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RAG1-associated expression of the MBP1–11-specific transgenic T cell receptor in H-2^b mice

Abigail C. Buenafe^{2,*}, Courtney Sherwood¹, Nicole Moes¹, and Richard E. Jones^{1,2,*}

¹ Neuroimmunology Research, Veterans Affairs Medical Center, Portland, OR 97239

² Department of Neurology, Oregon Health and Science University, Portland, OR 97239

Abstract

We pursued a breeding strategy intended to generate disease resistant mice with exclusive expression of the H-2^u-restricted myelin basic protein (MBP) 1–11 peptide-specific transgenic (Tg) T cell receptor (TCR) on the T cell deficient RAG1KO (H-2^b) background. Utilizing specific screening assays for the offspring, analyses of the F1 intercross and subsequent crosses revealed that the TgTCR-associated clonotypic marker detected by the 3H12 mAb could only be found in association with the H-2^b homozygous background in offspring possessing a functional *rag1* gene. Moreover, expression of the MBP-specific TgTCR could not be found in H-2^b homozygous offspring that were RAG1 deficient (*rag1*^{−/−}). PCR analysis of genomic DNA from these 3H12-negative offspring verified the presence of the TCR transgenes. Thus, the presence of a functional *rag1* gene was required for the expression of the MBP-specific TgTCR on the H-2^b background. Given the role for RAG1, the results have important implications for T cell repertoire development.

Keywords

Transgenic T cell receptor (TgTCR); recombinase activating gene 1 (RAG1); myelin basic protein (MBP)

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease model that shares many important features with multiple sclerosis. Such shared features include the significant involvement of T cells specific for central nervous system (CNS) components and the development of acute and/or chronic inflammatory lesions in the brain and spinal cord. The characterization of T cell receptors on T cells that are capable of initiating EAE pathogenesis together with the technology of generating TCR transgenic (TgTCR) mice has opened numerous avenues for the investigation of disease mechanisms in EAE and many other autoimmune disorders. As reviewed by Lafaille (2004), TgTCR mice have now been generated with specificity for many major CNS myelin proteins recognized by EAE pathogenic T cells including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG).

*Corresponding authors: Abigail C. Buenafe, Ph.D., Tykeson MS Research UHS-46, 3181 SW Sam Jackson Park Rd, Oregon Health and Science University, Portland, OR 97239; tel: 1 503 494 4424; fax: 1 503 494 9537; e-mail: buenafea@ohsu.edu. Richard E. Jones, Ph.D., Portland VA Medical Center, R&D-23, 3710 SW Veterans Hospital Rd., Portland, OR, 97239; tel: 503 220 8262 x55177; e-mail: jonesric@ohsu.edu..

In a recent study, we addressed the question of whether epitope spreading was a necessary component of EAE progression by generating MBP-specific TgTCR mice possessing the *scid/scid* genotype on the H-2^u background (Jones et al., 2003). These mice, originally designated TgSCID^{+/+} (Jones et al., 2003), are referred to as TgSCID mice in this manuscript. TgSCID mice were immunodeficient and incapable of rearranging endogenous TCR genes. However, there have been reports that the immunodeficient *scid/scid* genotype is leaky and that low level TCR gene rearrangement can occur (Nonoyama et al., 1993). We therefore sought to use a breeding strategy to place the MBP1–11-specific TgTCR on a RAG1 deficient background that would provide complete inhibition of endogenous TCR gene rearrangement. The RAG1KO strain (H-2^b) was chosen for breeding with TgSCID because the RAG1 deficient condition is non-leaky (Mombaerts et al., 1992).

