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Vaccination with BV8S2 Protein Amplifies TCR-Specific Regulation and Protection Against Experimental Autoimmune Encephalomyelitis in TCR BV8S2 Transgenic Mice[1]

Halina Offner,[2]*† Kirsten Adlard,* Bruce F. Bebo, Jr.,*† Jeanette Schuster,* Gregory G. Burrows,*†‡ Abigail C. Buenafe,† and Arthur A. Vandenbark*†§

TCR determinants overexpressed by autopathogenic Th1 cells can naturally induce a second set of TCR-specific regulatory T cells. We addressed the question of whether immune regulation could be induced naturally in a genetically restricted model in which a major portion of TCR-specific regulatory T cells expressed the same target TCR BV8S2 chain as the pathogenic T cells specific for myelin basic protein (MBP). We found vigorous T cell responses to BV8S2 determinants in naive mice that could be further potentiated by vaccination with heterologous BV8S2 proteins, resulting in the selective inhibition of MBP-specific Th1 cells and protection against experimental encephalomyelitis. Moreover, coculture with BV8S2-specific T cells or their supernatants reduced proliferation, IFN-γ secretion, and encephalitogenic activity of MBP-specific T cells. These results suggest that immune regulation occurs through a nondeletional cytokine-driven suppressive mechanism. ***The Journal of Immunology,*** **1998, 161: 2178–2186.**

Immunoregulation directed at TCR determinants represents a powerful mechanism for inhibiting the development and expression of autopathogenic Th1 cells directed at tissue-specific Ags (1). The prototype model that elucidated this type of anti-idiotypic regulation is experimental autoimmune encephalomyelitis (EAE),[3] a paralytic disease of the central nervous system mediated by encephalitogenic Th1 cells (2). In mice and rats, pathogenic T cells specific for myelin basic protein (MBP) preferentially utilize the BV8S2 gene paired with the AV2 or AV4 genes (3–5). The overexpression of BV8S2 during EAE apparently causes the spontaneous induction of anti-BV8S2-specific T cells (6), which can be expanded and in which the inhibitory function can be potentiated in vivo by vaccination with TCR peptides that resemble naturally processed TCR determinants. Thus, injection of low doses of recombinant BV8S2 proteins or BV8S2 peptides can prevent and treat EAE in Lewis rats and B10.PL mice (7–10). The importance of this regulatory mechanism is further documented by the development of more severe EAE in rats and mice that have been tolerized to TCR peptides or proteins (11–13).

The generation of TCR transgenic (Tg) mice specific for MBP provides a system uniquely useful for evaluating the mechanisms of disease development and regulation in EAE. Goverman et al. (14) produced mice that were Tg for either or both of the TCR α (AV2)- and β (BV8S2)-chains of a T cell clone specific for MBP-NAc_{1-11} peptide, which is encephalitogenic for mice expressing the H-2^u haplotype (15). Although spontaneous proliferation responses to MBP-NAc_{1-11} were not reported in single Tg mice, unimmunized double Tg mice did have T cell responses to this peptide, and some mice, mostly juveniles, kept in nonsterile conditions developed spontaneous clinical EAE (14, 16). The rate of spontaneous EAE could be dramatically enhanced from ~15 to 100% by crossing TCR$\alpha\beta$ Tg mice with RAG-1-deficient mice that could not rearrange endogenous TCR genes (17). These results suggested that endogenous rearrangement may provide a relatively small pool of regulatory T cells, possibly including those involved in network interactions, that appears somewhat later than the encephalitogenic T cells and naturally prevents EAE. In double TCR Tg mice, these regulatory T cells would be expected to rearrange either one or both of their TCR chains and express only one or neither of the TCR$\alpha\beta$ transgenes.

In the current study, we addressed the question of whether immune regulation directed at TCR determinants could be induced in single Tg mice expressing a TCR β-chain specific for MBP-NAc_{1-11} peptide (developed by Dr. Joan Goverman (14)). In these mice, essentially all of the T cells express the Tg BV8S2 chain, paired with naturally rearranged TCR α-chains. T cells specific for MBP-NAc_{1-11} are enriched and express AV2 or AV4 chains that are known to pair preferentially with BV8S2 (4–5). Conceivably, these mice could also produce a distinct population of TCR peptide-specific T cells that express the Tg BV8S2 chain paired with a different naturally rearranged TCR α-chain. This would create a situation in which a substantial portion of regulatory T cells would express the same BV8S2 chain as the pathogenic T cell targeted for regulation. Indeed, in a previous study in single Tg mice, sequence analysis of T cells specific for the MBP-NAc_{1-11} peptide demonstrated uniform expression of the transgene and preferential expression of the AV2S3 gene (18). Moreover, a BV8S2-specific regulatory T cell line derived from the same mice predominantly expressed the BV8S2 transgene as well as the same AV2S3 gene as the MBP-NAc_{1-11}-specific T cells, differing only in the AV

*Neuroimmunology Research R&D-31, Portland Veterans Affairs Medical Center, and Departments of †Neurology, ‡Biochemistry and Molecular Biology, and §Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, OR 97201

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[2] Address correspondence and reprint requests to Dr. Halina Offner, Neuroimmunology Research R&D-31, Portland Veterans Affairs Medical Center, 3710 SW Veterans Hospital Road, Portland, OR 97201. E-mail address: offner.halina@portland.va.gov

[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; Tg, transgenic or transgene; Mo-, mouse; Rt-, rat; GST, glutathione *S*-transferase; CDI, cumulative disease index; MCS, mean clinical score; SI, stimulation index; LN, lymph node; NAc, N-acetylated; RAG, recombination-activating gene.

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CDR3 sequence. These data indicated the presence of multiple T cell clonotypes that both expressed the BV8S2 transgene and recognized a dominant BV8S2 epitope.

According to network theory, overexpression of the BV8S2 transgene would be expected to enhance the spontaneous recognition of BV8S2 determinants. In this genetically restricted model, it is intriguing to consider what kind of regulation would result when the regulatory T cells express the same BV gene as the pathogenic target T cells. Would the BV8S2 T cells self-regulate, and if not, what factors would contribute to a selective regulatory mechanism? In the current study, we have established that the BV8S2 transgene engenders an elevated response both to MBP-$NAc_{1–11}$ peptide and to self or heterologous BV8S2 proteins. Responses to MBP-$NAc_{1–11}$ or BV8S2 Ags were potentiated by immunization, leading either to a relapsing form of EAE or, in the case of the heterologous rat BV8S2 protein, to protection against EAE.

