indicator of productive TCR-MHC interactions. (C) FACS analysis of lymph node cells shows that, despite poor positive selection, CD4 T cells accumulate in the OV $\alpha\beta$ TCR transgenic mouse.

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lymph node cellularity was, on average, higher in the MBP $\alpha\beta$ TCR RAG1 deficient mice.

OV $\alpha\beta$ TCR T cells have a lower affinity than MBP $\alpha\beta$ TCR T cells for the I-A^u:Ac1-11 complex

The original T cell cultures and the hybridomas generated from these T cells both showed that the OV $\alpha\beta$ TCR had a functionally lower affinity for the I-A^u:Ac1-11 complex than the MBP $\alpha\beta$ TCR. Before evaluating in vivo function, it was necessary to determine if the naive T cells collected from the two TCR transgenic mice retained this functional difference. Furthermore, by using a blocking mAb it was possible to directly compare the relative avidities of the two TCRs [21].

CD4 T cells from OV $\alpha\beta$ and MBP $\alpha\beta$ TCR transgenic RAG1^{-/-} mice were purified by negative selection and then challenged with irradiated I-A^u splenocytes loaded with titrated

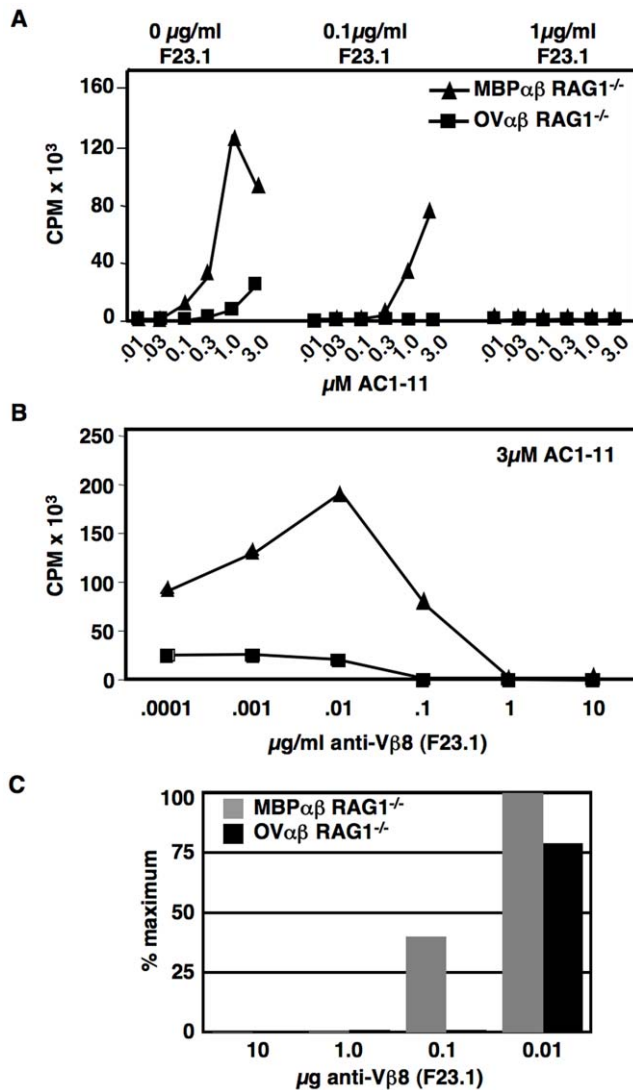


Figure 4. The relative affinity of the $OV\alpha\beta$ and $MBP\alpha\beta$ TCRs. Antibody blocking studies show that the $OV\alpha\beta$ TCR is of much lower affinity for the $I-A^u:Ac1-11$ peptide ligand as compared to the $MBP\alpha\beta$ TCR. (A) Purified $CD4^+$ T cells from $MBP\alpha\beta$ TCR $RAG1^{-/-}$ and $OV\alpha\beta$ TCR $RAG1^{-/-}$ mice were incubated with the indicated amount of antibody against $V\beta 8$ (F23.1) and then challenged with titrated amounts of the Ac1-11 peptide presenting by B10.PL APCs. (B) Purified T cells were activated with $3\mu M$ Ac1-11, presented by B10.PL APCs, in the presence of titrated amounts of blocking antibody. Assays were pulsed after 48 hours and harvested at 72 hours. Each condition was done in triplicate and the data was averaged. One representative experiment of three is shown. (C) For clarity, the data from (B) are presented as the percent of the maximal response of the T cells without the blocking antibody.
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amounts of the Ac1-11 MBP peptide. As we found with the original T cell lines as well as with the hybridomas, naive $OV\alpha\beta$ T cells respond much less well to the $I-A^u:Ac1-11$ complex than the MBP TCR expressing T cells (Fig. 4A). To test the relative affinity of the two TCRs for $I-A^u:Ac1-11$ complex, we first incubated purified T cells with different doses of F23.1 [30], an anti- $V\beta 8.2$ mAb that we have previously shown does not cause activation of T cells at the concentrations used for these experiments [21]. We then examined the response to titrated doses of peptide. As can be seen in Fig. 4A, $1\mu g/ml$ of F23.1 completely blocked the response