Because the TgSCID mice develop spontaneous EAE at an early age, it is necessary to protect these mice by the transfer of histocompatible, normal splenocytes (immunocompetent, non-SCID) by the age of five weeks in order to generate healthy breeders for this colony. The transfer of normal wildtype splenocytes provides regulatory T cells that are able to down-regulate the function of organ-specific autoimmune TgTCR T cells (Ollivares-Villagomez et al., 1998). Offspring generated at the F1 intercross stage of our strategy would be expected to include mice in which the TgTCR was associated with the H-2^b background. MBP1–11-specific TgTCR T cells that could be isolated from such TgTCR RAG1KO H-2^b mice would be expected to be free of endogenous T cell specificities. We recognized that such TgTCR RAG1KO H-2^b mice might be very useful experimentally since they would possess naïve TgTCR T cells (due to non-reactivity with MBP1–11 in association with H-2^b), and they would be expected to resist development of spontaneous EAE as a result, thereby facilitating their breeding. In a similar fashion, McHugh et al. (2001) reported that a TgTCR associated with autoimmune gastritis was expressed on over 80% of CD4 single positive thymocytes in mice bearing the non-restricting H-2^b haplotype. Moreover, although peripheral expression of the TgTCR was comparable to that in H-2^d mice, spontaneous autoimmune gastritis developed only in mice with the appropriately restricting H-2^d haplotype, and not in H-2^b mice. The current report describes our breeding and screening strategy for the generation of the TgTCR RAG1KO mice on the H-2^b background, analysis of the resulting offspring, and an unexpected absence of TgTCR expression associated with the absence of functional RAG1.

MATERIALS AND METHODS

Mice

RAG1KO mice, B10.PL, C57BL6, and C.B-17scid mice were obtained from Jackson Laboratories, Bar Harbor, ME. TgSCID^{+/+} (original designation) and TgSCID^{-/-} mice were derived from in-house colonies and have been described (Jones et al., 2003). In the current manuscript, TgSCID refers to the TgSCID^{+/+} mice. All other mice were generated from breedings between TgSCID and RAG1KO mice and subsequent offspring intercrosses and backcrosses, as described in this paper. Mice were housed under SPF conditions at the Portland Veterans Affairs Medical Center, Veterinary Medical Unit. All procedures were approved and performed according to institutional guidelines.

Proliferation Assay

An MBP1–11-specific T cell line was generated from spleen cells of TgSCID mice that were transgenic for an MBP1–11-specific TCR (Lafaille et al., 1994). Spleen cells were initially stimulated with 2.5 ug/ml ConA in Stim Medium (RPMI 1640 containing 1 mM Na Pyruvate, 2 mM L-glutamine, 57.2 uM 2-ME, 10% FBS) for 2 days. Cell aliquots were stored frozen in liquid nitrogen following 5 days in expansion medium (RPMI 1640

containing 1 mM Na Pyruvate, 2 mM L-glutamine, 57.2 μ M 2-ME, 10% FBS, 20 U/ml recombinant IL-2). For proliferation assays, MBP1-11-specific T cells were plated at 0.02×10^6 cells per well with 0.8×10^6 antigen-presenting cells (APC) processed from irradiated thymi + spleens of either B10.PL (H2^u) or C57BL6 (H2^b) mice. MBP1-11, purified protein derivative of Mycobacterium tuberculosis strain H37Ra (PPD, 5 μ g/well)(Statens Serum Institute, Denmark), Con A (10 μ g/ml), or medium were added to triplicate wells and cultures were maintained for 3 days in 7% CO₂ at 37°C. [³H]-Thymidine was added to each well approximately 18 hours prior to harvest and counting in a Betaplate liquid scintillation counter (Perkin-Elmer-Wallac).

Genomic DNA (gDNA) isolation

gDNA was prepared from the tail tissue of mice using the REDEExtract-N-Amp kit (Sigma Chemical Co., St. Louis, MO) according to manufacturer's instructions. Two to 4 μ l of extract was used in each PCR reaction.

PCR Analysis

PCR amplification with the REDEExtract-N-Amp kit (Sigma Chemical Co., St. Louis, MO) was used for genotyping of mouse offspring. The RAG1 status of the offspring was determined with the use of two sets of primers (Liao et al., 1998) specific for either the *rag1* gene wildtype sequence or the RAG1KO gene sequence. Primers for the *rag1* wildtype gene: RAG1for (5'-CCAGTAGATACCATTGCGAAGAGG-3') + RAG1rev (CACGTTCTGTGAACCATGCTCTATC-3'). Primers for the RAG1KO gene: Neorev (5'-CCGCTTCCATTGCTCAGCGG-3') + RAG1rev. Both sets of primers were used at an annealing temperature of 56°C. The use of both sets of primers allowed the identification of offspring that were homozygous for either gene or heterozygous for both genes. We also designed two sets of primers to assess the H-2 allotype of the offspring. Non-cross reactive primers were designed by alignment of the *H-2K^b* and the *H-2K^u* genes. *H-2K^b* primers (anneal temp 58°C): H2Kb.3 for (5'-TCTCACACTATTCAGGTGATCTCTG-3') + H2Kb/u.2rev (5'-ATCTGCGGAGCCACTCC-3'). *H-2K^u* primers (anneal temp 62°C): H2Ku.2for (5'-ACGATCCAGGTGATGTACG-3') + H2Kb/u.2rev. The use of both primer sets allowed the identification of homozygous or heterozygous offspring.