Materials and Methods

Mice

Tg mice bearing the functionally rearranged BV8S2 gene specific for MBP-$NAc_{1–11}$ on the B10.PL background were kindly provided by Dr. Joan Goverman (University of Washington Seattle, WA). Male Tg mice were bred with B10.PL females and the offspring tested for expression of the Tg by FACS analysis of blood cells stained for BV8S2 as described previously (14). For some experiments, mice expressing the BV8S2 Tg were compared with littermates that did not express the Tg. The colony was housed and cared for at the Animal Resource Facility (Portland Veterans Affairs Medical Center) according to institutional guidelines.

Antigens

N-acetylated MBP-1–11 peptide (Ac-ASQKRPSQRSK) was synthesized using solid phase techniques and was purified by HPLC at the Beckman Institute, Stanford University (Stanford, CA). Mouse basic protein (Mo-BP) was extracted from mouse brains (Pel-Freeze Biologicals, Rogers, AK) and purified as previously described (19). Glutathione *S*-transferase (GST) and GST-BV8S2 proteins were expressed and purified as described previously (12). The GST-BV8S2 fusion protein contains the complete BV, BD, and BJ regions and the first 19 residues of the BC region from the TCR of an encephalitogenic rat T cell clone fused to the C-terminal end of GST. To control for the GST-BV8S2 protein, the GST protein was produced and purified using the same expression system. In previous experiments, neonatal injection of the GST protein in IFA did not alter the course of EAE induced with guinea pig MBP/CFA in rats (12), nor did GST affect the course of EAE induced with MBP-$NAc_{1–11}$/CFA in BV8S2 Tg mice (see below). The GST protein was included as a control in all tissue culture experiments utilizing the GST-BV8S2 protein. The BV8S2-his6 protein contains the identical BV, BD, and BJ regions plus the first amino acid of the BC region followed by a C-terminal histidine tag. The mouse BV8S2 protein (JB9) consists of the mouse BV8S2 transgene product, including the BV, BD, and BJ regions, and the first 19 residues of the BC region. To control for the BV8S2-his6 proteins, the identical host harboring a plasmid lacking the coding sequence of the BV8S2 protein was used in the same expression and purification process to produce a fraction that did not contain detectable protein, but would contain contaminants. This fraction had no effect on the course of EAE or on lymphocyte proliferation responses in rats (our unpublished data). A truncated form of the rat AV2S3 protein (from an encephalitogenic T cell clone specific for $MBP_{72–89}$ peptide) without the constant region was cloned and expressed in *Escherichia coli* as a control in tissue culture experiments (Buenafe et al., manuscript in preparation).

Induction of active EAE and protection with BV8S2 protein

EAE was induced in Tg male mice by injecting 400 μg of MBP-$NAc_{1–11}$/CFA containing 200 μg of *Mycobacterium tuberculosis* s.c. over four sites on the flank. For protection experiments (six total), mice were injected with 12.5 μg of recombinant rat or mouse BV8S2 proteins/IFA (experimental) or saline/IFA (sham control) i.p. on days -7 and $+3$ relative to injection of the MBP-$NAc_{1–11}$, according to the protocol developed by Kumar and Sercarz (9). In three experiments, mice were boosted weekly with 12.5 μg BV8S2 protein or saline given s.c. Mice were assessed daily for clinical signs of EAE according to the following scale: 0 = no signs; 1 = limp tail; 2 = moderate hind limb weakness (waddling gait); 3 = moderately severe hind limb weakness; 4 = severe hind limb weakness; 5 = paraplegia; 6 = quadriplegia, moribund condition.

The primary episode was defined as the period beginning on the first day of clinical signs and ending on the second day after the daily clinical scores returned to the lowest score before a second increase in clinical severity (relapse) or no further change in daily scores (chronic EAE). The cumulative disease index (CDI) was determined for each mouse for the primary episode and relapse by summing the daily clinical scores during each episode, and the mean CDI $\pm$ SEM was calculated for the control and experimental groups. For passive EAE, the CDI was determined for the first 10 days of clinical EAE (10-day CDI). The mean clinical score (MCS) was calculated for each mouse by dividing the CDI by the duration (days) of the primary episode or relapse, and the mean $\pm$ SEM was calculated for the control and experimental groups, or for the clinically affected mice only (see tables). Representative mice from control and protected groups were sacrificed for histologic analysis of spinal cords. Spinal cords were removed, fixed in formalin, and embedded in paraffin, and sections from throughout the cord were stained with hematoxylin and eosin.

Isolation of lymphocytes

Spinal cord cells were isolated as previously described (20). Briefly, spinal cords were isolated by insufflation, washed in RPMI 1640 to remove contaminating blood cells, then passed through a wire mesh screen to obtain a single-cell suspension. This suspension was then washed and resuspended in 80% isotonic Percoll. T cells were isolated at the 40/80% interface of a Percoll step gradient. Lymph nodes (LN) and spleens were removed surgically, and single cell suspensions were prepared as described previously (21).

T cell lines

MBP-$NAc_{1–11}$-specific T cell lines were derived from the draining LN of BV8S2 Tg mice immunized with 400 μg $NAc_{1–11}$ peptide/CFA over four sites on the flank (coculture experiments) or with 200 μg GST-BV8S2/CFA (cytokine experiments) as described (12). Both immunizations contained 200 μg *M. tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI). LN cells were isolated 14 to 16 days after immunization and cultured for 3 days in the presence of 50 μg/ml Ag at 5×10^6 LN cells/ml in stimulation medium containing 1% FBS, 0.05 mM 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics in RPMI. T cell lines were then expanded in growth medium (stimulation medium supplemented with 10% FBS and 20 U/ml human rIL-2) for 7 to 10 days, followed by stimulation of 5×10^5 T cells/ml with Ag plus 10^7 irradiated Tg thymocytes/ml as APCs.