of T cells from both mice. At a 10-fold lower dose of blocking mAb, however, $MBP\alpha\beta$ T cells respond strongly, while $OV\alpha\beta$ T cells are still completely blocked. We next used titrated amounts of the blocking mAb, while keeping the peptide concentration constant (Fig. 4B). Again, the response of T cells from the $OV\alpha\beta$ mouse line was blocked by much less mAb. Since the absolute level of proliferation greatly differed between the two T cell types, we also expressed the data as the percent of the maximum response (Fig. 4C). Here it is clear that even just $10ngs$ of the anti- $V\beta 8.2$ mAb is sufficient to partially block the response of the $OV\alpha\beta$ T cells, whereas the $MBP\alpha\beta$ T cells are not affected. Together these data show that the affinity of the $OV\alpha\beta$ TCR for the $I-A^u:Ac1-11$ complex is substantially less than the affinity of the MBP TCR for the same ligand.

Development of spontaneous EAE in mice transgenic for low-affinity MBP-specific TCR

RAG-sufficient mice carrying the transgenes for the $OV\alpha\beta$ TCR or the $MBP\alpha\beta$ TCR were monitored for a period up to twelve months. The incidence of spontaneous EAE was recorded from the onset of obvious hind leg paraparesis (gait disturbance). The animals were sacrificed when full hind leg paralysis was observed. Consistent with reported results, approximately 40% (4 of 10 mice, Fig. 5 A,B) of $MBP\alpha\beta$ TCR mice developed EAE, with an average onset of approximately 11 weeks after birth [14,31]. In contrast, only one $OV\alpha\beta$ mouse was observed to develop EAE (Fig. 5 A,B). Onset of the disease in this mouse occurred 16 weeks after birth. A group of nine $OV\alpha\beta$ TCR mice was monitored for a total of twelve months and more than 50 mice were monitored for a shorter period of time (~6 months). Of this group, only one additional mouse (12 weeks old) showed any overt signs of the disease.

The greatly reduced frequency of EAE in the $OV\alpha\beta$ TCR mice is most likely due to one, or a combination of, three factors. First, the lower frequency of $OV\alpha\beta$ TCR T cells as compared to

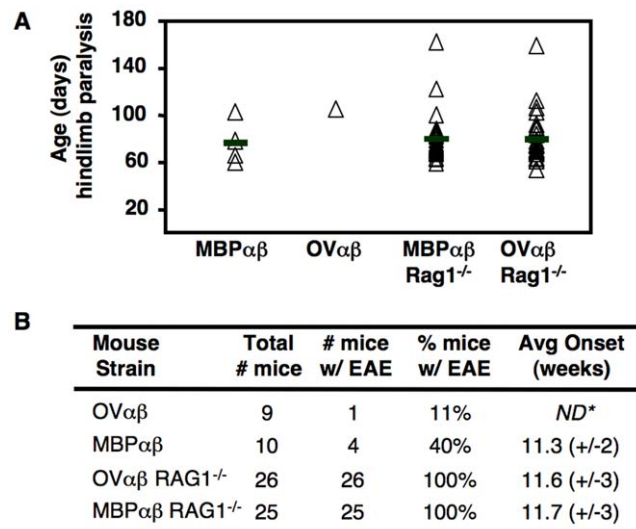


Figure 5. Development of spontaneous EAE in TCR transgenic mice. (A) RAG1 deficient mice were monitored for twelve months for overt signs of EAE (hind leg paraparesis). The day of the onset of EAE in individual mice is indicated on the chart. (B) More than 60% of $MBP\alpha\beta$ mice and nearly all $OV\alpha\beta$ TCR mice remained healthy throughout the course of the study, whereas 100% of the TCR transgenic RAG1 deficient became sick. Onset of disease was at an average of eleven weeks of age in all three susceptible mice.
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MBP $\alpha\beta$ T cells may play a role; second, the lower affinity of the OV $\alpha\beta$ TCR may make it inherently less encephalitogenic; or third, regulatory T cells (presumably CD4⁺CD25⁺ T cells) might be more effective at controlling the lower affinity OV $\alpha\beta$ T cells. We sought to differentiate between these possibilities by studying the RAG1 deficient MBP $\alpha\beta$ and OV $\alpha\beta$ TCR transgenic mice.