PCR amplification was also used to detect the presence of the TCR transgenes in offspring. We used sequences within the CDR3 region of the TgTCR genes to design primers that would be specific for either the AV4 TgTCR or the BV8 TgTCR. AV4 TgTCR primers: AV4Tgfor (5'-CGGAGAAGGGCCACAGTTC-3') + AV4TgCDR3rev (5'-CTCATTTCCATAGTTCTCACTCAGAGC-3'). BV8 TgTCR primers: mBV8 (CATGGGCTGAGGCTGATCCATT-3') + BV8TgCDR3rev (5'-GTGTTCCCCCAGATGCAT-3'). Both sets of TgTCR primers were used at an annealing temperature of 60°C. All PCR samples in this paper were amplified for 30 to 35 cycles.

Flow Cytometry

Peripheral blood samples were collected into EDTA (3 mg/ml) from the tail vein of offspring in order to stain for surface expression of CD4 and the TgTCR clonotypic marker recognized by the 3H12 mAb, as described (Olivares-Villagomez et al., 1998). Briefly, red blood cells were lysed with 0.15M NH₄Cl buffer before staining. Washed samples were first incubated with the 3H12 mAb (10 μ l supernatant) for 30 mins at 4°C, washed, and then stained with PE-labeled anti-mouse IgM mAb (BD Pharmingen) for an additional 20 mins at 4°C. Control samples were stained with PE-labeled anti-mouse IgM mAb without prior incubation with 3H12. After washing, samples were incubated with FITC-labeled anti-CD4 mAb (BD Pharmingen) for 20 mins and then washed. Propidium iodide (100 μ l) was added

to each tube in order to exclude non-viable cells. Samples were analyzed on a FACScan (Becton-Dickinson).

RESULTS

MBP1–11-specific TgTCR T cells do not respond to antigen presented by H-2^b APCs

As a result of interbreeding TgSCID mice (H-2^u) with RAG1KO mice (H-2^b), some offspring were generated in which the TgTCR was placed on the H-2^b background. These TgTCR H-2^b mice are the focus of this report. Specifically, placing the TgTCR on the RAG1KO H-2^b background would provide a convenient source of transferable TgTCR⁺ T cells without the presence of endogenous T cells of other specificities or B cells. In addition, the isolated TgTCR⁺ T cells would presumably be naïve due to an absence of stimulation *in vivo*. As demonstrated in Figure 1, we verified that MBP1–11-specific TgTCR⁺ T cells failed to proliferate to MBP1–11 peptide presented by H-2^b APCs isolated from C57BL6 mice. TgTCR⁺ T cells responded to MBP1–11 presented by H-2^u APCs (B10.PL) over a range of concentrations (one concentration shown), but did not respond to the irrelevant antigen PPD presented by either type of APC. Both H-2^b and H-2^u APCs were able to facilitate proliferation of TgTCR⁺ T cells in the presence ConA, which is an APC-dependent response (Ahmann et al., 1978), but not an H-2-restricted response.

Breeding scheme and screening of TgTCR⁺ x RAG1KO offspring

Figure 2 outlines the initial breeding strategy that placed the TgTCR on the RAG1[−] background. TgSCID mice (*rag1*^{+/+}, *H-2*^{u/u}, *tgtr*^{+/+}) were bred with RAG1KO mice (*rag1*^{−/−}, *H-2*^{b/b}, *tgtr*^{−/−}) in the parental cross as indicated. Although the *scid/scid* genotype was present in the TgSCID parent, it was not selected for in subsequent offspring crosses and was eventually lost (data not shown). All offspring in the F1 generation were expected to be heterozygous for the genes of interest (*rag1*, *H-2*, *tgtr*) and thus were not genotyped. Offspring from the F1 generation were intercrossed to produce the F2 generation. Figure 2 shows the genotype frequencies expected (i.e. predicted) of a normal Mendelian inheritance pattern for the H-2 allotypes and *tgtr* genes and the TgTCR phenotype expected with each of the expected genotypes.