Proliferation assay

Proliferative responses of the T cell lines were evaluated in 96-well microtiter plates by incubating 4×10^5 LN or spleen cells or 2×10^4 T lymphocyte line cells/well with 10^6 irradiated thymocytes/well plus Ag at varying concentrations ranging from 1 to 100 μg/well. Cultures were incubated for 72 h at 37°C and 7% CO_2, the last 18 h in the presence of 0.5 μCi [^{3}H]thymidine. Cells were harvested onto glass fiber filters, and thymidine uptake was determined by liquid scintillation. Mean cpm $\pm$ SEM was calculated from triplicate wells. The stimulation index (SI) was obtained by dividing cpm from Ag-stimulated wells by cpm from wells with no Ag. SI in cultures stimulated with GST alone was subtracted from the SI induced with GST-BV8S2 protein.

Phenotyping

Phenotyping of T cells was performed on a FACScan (Becton Dickinson, Mountain View, CA) as previously described (22). Spinal cord T cells were double stained with soluble mouse OX40 ligand/human Ig fusion protein (a gift from Dr. Andrew D. Weinberg, Chiles Research Center, Providence Hospital, Portland, OR) for 15 min at room temperature, washed, and incubated with a FITC-conjugated anti-human Ig (mouse and rat preabsorbed, PharMingen, San Diego, CA) for 15 min at room temperature. OX40 ligand-stained cells were subsequently stained with anti-mouse CD4-phycoerythrin (PharMingen). Two-color immunofluorescence analysis was performed on a FACScan (Becton Dickinson). Quadrants were drawn based on background staining with control Abs.

Measurement of cytokine secretion

LN and spleen cells were suspended at 4×10^6 cells/ml in stimulation medium with and without specific Ags. Ag specific T cell lines were suspended at 0.5×10^6 cells/ml and cocultured with 5×10^6 irradiated spleen cells as APC in stimulation medium as above. Cell culture supernatants were recovered at 72 h and frozen at -70°C until needed for the cytokine assay. Measurement of cytokines was performed by ELISA developed in

our laboratory using cytokine specific capture and detection Abs (PharMingen). Capture Abs for IFN-γ, IL-5, and IL-10 were diluted to 2 μg/ml in bicarbonate coating buffer (0.1 M $NaHCO_3$, pH 8.2). Standard curves for each assay were generated using recombinant mouse cytokines (PharMingen), and the concentration of cytokines in the cell supernatants was determined by interpolation from the appropriate standard curve.

Assessment of Ab responses

Ab reactivity to Mo-BP, MBP-$NAc_{1–11}$ peptide, and rat and mouse recombinant BV8S2 proteins was determined by indirect ELISA as described previously (22). Briefly, mouse antisera from BV8S2-protected and control Tg mice with EAE were incubated in Ag-coated wells, and bound Ab was detected spectrophotometrically with peroxidase-labeled rabbit anti-mouse Ab and *o*-phenylenediamine as a substrate. Differences between groups were determined using Student's *t* test.

Coculture of MBP-$NAc_{1–11}$- and rat (Rt)-BV8S2-specific T cell lines

To study the effects of coculture on proliferation of encephalitogenic T cells, 1 or 2×10^4 MBP-$NAc_{1–11}$-specific T cells were stimulated in the presence or absence of 2×10^4 Rt-BV8S2-specific T cells with $NAc_{1–11}$ (0.5 μg/ml) or Mo-BP (5 μg/ml) plus 10^6 irradiated Tg thymocytes as APC in 96-well microtiter plates. Likewise, 2×10^4 BV8S2-specific T cells were stimulated with $NAc_{1–11}$ plus APC. Cultures were incubated for 72 h at 37°C with 7% CO_2, and proliferation was measured by thymidine uptake as described above. To determine the effects of coculture on encephalitogenicity, 1.5×10^6 MBP-$NAc_{1–11}$-specific T cells were cultured in the bottom compartment of a transwell system with $NAc_{1–11}$ peptide (10 μg/ml) and 3×10^7 irradiated Tg splenocytes as APC in multiple wells; the top well, which was separated from the bottom well by a semipermeable membrane, contained 3×10^6 Rt-BV8S2 T cells plus Rt-BV8S2 (10 μg/ml) plus 3×10^7 APC. Wells were cultured in parallel containing each cell type alone plus Ag plus APC. The cultures were incubated for 72 h, 2×10^7 MBP-$NAc_{1–11}$ T cells from control wells were transferred by i.p. injection into three Tg and three parental non-Tg recipient mice, and the same number of MBP-$NAc_{1–11}$-specific T cells from the bottom compartment of the cocultured transwells were transferred into three Tg recipients. The recipient mice were boosted with pertussigen at the same time (75 ng) and 48 h (200 ng) after transfer of the T cells and were scored daily for signs of EAE as described above. To evaluate the effects of coculture on IFN-γ release, cell culture supernatants from cocultured and control wells were recovered at 72 h, and measurement of IFN-γ was performed by ELISA as described above.

Results

Effects of the BV8S2 Tg on spontaneous recognition of MBP-$NAc_{1–11}$ and BV8S2 determinants

Introduction of the BV8S2 Tg would be expected to limit the TCR BV repertoire and, depending on the choice of paired AV genes in the CD4 compartment, enrich the pool of circulating T cells specific for MBP-$NAc_{1–11}$. In this study, we sought to determine whether there was an increased spontaneous recognition of BV8S2 determinants as a result of the overexpression of BV8S2 in the Tg mice. In a previous study in BV8S2 Tg mice (18), evaluation of the TCR BV repertoire revealed that >90% of T cells expressed BV8S2, in contrast to <20% BV8S2 T cells in non-Tg B10.PL littermates. A similar evaluation of the native TCR AV repertoire revealed no significant skewing in the BV8S2 Tg mice compared with the non-Tg B10.PL littermates, indicating no general preferential pairing of AV genes with the overexpressed BV8S2 gene.

To evaluate the effects of the BV8S2 Tg specific for MBP-$NAc_{1–11}$ on spontaneous T cell responses, LN and spleen cells from naive BV8S2 Tg mice and non-Tg B10.PL littermates were assessed for Ag-driven proliferation and cytokine release upon stimulation with MBP-$NAc_{1–11}$ peptide. As shown in Figure 1, there were highly significant T cell proliferation responses to the encephalitogenic MBP-$NAc_{1–11}$ peptide in the LN and spleens from naive Tg mice but not from naive B10.PL littermates. As we have shown in a previous study, the BV8S2 Tg mice favored positive selection of MBP-$NAc_{1–11}$-specific T cells expressing the BV8S2 Tg in combination with the AV2S3 or AV4 genes (18).