It has been shown that if all endogenous TCR expression is removed from a mouse line carrying the transgenes for a different AC1-11/I-Au specific TCR, 100% of the mice develop EAE [15]. This result is due, at least in part, to the removal of residual CD4⁺CD25⁺ Treg cells that develop due to the expression of non-transgene encoded TCRs [16]. Therefore, we monitored RAG1 deficient mice carrying the transgenes for the high affinity MBP $\alpha\beta$ TCR for signs of EAE. In contrast to RAG1 sufficient MBP $\alpha\beta$ TCR mice, 100% of the MBP $\alpha\beta$ TCR, RAG1 deficient mice (24 mice) developed EAE (Fig. 5 A, B). Interestingly, the RAG1 deficient mice developed EAE at the same age (11 weeks) as their RAG1 sufficient counterparts (Fig. 5A, Ref. [14,31]). We also evaluated our RAG1 deficient OV $\alpha\beta$ TCR transgenic mice. In sharp contrast to what we found in the OV $\alpha\beta$ RAG1 sufficient mice, 100% (25/25 mice) of the OV $\alpha\beta$ RAG1 deficient mice developed EAE (Fig. 5A, B). Remarkably, the age that the mice developed the disease was, on average, nearly identical to both the MBP $\alpha\beta$ RAG1 sufficient and MBP $\alpha\beta$ RAG1 deficient mice (Fig. 5A, B).

Discussion

The underlying reasons for the onset of T cell mediated autoimmune diseases are complex and involve a variety of genetic and environmental factors. The presence of self-peptide:self-MHC specific T cells is, however, clearly essential. In general, a minimal affinity between self-MHC:self-peptide complexes and the TCR appears to be an important factor for the function T cells [32]. T cells that initiate autoimmune diseases, however, presumably have avidities for self-ligands that are closer to pathogen-derived antigen specific responses [33].

Autoimmunity is commonly thought to require high affinity “driver” T cell clones that initiate disease and cause inflammation [34]. This inflammation results in a DTH-like response that recruits macrophages and other non-specific T cells. The damage caused by the inflammation can also lead to epitope spreading, resulting in the activation of T cells specific for epitopes distinct from the disease-causing epitope. This secondary response, which appears to occur within the CNS, rather than peripheral sites [11,35], may involve lower affinity T cell clones [36]. Self-antigen specific driver clones, therefore, must be present in all mouse strains that are susceptible to EAE. Nonetheless, EAE typically does not occur spontaneously; rather it must be stimulated by various immunization protocols. Even TCR transgenic mice that have nearly clonal populations of high affinity self-reactive T cells are completely [15] or at least partially [14] protected from the onset of spontaneous EAE. It has been previously shown for one of these models that when the TCR transgenes were introduced into mice deficient for RAG1 expression, EAE developed spontaneously [15]. Therefore, a minor population of lymphocytes that required RAG1 for development played a critical role in suppressing disease. Later, the suppressor cells were definitively determined to be CD4⁺ T cells expressing non-transgene encoded TCRs [16].

Our studies of a low-affinity MBP AC1-11 peptide/I-A^u specific TCR-transgenic model were designed to determine if the affinity of the TCR-MHC/peptide interaction played a role in the pathogenesis of EAE. We first characterized antigen specific T

cells from mice carrying only the MBP TCR β chain. Interestingly, our analysis revealed that the TCR repertoire of the AC1-11 MBP peptide reactive T cells in these mice was limited to only two different TCRs. These data imply that central tolerance is highly efficient at removing all other TCRs reactive for this ligand, but for unknown reasons, fails to remove thymocytes expressing these two TCRs. This TCR α chain was used to generate a new TCR transgenic mouse model, which was compared to an existing mouse model that expresses a related high affinity TCR.

It is not known how disease causing, self-reactive T cells escape intrathymic negative selection [37]. One possibility is that thymocytes expressing TCRs that have very high avidities for self-antigens actually are deleted due to the ectopic expression of peripheral “tissue-specific” proteins in the thymus [38,39,40,41]. Indeed, it has previously been shown that mice lacking the last five exons of MBP (shiverer mice) mount T cell responses to additional MBP-derived epitopes [42,43]. These data demonstrated that thymic expression of the MBP is directly responsible for tolerance of at least some MBP-reactive thymocytes. Tissue specific antigens, however, may be expressed at low levels and, therefore, may not be efficient at negative selection of lower affinity self-reactive clones [44]. A variant on this idea is that the form of the self-antigen expressed in the thymus is somehow distinct from the peripherally expressed antigen [11]. In the case of the shiverer mice, T cell responses against the AC1-11 MBP derived peptide in shiverer mice were similar to those in wild type mice suggesting that the relatively unstable I-A^u:Ac1-11 complex is not efficient at driving clonal deletion [42,43].