Primers used for screening of offspring were first verified for specificity. Primers specific for either the *rag1* wildtype gene (RAG1wt) or the mutant *rag1neo* gene (RAG1neo) were tested on TgSCID (wildtype) or RAG1KO mice. As shown in Figure 3A, DNA isolated from a TgSCID mouse was positive for the 269 bp PCR product amplified by the RAG1wt primers but negative for the RAG1neo PCR product, as expected. Conversely, RAG1KO mouse DNA was negative for the RAG1wt PCR product but positive for the 473 bp RAG1neo PCR product. Thus, RAG1wt and RAG1neo primer sets could be used to distinguish offspring that were homozygous for either genotype or heterozygous for both.

Primers specific for the H-2K^b and H-2K^u haplotypes were tested on 3 different mouse strains: TgSCID (H-2K^u), RAG1KO (H-2K^b), and CB.17SCID (H-2K^d). As shown in Figure 3B, the 231 bp PCR product amplified by the H-2K^b primers (indicated by arrow) was present only in RAG1KO DNA and not in TgSCID or CB.17 DNA. In Figure 3C, the 231 bp PCR product amplified by the H-2K^u primers (see arrow) was present only in TgSCID DNA and not in RAG1KO or CB.17SCID DNA. Thus, we verified that our H-2K^b and H-2K^u primers could be used to distinguish between mice homozygous or heterozygous for the H-2K^b or H-2K^u genotypes in offspring. Amplification with β-actin primers in Figure 3C served as a positive control to verify the presence of intact DNA in the PCR reactions.

Of note, since TgTCR expression is typically a dominant trait, phenotypic screening for the TgTCR clonotypic determinant (recognized by the 3H12 mAb) would not distinguish

between TgTCR-expressing offspring that were *tgtr*^{+/+} versus *tgtr*^{+/-}. Therefore, the resultant frequency analyses presented (below, and see Figure 2) for 3H12⁺ offspring represent the combined frequencies of TgTCR expressers of the homozygous and heterozygous *tgtr*. Phenotypic screening for cell surface expression of TgTCR was performed by staining with 3H12 mAb, specific for a TgTCR clonotypic marker. Figure 4 demonstrates the typical 3H12 and CD4 expression pattern observed by flow cytometry in a positive control TgSCID mouse (panel A) and a negative control RAG1KO mouse (panel B), and in the *H-2^{b/b}* homozygous offspring that were *rag1*^{+/-} (panel C) versus *rag1*^{-/-} (panel D)(see below). The *rag1*^{+/-} *H-2^{b/b}* mouse (C), but not the *rag1*^{-/-} *H-2^{b/b}* mouse (D), offspring demonstrated positive staining of CD4⁺ cells that expressed the TgTCR marker recognized by 3H12. Incidentally, this particular *rag1*^{-/-} *H-2^{b/b}* mouse (mouse #358) was subsequently found to carry the MBP1–11 TCR transgenes (see below and Fig. 5).

Frequency analysis of TgTCR⁺ x RAG1KO offspring crosses

Two different breeding strategies were used to generate TgTCR⁺ mice on the *H-2^b* background. Table I summarizes the observed frequencies from the F2 generation (expected frequencies in parentheses) for the *rag1* genotypes and for the *H-2* allotypes associated with each of the *rag1* genotypes (top half of Table I). Also shown (bottom half, Table I) are the frequencies for observed and expected TgTCR⁺ expression (based on 3H12⁺ phenotypic staining) associated with each *H-2* allotype combination according to *rag1* genotype. Analysis of 103 total offspring in the F2 generation demonstrated that inheritance of the *rag1* genotypes (observed frequency) followed a normal Mendelian pattern and closely approximated the expected frequencies (Table 1, upper left). Subsequent analysis of the *H-2* genotypes within each of the *rag1* genotype subsets indicated that of the *rag1* genotypes, only the *rag1*^{+/+} subset (wildtype *rag1*) showed *H-2* frequencies that approximated the expected frequencies (Table 1, upper). In the *rag1*^{-/-} (RAG1KO) and the *rag1*^{+/-} conditions, *H-2^{u/u}* offspring were underrepresented by 1.6–1.8-fold while *H-2^{b/b}* offspring were overrepresented by 1.7–2.0-fold. We screened 100% (24/24) of the *rag1*^{-/-} and 32% (18/56) of the *rag1*^{+/-} mice (bottom half of Table I). Among the *rag1*^{-/-} offspring, a striking bias was found against 3H12⁺ expression in *H-2^{b/b}* mice (bold). Out of 11 *rag1*^{-/-} mice that were also homozygous for *H-2^{b/b}*, none were 3H12⁺ although 0.75 was the expected TgTCR⁺ frequency within each subset. Among *rag1*^{+/-} animals, 3H12⁺ expression appeared to be closer to the expected frequency in the *H-2^{u/u}* and *H-2^{b/b}* subsets.