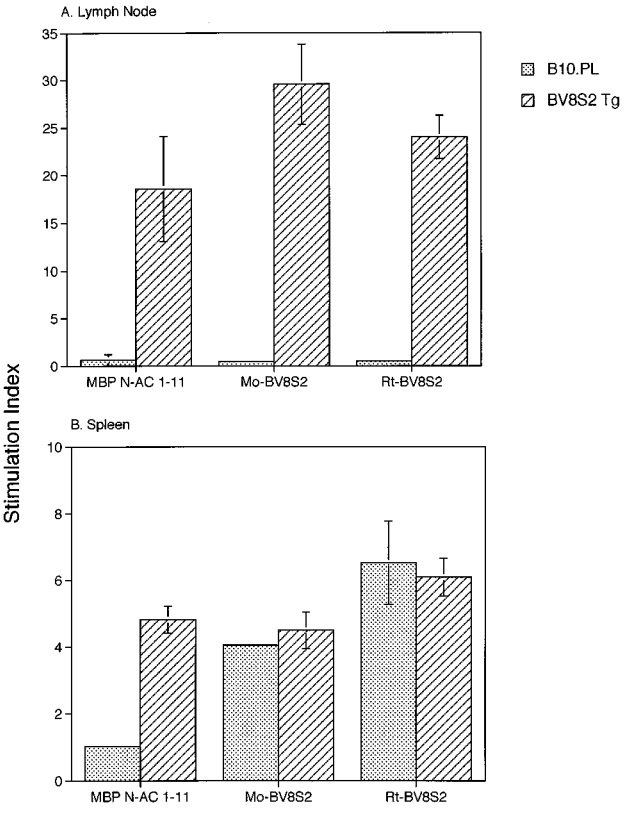


FIGURE 1. Proliferation responses of LN cells (*A*) and splenocytes (*B*) to MBP-$NAc_{1–11}$ peptide and recombinant Mo-BV8S2 and Rt-BV8S2 proteins in 10- to 12-wk old naive BV8S2 Tg mice and non-Tg littermates. SI = cpm in response to Ag/cpm of unstimulated cells. Unstimulated LN cells gave background values of 250 cpm (non-Tg mice) and 2120 cpm (Tg mice); splenocytes gave background values of 3483 cpm (non-Tg) and 3440 cpm (Tg mice).

Despite the increased natural recognition of MBP-$NAc_{1–11}$, spontaneous EAE was never observed in the BV8S2 single Tg mice.

The enhanced proliferation response of MBP-$NAc_{1–11}$-specific T cells in the absence of spontaneous clinical EAE in naive Tg mice suggested a lack of maturation to encephalitogenic Th1 effector cells known to produce elevated levels of inflammatory cytokines including IFN-γ. Indeed, MBP-$NAc_{1–11}$-specific T cells from naive Tg mice produced low to absent levels (<1 ng/ml) of both Th1 and Th2 cytokines in LN and spleens (Table I), despite strong proliferation responses (Fig. 1).

Of importance to the basic question posed in this study, LN cells from naive Tg mice also had strikingly higher responses than LN cells from non-Tg B10.PL littermates to both the overexpressed Mo-BV8S2 and the heterologous Rt-BV8S2 proteins (Fig. 1*A*). Lower but comparable responses to both BV8S2 proteins were also observed in spleens from both Tg and non-Tg mice (Fig. 1*B*). These data indicate a background recognition of BV8S2 determinants in B10.PL mice that is strongly and spontaneously enhanced in the LN of BV8S2 Tg mice. However, unlike T cells specific for MBP-$NAc_{1–11}$ peptide, which produced very low levels of cytokines, spleen cells from naive Tg mice secreted significantly elevated levels of both IFN-γ and IL-10 upon stimulation with either Mo- or Rt-BV8S2 proteins (Table I).

Immunization of BV8S2 Tg mice with MBP-$NAc_{1–11}$ in CFA induced a Th1 response and EAE

To induce clinical signs of EAE, BV8S2 Tg mice were immunized with MBP-$NAc_{1–11}$ peptide in CFA. This immunization, in the

Table I. *Lymphokine production from BV8S2 Tg B10.PL mice*

		Response (ng/ml) to:					
		MBP-NAc_{1-11}		Rt-BV8S2		Mo-BV8S2	
	Cells	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10
Immunization							
Naive	LN	0.1 ± 0.1	0.1 ± 0.1	<0.1	0.1 ± 0.1	0.9 ± 0.8	0.6 ± 0.2
	SPL	0.5 ± 0.5	0.3 ± 0.2	2.0 ± 0.1	0.8 ± 0.0	3.4 ± 0.1	1.4 ± 0.0[a]
Rt-BV8S2/IFA	LN	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	ND	ND
	SPL	0.4 ± 0.0	0.7 ± 0.0	3.3 ± 0.1[b]	1.5 ± 0.0[b]	ND	ND
MBP-NAc_{1-11}/CFA (EAE)	LN	15.4 ± 0.6[b]	0.2 ± 0.0	ND	ND	10.9 ± 0.6[b]	0.2 ± 0.0
	SPL	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.6 ± 0.0	ND	ND
MBP-NAc_{1-11}/CFA	LN	0.8 ± 0.1[c]	0.1 ± 0.1	>4.0[b]	0.5 ± 0.0	ND	ND
Rt-BV8S2/IFA (protected)	SPL	0.5 ± 0.0	0.2 ± 0.0	1.3 ± 0.0[c]	3.2 ± 0.0[b,c]	ND	ND
T cell lines							
MBP-NAc_{1-11}/CFA[d]		69.3 ± 3.3	0.8 ± 0.0	>32	1.4 ± 0.2	27.3 ± 1.2	0.8 ± 0.1
Mo-BV8S2/CFA[e]		5.8 ± 0.2	22.5 ± 0.7	ND	ND	ND	ND

[a] Significant increase vs response induced by MBP-NAc1-11. SPL, spleen.
[b] Significant increase vs naive response.
[c] Significant change in protected vs sick mice.
[d] Mice were immunized with MBP-NAc_{1-11}/CFA, and three separate T cell lines were selected from LN with MBP-NAc_{1-11}, Rt-BV8S2, or Mo-BV8S2.
[e] Mice were immunized with Mo-BV8S2/CFA, and a T cell line was selected from LN with MBP-NAc_{1-11}.