Whatever the exact mechanism of clonal escape might be, it was reasonable to hypothesize that thymocytes expressing the lower affinity OV $\alpha\beta$ TCR would develop at least as efficiently those expressing the as the higher affinity MBP $\alpha\beta$ TCR. Indeed, the lower affinity for self-antigen would arguably make the OV $\alpha\beta$ TCR thymocytes less susceptible to clonal deletion. We found, however, that the low affinity OV $\alpha\beta$ TCR was actually very poorly positively selected. There was no indication of negative selection (i.e. reduced cellularity, upregulation of CD69, etc.) in either mouse. Therefore, in contrast to finding that aberrant negative selection plays a significant role in allowing the escape of disease causing T cells, we find that positive selection is a major contributor. In other words, in our model system, high affinity, self-reactive T cells are efficiently positively selected, whereas, low affinity, less “dangerous” clones, fall by the wayside. This difference in positive selection may also account for the four-fold excess of high affinity T cell clones that we consistently find in the TCR β only mice.

Consistent with previous studies [14,31], in our analysis, we also found that ~40% of mice carrying the transgenes for the high affinity MBP $\alpha\beta$ TCR spontaneously developed EAE at approximately eleven weeks of age. This level of penetrance is consistent with what we have seen over several years of maintaining this mouse strain. Of the nine OV $\alpha\beta$ TCR mice that we monitored for the complete twelve months, only one mouse developed EAE. Overall, approximately fifty OV $\alpha\beta$ TCR mice were produced and were monitored for up to six months, with only one additional mouse showing clear signs of paralysis, which occurred at 12 weeks. The minimal number of sick OV $\alpha\beta$ TCR mice is due either to the reduced affinity of the TCR and/or due to the reduced numbers/percentage of MBP specific T cells. Clearly the total number of potentially encephalitogenic T cells in the OV $\alpha\beta$ TCR mice far exceeds what can be found in a wild type mouse, but, nonetheless, it is several fold fewer than the more disease prone MBP $\alpha\beta$ TCR transgenic mice. Eliminating all endogenous TCRs by breeding to RAG1 deficient mice, however, demon-

strated that both versions of the MBP specific T cells were likely kept in check by the presence of RAG-dependent regulatory cells. Based on previous work, these regulatory cells are, at least in part, CD25⁺CD4⁺ Tregs [16,17]. These data, therefore, suggest one of two possibilities, which are not exclusive. First, the absolute ratio of Tregs to self-reactive T cells may be critical, with relatively subtle alterations resulting in disease. Alternatively, Tregs may be far more efficient at controlling self-reactive T cells with lower affinity TCRs. We anticipate that cell transfer studies will allow us to distinguish between these two mechanisms.

Rather surprisingly, the onset of EAE for the MBP $\alpha\beta$ TCR mice on a wild type background and both the MBP $\alpha\beta$ TCR and OV $\alpha\beta$ TCR mice on a RAG1 deficient background was nearly identical, with a mean of eleven weeks. Even the two OV $\alpha\beta$ TCR RAG1 sufficient mice in which disease was observed became sick around this timeframe (12 and 16 weeks). These data suggest that some initiating event, perhaps release of myelin basic protein due to apoptosis of nerve cells, reproducibly occurs soon after the mice enter adulthood. Perhaps this is related to the finding that self-reactive T cells contribute to neurogenesis [45]. Importantly, if RAG dependent cells, such as Tregs, are present during this “window of opportunity”, they are capable of preventing autoimmune disease.

Overall, our analysis of high and low affinity MBP Ac1-11 specific T cells reveals several important aspects concerning the

onset of the autoimmune disease, EAE. First, the frequency of different clonotypes that are capable of recognizing the dominant Ac1-11 epitope appears to be very limited. Second, positive selection, rather than negative selection, appears to have a major impact on the frequency of different clonotypes. Third, regulatory cells appear to be very efficient at controlling self-reactive T cells expressing a low affinity TCR. And finally, potentially encephalitogenic T cells appear to have relatively short window of opportunity to cause disease. Intriguingly, if regulatory cells are present during this window of opportunity, which is around eleven weeks after birth, more often than not encephalitogenic T cell responses are suppressed and this suppression lasts a lifetime.

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Author Contributions

Conceived and designed the experiments: JL OV DBS. Performed the experiments: JL OV. Analyzed the data: JL OV DBS. Wrote the paper: JL OV DBS.

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