In order to obtain additional *rag1*^{+/-}, *H-2^{b/b}* offspring that could be screened for TgTCR expression, we also analyzed offspring obtained from intercrosses between mice that were *rag1*^{+/-}, *H-2^{b/b}*, *tgtr*^{+/-} (summarized in Table II, second intercross set). As expected, 100% of the offspring in this intercross were homozygous for *H-2^{b/b}* (not shown). Analysis of 58 total offspring in this group demonstrated again that the observed distribution among the *rag1* genotypes followed a simple Mendelian pattern and were similar to the expected frequencies (not shown). Consistent with the analysis above, 3H12⁺ expression was found in none of the 16 *rag1*^{-/-} offspring although 0.75 was the expected frequency. As summarized in Table II, between the two different breeding schemes analyzed, 3H12⁺ expression was not detected in any of the 27 total *rag1*^{-/-}, *H-2^{b/b}* offspring screened. However, among the 31 total *rag1*^{+/-}, *H-2^{b/b}* offspring screened, 17 were found to be 3H12⁺. We also backcrossed a *rag1*^{-/-}, *H-2^{b/u}*, *tgtr*^{+/+} mouse to a RAG1KO mouse (*rag1*^{-/-}, *H-2^{b/b}*, *tgtr*^{-/-}) and screened the *rag1*^{-/-} *H-2^{b/b}* offspring in this set (not shown). Of the 3 *rag1*^{-/-} *H-2^{b/b}* offspring, all were negative for 3H12⁺ expression (not shown).

Detection of TCR transgenes in the gDNA of 3H12⁻ offspring

Since screening for TgTCR expression with the 3H12 mAb was not a genotype assay, this raised the possibility that mice that were negative for 3H12 staining could still possess the

TCR transgenes. Because the *rag1*^{-/-} *H-2^{b/b}* 3H12⁻ offspring were also negative for CD4 staining, we concluded that the absence of 3H12 reactivity was due to an absence of CD4⁺ T cells and was not due to other less likely reasons, such as a mutational change in the epitope.

To detect the presence of the AV4 and BV8 TCR transgenes we took advantage of transgene CDR3 sequences to design primers that would amplify the transgenes but not endogenous TCR genes. Figure 5 demonstrates the specificity of primer sets used to amplified the correctly sized 177 bp BV8Tg fragment and 178 bp AV4Tg fragment in gDNA from control mice bearing the TgTCR (TgSCID or *rag*^{+/-} *H-2^{b/b}* 3H12⁺) but not in mice without the TgTCR (TgSCID^{-/-} or RAG1KO). Figure 5 also demonstrates that in the analysis of gDNA from 9 total *rag1*^{-/-} *H-2^{b/b}* 3H12⁻ offspring screened for the AV4Tg and BV8Tg by PCR, 8 of these were found to still bear the TCR transgenes. Thus, we have concluded that a number of the *rag1*^{-/-} *H-2^{b/b}* 3H12⁻ offspring in fact do possess the TCR transgenes but fail to express them, at least in the periphery.

DISCUSSION

Our specific findings in these studies indicate that expression of the MBP1-11-specific TgTCR in mice homozygous for the non-restricting *H-2^b* haplotype was dependent on the presence of a functional *rag1* gene. In contrast, TgTCR expression in the presence of the *H-2^u* haplotype was not dependent on a functional *rag1* gene. Since expression of the TgTCR in *H-2^{b/b}* mice was conditional upon RAG1 function, the possibility is raised that mechanisms involving gene rearrangement are involved in TgTCR expression on the *H-2^{b/b}* haplotype.