absence of pertussigen, induced an initial severe episode of clinical EAE in >80% of mice, followed by less severe relapses or chronic disease in a majority of mice (saline/IFA sham-treated control groups, Table II and Fig. 2). The proliferation response to MBP-NAc_{1-11} of LN cells from immunized Tg mice was similar to or less than that from naive unimmunized Tg mice. However, the cytokine profile of MBP-NAc_{1-11}-stimulated LN cells and T cell lines from the immunized mice was clearly Th1-like, with vastly elevated levels of IFN-γ (Table I). Interestingly, LN cells and T cell lines from mice immunized with MBP-NAc_{1-11} peptide in CFA also had strongly elevated (>10-fold) naturally induced IFN-γ-dominated Th1 responses to Mo- and Rt-BV8S2 proteins in vitro (Table I), even though neither of these BV8S2 proteins was included in the injection mixture. This result clearly demonstrated that expansion and maturation of TCR-reactive T cells occurred as a consequence of the activation of MBP-NAc_{1-11}-specific T cells.

Vaccination with rat but not mouse BV8S2 proteins protected against EAE

The naturally induced response to BV8S2 determinants in BV8S2 Tg mice was clearly unable to prevent induction of EAE upon immunization with MBP-NAc_{1-11}/CFA. To boost the response to Mo-BV8S2 determinants, 10- to 12-wk-old Tg mice were vaccinated with heterologous Rt-BV8S2 proteins that had induced strong proliferation and cytokine responses in naive Tg spleen cell cultures and that boosted T cell-mediated IFN-γ and IL-10 cytokine responses when injected with IFA (Table I). Mice vaccinated with Rt-BV8S2 proteins (either the GST-BV8S2 or the BV8S2-his6 forms) in IFA on days −7 and +3 relative to induction of EAE with MBP-NAc_{1-11}/CFA had a significantly lower incidence of EAE (38 vs 81%, composite of three separate experiments, $p < 0.05$), and as a group, a significantly lower CDI and MCS than control mice treated with saline/IFA (Table II and Fig. 2*A*). Mice treated with GST/IFA developed EAE indistinguishable from saline/IFA controls (not shown). Vaccinated mice that were not protected developed an intensity of clinical EAE similar to that in control mice, although in most vaccinated mice the CDI and MCS values were nominally less.

To sustain responses to BV8S2 proteins, mice in subsequent experiments were vaccinated on days −7 and +3 as before and then boosted weekly with Rt-BV8S2 protein, injected s.c. in saline (Fig. 2*B*). As shown in Table II, this boosting regime improved the degree of resistance to the primary episode of

Table II. *Inhibition of primary and relapsing EAE with recombinant BV8S2 proteins in BV8S2 Tg mice*[a]

Treatment (10–12 wk old)	Primary Episode					Relapse				
	Incidence	Onset	Duration	CDI	MCS	Incidence	Onset	Duration	CDI	MCS
Saline/IFA	13/16	15 ± 1	17 ± 2	49 ± 10	2.8 ± 0.5	ND	ND	ND	ND	ND
Rt-BV8S2/IFA (−7, +3, no boost)	6/16*	14 ± 1	18 ± 2	17 ± 7*	0.9 ± 0.4**	ND	ND	ND	ND	ND
Saline/IFA	10/10	13 ± 1	22 ± 1	63 ± 9	3.1 ± 0.4	6/9	35 ± 2	10 ± 1	22 ± 6	2.3 ± 0.5
Rt-BV8S2/IFA (−7, +3, boosted weekly in saline)	1/9***	15	22	7 ± 7***	0.3 ± 0.3***	2/9	35 ± 4	11 ± 3	6 ± 4*	0.6 ± 0.4*
Saline/IFA	6†/6	13 ± 1	13 ± 2	49 ± 30	4.5 ± 0.6	ND	ND	ND	ND	ND
Mo-BV8S2/IFA (−7,+3, boosted weekly in saline)	4‡/6	14 ± 0	9 ± 0	20 ± 9	2.8 ± 0.9	ND	ND	ND	ND	ND

[a] Onset and duration values included only clinically affected mice. CDI and MCS values include all mice in the respective groups, including mice protected from EAE.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; † four mice died; ‡ one mouse died.

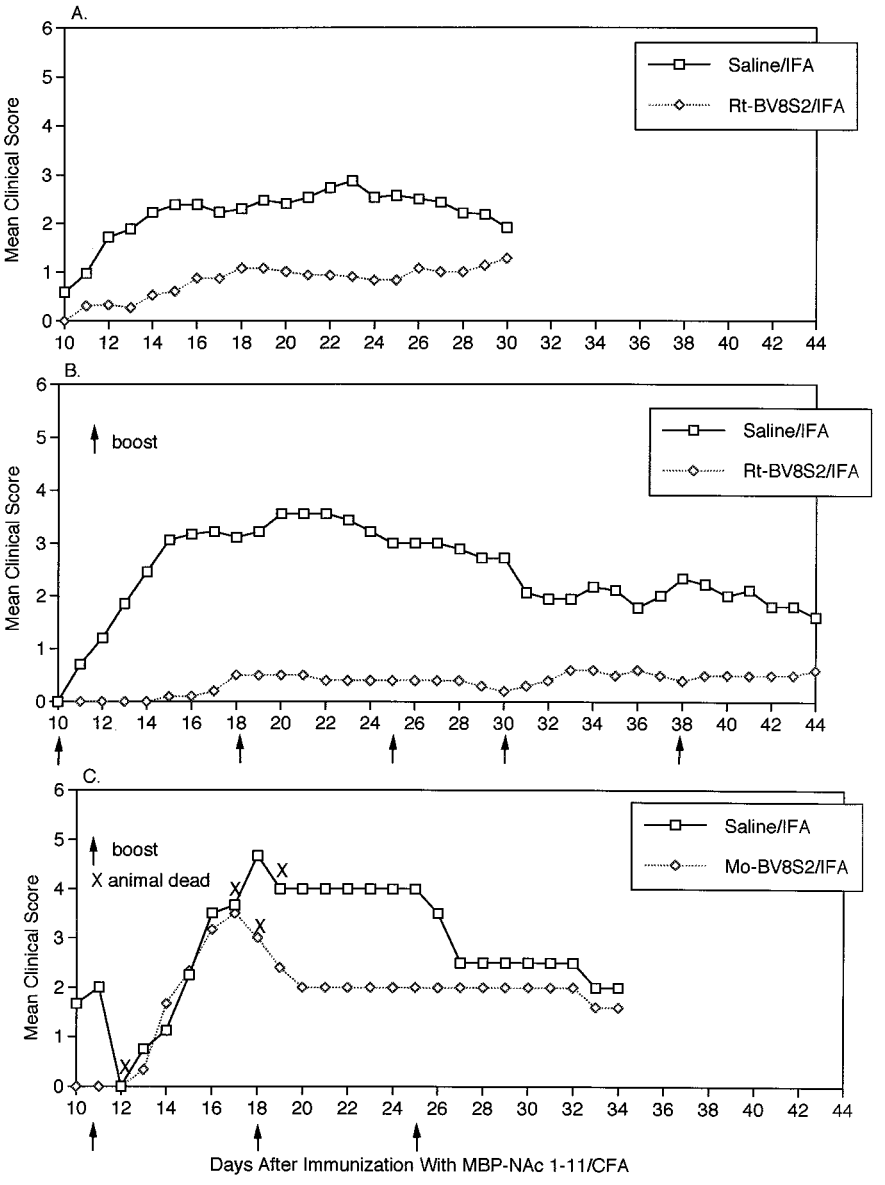


FIGURE 2. Course of clinical EAE in BV8S2 Tg mice treated with saline/IFA, Rt-BV8S2 protein/IFA, or Mo-BV8S2 protein/IFA. *A*, Tg mice treated with Rt-BV8S2 protein (12.5 μg of either GST-BV8S2 or BV8S2-his6 in IFA) on days −7 and +3 relative to challenge with MBP-NAc_{1-11}/CFA (composite of three separate experiments). *B*, Tg mice treated with Rt-BV8S2 protein (either GST-BV8S2 or BV8S2-his6) on days −7 and +3 relative to challenge with MBP-NAc_{1-11}/CFA and boosted weekly thereafter with Rt-BV8S2 protein in saline (composite of two separate experiments). *C*, Tg mice treated with Mo-BV8S2 protein on days −7 and +3 relative to challenge with MBP-NAc_{1-11}/CFA and boosted weekly thereafter with Mo-BV8S2 protein in saline.

EAE (11 vs 100% incidence, composite of two separate experiments), with highly significant differences in group disease severity scores. Moreover, mice vaccinated and boosted with Rt-BV8S2 proteins had significantly less severe relapses (Table II). Mice protected after vaccination with BV8S2 proteins had essentially no inflammatory lesions in spinal cord sections (Fig. 3), as well as a threefold reduction of both total and activated ($OX40^+$) inflammatory cells in the CNS compared with saline/IFA-treated controls (Fig. 4). Two of nine vaccinated mice that eventually developed mild EAE had intermediate levels of inflammatory cells in the CNS (Fig. 4).

In a consecutive experiment, treatment using the same regime with self Mo-BV8S2 protein in IFA was less effective in protecting mice against EAE than treatment with the heterologous Rt-BV8S2 proteins. As shown in Table II and Figure 2*C*, the severity of EAE in control mice was extreme (four of six mice died), and the treatment effect, although nominally less (only one of six mice died, with lower CDI and MCS scores), was not significant.

Responses to MBP-NAc_{1-11} peptide and BV8S2 proteins in vaccinated mice

The most meaningful immunologic difference between Rt-BV8S2-protected and control mice with EAE was a striking 19-fold reduction in IFN-γ release by LN cells stimulated with the MBP-NAc_{1-11} peptide (Table I). This 95% reduction in cytokine production was not reflected by differences in the proliferation response to MBP-NAc_{1-11}, which was essentially the same in protected and control LN cells (Fig. 5). Similarly, there was no difference in Ab response to Mo-MBP or MBP-NAc_{1-11} peptide in the two groups of mice (Fig. 6). However, unlike LN cells or an MBP-NAc_{1-11}-specific T cell line from the control mice with EAE, which produced very high levels of IFN-γ (69.3 ng/ml) and low levels of Th2 cytokines (IL-5, 1.2 ng/ml; IL-10, 0.8 ng/ml; Table I), an MBP-NAc_{1-11}-specific T cell line selected from mice vaccinated with Rt-BV8S2 protein had low proliferation response to the encephalitogenic peptide (1.2×), but was highly skewed toward production of Th2 cytokines (IL-5, >32 ng/ml, and IL-10, 22.5 ng/ml; Table I) instead of