With the absence of TgTCR expression in RAG1KO, *H-2^{b/b}* homozygous offspring, we first considered that this could be a lethal combination causing offspring with the desired genotype to be non-viable *in utero*. Alternatively, TgTCR⁺ RAG1KO *H-2^{b/b}* offspring could be viable and present, but not detected by our phenotypic screening methods. Subsequently, we determined that the TCR transgenes were in fact still present in the genomic DNA of many of these offspring. Presumably, the transgenes themselves remained functional since they were expressed in littermates that were RAG1⁺ or *H-2^{b/u}*. Because CD4⁺ T cells were not found in the periphery of 3H12⁻ offspring on the RAG1KO *H-2^{b/b}* background, we hypothesize that if TgTCR expressing T cells were present in the thymus of these mice, they did not survive thymic selection. In this case, TgTCR⁺ T cells would not exit from the thymus to appear in the periphery. An analysis of TgTCR expression in thymic subpopulations may shed light on the mechanism leading to the absence of TgTCR T cells. Flow cytometry analysis of 3H12 association with single (CD4⁺ or CD8⁺) or double positive (CD4⁺CD8⁺) thymic subpopulations may indicate if TgTCR expression is present at a specific developmental stage. Our preliminary data indicate that TgTCR may be expressed at least at the double negative (CD4⁻CD8⁻) thymic T cell stage in both RAG1⁻ and RAG1⁺ *H-2^{b/b}* mice, indicating that TgTCR expression may not be subject to selection to this point (not shown). Although TCR expression is relatively low at the double negative stage in normal mice, pre-rearranged TCR transgenes are readily expressed at this early stage since expression is not limited by RAG1 activity (Serwold et al., 2007). Whether these TgTCR T cells in our model are actively deleted or whether they perish from neglect (see review by Starr et al., 2003) remains to be determined, although active deletion does not seem to occur in the *H-2^{b/u}* offspring (see below).

We observed that RAG1 functional activity was required for the successful selection of the MBP-specific TgTCR⁺ CD4⁺ T cells on the *H-2^{b/b}* background and their migration to the periphery. This was surprising since the TgTCR genes do not require RAG1 regulated gene rearrangement for their expression. One possible explanation is that there could be a

regulatory role for endogenous T and/or B cells in thymic selection, particularly in a case where positive selection may not be favored. Flow cytometric analysis of 3H12 expression in *rag1*^{+/-} *H-2^{b/b}* offspring indicated the presence of 3H12⁻ CD4⁺ T cells in these mice that are likely to express endogenous TCR (see Fig. 4C). These endogenously generated lymphocytes, absent from RAG1-deficient mice, could provide an opportunity for cell-cell interaction with the TgTCR⁺ T cells or with MHC Class II⁺ cells in the thymic environment to promote selection of the TgTCR⁺ cells. At present we are not aware of evidence in the literature supporting such a scenario.

A second possibility for the expression of the MBP-specific TgTCR on the H-2^b background involves RAG1 promotion of endogenous TCR gene rearrangement, allowing for a second TCR to be expressed on the TgTCR T cell surface and providing an alternative TCR for positive selection on H-2^b. TgTCR expressing T cells would then be able to mature and appear in the periphery only when rescued by the expression of a selectable endogenous TCR. In this case, all TgTCR⁺ T cells found in the periphery of H-2^b offspring would be expected to express dual TCRs. Support for this scenario is present in the literature. The existence of dual TCRs in 10–30% of T cells in the normal repertoire of humans and mice has raised the question of their role in the circumvention of thymic tolerance and in autoimmunity (Padovan et al., 1993; Elliott and Altmann, 1995). In many cases, dual TCR expression has been observed as a consequence of the less stringent allelic exclusion associated with the TCR alpha locus. In a study involving the generation of TgTCR mice specific for the human collagen IV $\alpha 2$ chain (hC4), He et al. (2002) discovered that the hC4-specific TgTCR could not be positively selected on a RAG1⁻ H-2^s background although the original hC4-specific T cell hybrid was restricted by I-A^s. Only in the presence of a functional *rag1* gene could CD4⁺ T cells bearing the hC4-specific TgTCR be found. It was further demonstrated through Ab staining that multiple V α 's were associated with T cell fractions responding to the hC4 antigen. Other studies (Tarbell et al., 2002; Paterson et al., 1999) have demonstrated that TgTCR⁺ T cells normally undergoing negative selection could be positively selected in the presence of endogenous TCR as dual TCR T cells. Interestingly, an analysis of the CD4⁺CD25⁺ Treg population from MBP-specific TgTCR mice revealed that Treg activity in the MBP-specific TgTCR⁺ population was dependent on the presence of endogenous TCRA chains, highlighting the importance of dual TCR specificity to Treg function in this model (Hori, 2002).