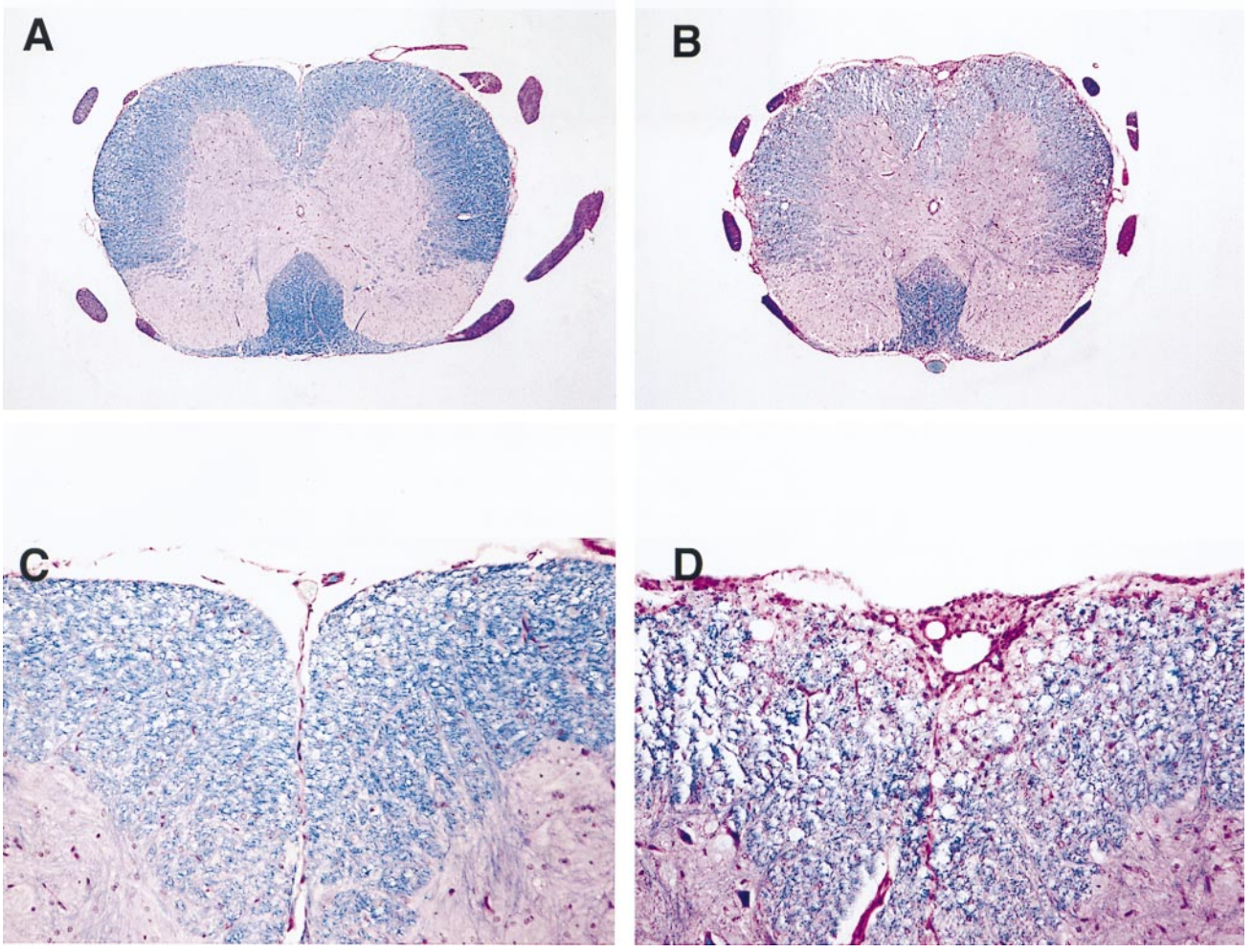


FIGURE 3. Histologic sections of spinal cords from control (saline/IFA-treated) Tg mice with EAE (*A* and *C*) and from Rt-BV8S2/IFA-protected Tg mice (*B* and *D*). Spinal cords were fixed, cross-sectioned, and stained with hematoxylin and eosin (magnification: *A* and *B*, 50×; *C* and *D*, 200×).

inflammatory cytokines (IFN-γ, 5.8 ng/ml; Table I) associated with induction of EAE.

LN cells from the Rt-BV8S2-protected and control mice with EAE had similar proliferation responses to the Rt-BV8S2 protein, with substantial cross-recognition of the Mo-BV8S2 protein, but minimal recognition of the Rt-AV2S3 protein, as expected (Fig. 5). The BV8S2-reactive LN T cells from protected and control mice produced similar high levels of IFN-γ (>4–11 ng/ml) with relatively low levels of IL-10 (Table I). However, the most obvious difference in response to BV8S2 proteins occurred in the spleen cells from protected mice, which produced 5× higher levels (3.2 ng/ml) of IL-10 than spleen cells from control mice (0.6 ng/ml), even though proliferation responses were not different (not shown). In addition to elevated splenic IL-10 responses to BV8S2, protected mice had higher Ab levels to both Rt- and Mo-BV8S2 proteins than control mice with EAE (Fig. 6).

MBP-NAc$_{1–11}$ T cells are inhibited when cocultured with Rt-BV8S2-specific T cells or supernatants

To evaluate the direct regulatory influence of BV8S2-specific T cells, an MBP-NAc$_{1–11}$-specific T cell line was activated with the MBP peptide and APC in the presence of Rt-BV8S2-specific T cells, but without added Rt-BV8S2 protein. In these coculture experiments, the proliferation response to the MBP-NAc$_{1–11}$ peptide was significantly inhibited (>50%) in the presence of the BV8S2-specific T cells (Fig. 7). Moreover, MBP-NAc$_{1–11}$-specific T cells incubated in transwells exposed to soluble factors produced by activated Rt-BV8S2-specific T cells had a similar reduction (40%) in secretion of IFN-γ and a significantly reduced ability to transfer clinical EAE to naive recipient mice (Fig. 7). These data clearly demonstrate the inhibitory capacity of soluble factors produced by BV8S2-specific T cells on the activation and encephalitogenic activity of MBP-NAc$_{1–11}$-specific T cells.

Discussion

This study was designed to address the fundamental question of whether regulation directed at TCR determinants could exist and function in genetically restricted animals in which the majority of T cells, including those with regulatory specificity, expressed the target V gene. The single TCR β-chain Tg mice used in this study predominantly expressed the Tg on >90% of mature T cells, and because the mice had a functional RAG gene, the experiment allowed for rearrangement of TCR α-chains. Such TCR α-chains, when paired with the BV8S2 Tg chain specific for MBP-NAc$_{1–11}$, could form new specificities, including that directed at a dominant determinant within the BV8S2 molecule itself (8). The positive identification of T cells expressing BV8S2 that are also specific for BV8S2 in the Tg mice does not preclude regulatory T cell populations that express other BV or AV genes, as was implied by enhancement of spontaneous EAE in TCRαβ double Tg mice lacking a functional *RAG-1* gene (17). However, our demonstration of BV8S2-specific T cells that differed from