Expression of the MBP-specific TgTCR in RAG1⁺ H-2^b mice generally ranged from 50–75% of CD4⁺ T cells in the periphery. One would expect these MBP1–11-specific TgTCR⁺ T cells to be naïve T cells since antigen recognition is restricted to H-2^u. However, if these are dual TCR T cells, there may be some question as to their naïve status. Still, a study by Hah et al (2005) found that tolerance induction through one TgTCR did not affect the activation kinetics when stimulating the same T cells through a second TgTCR with a different specificity. Other studies have found some level of cross-tolerance or cross-antagonism in dual TCR systems (Zhou et al., 2004; Yang et al., 2003; Robertson et al., 1999). Further investigation will be required to assess the activation status of the MBP-specific TgTCR T cells selected in RAG1⁺ *H-2^{b/b}* mice.

We were also able to detect MBP-specific TgTCR⁺ T cells in the periphery of *rag1*^{-/-} offspring that were heterozygous for *H-2^{b/u}*, demonstrating that RAG1 activity is not necessary for TgTCR expression. In this case, the mice were unable to undergo endogenous TCR gene rearrangement so it is likely that TgTCR⁺ T cells utilized the TgTCR for positive selection in the context of H-2^u, but not H-2^b. TgTCR was expressed on all CD4⁺ T cells in this set of offspring and the presence of T cells expressing endogenous TCR was not required. The occurrence of spontaneous EAE was noted in TgTCR⁺ *H-2^{b/u}* offspring but not in any TgTCR⁺ *H-2^{b/b}* offspring.

In summary, we have generated MBP1–11-specific TgTCR mice on the *H-2^{b/b}* background and we have found that in the absence of *H-2^u*, phenotypic expression of the TgTCR in the periphery of *H-2^b* mice requires the presence of a functional *rag1* gene. The mechanism(s) of this RAG1 regulatory role of TgTCR expression on a different *H-2* background are of interest since this process may lead to the peripheral expression of TCRs that are only marginally selected or not generally selected in the thymus. Such a process could diversify the T cell repertoire as well as allow the escape of potentially autoreactive T cells. Finally, the ability of such mechanisms to promote the generation of dual TCR regulatory T cells and the role of such regulatory T cells are also of significant interest since the dual TCR feature may be advantageous or even required in particular regulatory mechanisms.

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Figure 1.

MBP1-11-specific TgTCR T cells do not proliferate to antigen in the context of H-2^b antigen presenting cells. A MBP1-11-specific T cell line derived from TgSCID mice was stimulated with different concentrations of MBP1-11 peptide (3 ug/ml shown) in the presence of either H-2^u APC (from B10.PL) or H-2^b APC (from C57BL6). Tg TCR T cells were also stimulated with ConA (10 ug/ml) and PPD (5 ug/well). Data are presented as counts per minute (CPM) of incorporated [³H]-thymidine.

Figure 2. Breeding scheme and expected resulting genotypes from F1 and F2 offspring. The proportions for the *rag1* genotypes in the F2 offspring are shown with the proportions for the H-2 and TgTCR genotypes (boxed). 3H12 phenotypic screening for TgTCR expression could not distinguish between the homozygous or heterozygous transgenic genotypes that were consequently combined (brackets) for the phenotypic frequency analysis.

Figure 3.

(A) The RAG neo primer specifically amplified the mutated *rag1* gene in RAG1KO mice but not the *rag1* wildtype gene in TgSCID mice, while the RAG wt primer amplified the wildtype *rag1* gene in TgSCID mice but not the RAG1KO gene. (B) H-2K^b primers specifically amplified a 231 bp PCR product (arrow) in RAG1KO (H-2K^b) but not in TgSCID (H-2K^u) or CB.17SCID (H-2K^d) mice. (C) H-2K^u primers specifically amplified a 231 bp PCR product (arrow) in TgSCID but not RAG1KO or CB.17SCID mice. α -actin (α -act) primers served as a positive control.

Figure 4.

Phenotypic screening for TgTCR expression. TgSCID (A) served as a positive control and RAG1KO (B) served as a negative control for 3H12 staining. *rag1*^{+/-}*H-2*^{b/b}(C), but not *rag1*^{-/-}*H-2*^{b/b}(D), offspring were found to express the TCR transgene-associated clonotypic marker recognized by the 3H12 mAb. Data are shown as fluorescent intensity of anti-CD4-FITC staining (x-axis) relative to staining with the 3H12 mAb plus PE-labeled anti-mouse IgM secondary Ab (y-axis).

Figure 5. *rag1^{-/-} H-2^{b/b}* offspring that were peripherally negative for the TCR transgene-associated surface marker 3H12 were found to bear the TCR transgenes in genomic DNA. Primers were designed to specifically detect the presence of the MBP1–11-associated BV8 (A) and AV4 (B) TCR transgenes by PCR. Out of 9 TgRAG offspring (*rag1^{-/-} H-2^{b/b}*) that stained negative with the 3H12 mAb, 8 were found to be positive for both the BV8 and AV4 transgenes. Positive controls for the TCR transgenes included a TgSCID mouse and a TgRAG 3H12⁺ offspring. Negative controls included TgSCID^{-/-} and RAG1KO mice.

Table 1

Offspring analysis from the F2 generation resulting from an intercross of *ragI*^{+/-} *H-2*^{b/u} *tgtr*^{+/-} mice.

	Observed frequency (Expected frequency)	<u>H-2^a</u>		
		<u>u/u</u>	<u>u/b</u>	<u>b/b</u>
<i>ragI</i> ^{-/-}	24/103=0.23 (0.25)	4/103=0.039 (0.0625)	9/103=0.087 (0.125)	11/103=0.107 (0.0625)
<i>ragI</i> ^{+/-}	56/103=0.54 (0.5)	7/103=0.068 (0.125)	23/103=0.22 (0.25)	26/103=0.25 (0.125)
<i>ragI</i> ^{+/+}	23/103=0.22 (0.25)	6/103=0.058 (0.0625)	12/103=0.12 (0.125)	5/103=0.048 (0.0625)
		<u>u/u</u>	<u>u/b</u>	<u>b/b</u>
<i>ragI</i> ^{-/-}	3H12⁺ b	3/4=0.75 (0.75)	8/9=0.89 (0.75)	0/11=0 (0.75)
<i>ragI</i> ^{+/-}	3H12⁺	3/5=0.6 (0.75)	0/1=0 (0.75)	8/12=0.67 (0.75)
<i>ragI</i> ^{+/+}	3H12⁺	nd ^c	nd	nd

^a H-2 genotype observed frequencies associated with each of the *ragI* genotypes were calculated using the total number of F2 mice screened (n=103).

^b 3H12⁺ observed frequencies associated with each *ragI* × H-2 genotype combination were calculated using the number of mice within each subset that underwent 3H12 screening.

^c nd, not done.

Table II

Summary from 2 breeding schemes of expected vs. observed frequencies of 3H12⁺ staining in TgRAG offspring that are *H-2^{b/b}* and either homozygous (*rag1^{-/-}*) or heterozygous (*rag1^{+/-}*) for the RAG1KO gene.

	3H12 ⁺ in <i>rag1^{-/-} H2^{b/b}</i>		3H12 ⁺ in <i>rag1^{+/-} H2^{b/b}</i>	
	<u>expected</u>	<u>observed</u>	<u>expected</u>	<u>observed</u>
<i>rag1^{+/-} H2^{b/u} tgtr^{+/-}</i> F1 intercross ^a	0.75	0 (0/11)	0.75	0.67 (8/12)
<i>rag1^{+/-} H2^{b/b} tgtr^{+/-}</i> intercross	0.75	0 (0/16)	0.75	0.47 (9/19)

^aData from Table I.