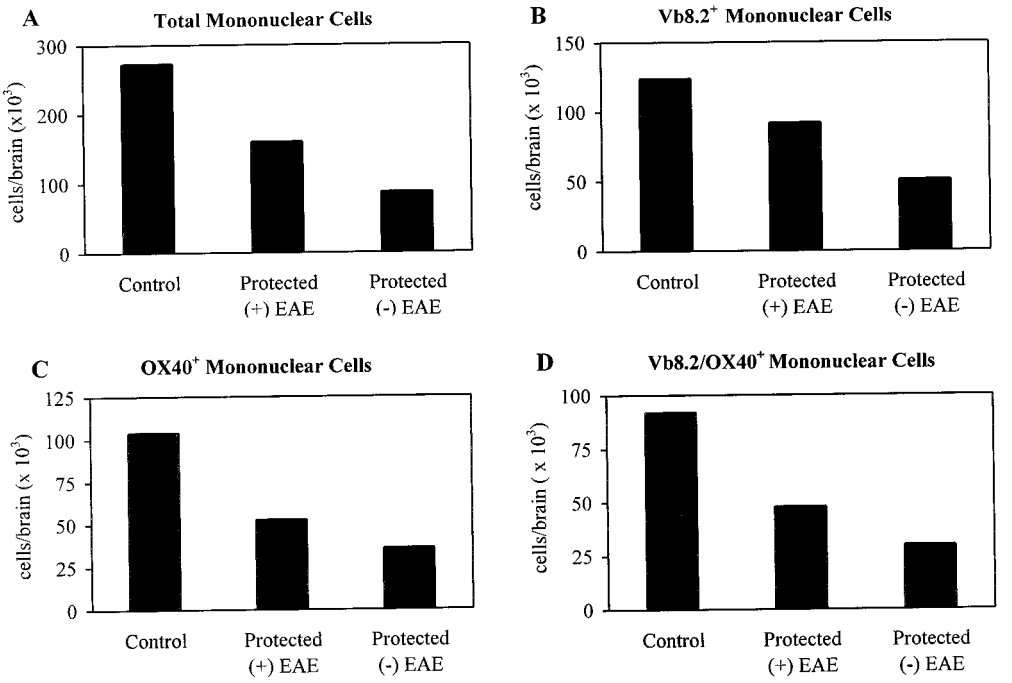


FIGURE 4. Recovery and characterization of inflammatory cells from the CNS of Rt-BV8S2-protected, BV8S2-treated but clinically affected, and sham-treated Tg mice with EAE. Spinal cords were collected from groups of two to five mice after recovery from relapsing EAE (day 60 after injection of MBP-NAc_{1-11}/CFA), and inflammatory cells were collected, counted, and analyzed by FACS for expression of BV8S2 and OX40 activation marker.

MBP-NAc_{1-11}-specific T cells by only the AV CDR3 sequence indicated that the single TCR Tg mice had considerable capacity to form productive combinations of novel AV genes with the BV8S2 transgene. BV8S2-specific T cells expressing other BV genes may have been rearranged and may have contributed to regulation, but did not constitute more than a small minority of the BV8S2-reactive population. However, formal proof of autoregulation will require highly characterized T cell clones.

In this study, we characterized the induction and function of both encephalitogenic and regulatory T cell specificities in BV8S2 Tg mice. The results demonstrate conclusively that overexpression of the BV8S2 transgene 1) engendered a natural pre-effector T cell population specific for MBP-NAc_{1-11} that acquired encephalitogenic capability only after immunization with this peptide in CFA; and 2) naturally induced a second population of T cells specific for BV8S2 determinants that upon activation could directly inhibit the activation and encephalitogenic activity of MBP-NAc_{1-11}-specific

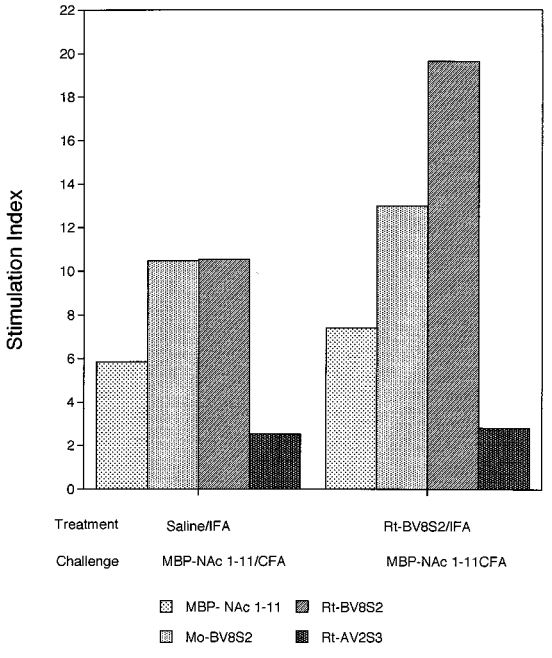


FIGURE 5. Proliferation responses of LN cells from sham-treated control and Rt-BV8S2-protected Tg mice to MBP-NAc_{1-11} peptide and Mo- and Rt-BV8S2 proteins. LN cells were collected from groups of three to five Tg mice after recovery from the initial episode of EAE in control mice and cultured with the indicated Ag as described in *Materials and Methods*. SI = cpm in response to Ag/cpm of unstimulated cells. Unstimulated LN cells gave background values of 780 cpm (sham-treated mice) and 500 cpm (Rt-BV8S2-treated mice).

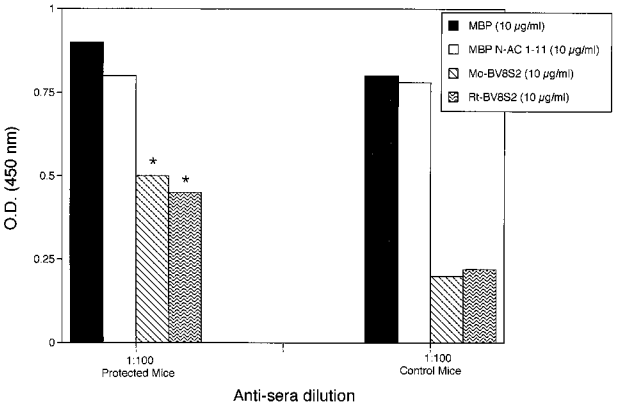
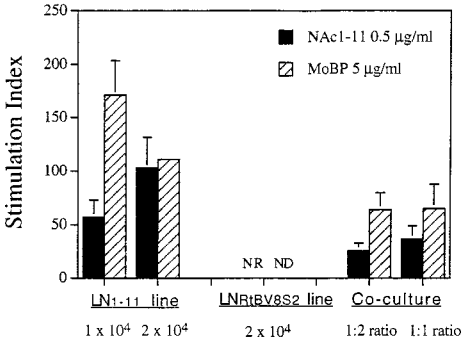


FIGURE 6. Ab responses to MBP and recombinant BV8S2 proteins. Serum was collected from groups of three to five Tg mice after the first clinical episode of EAE and analyzed for Ab response by ELISA, as described in *Materials and Methods*. * Indicates a significantly ($p < 0.05$) elevated Ab level in protected vs control mice.



IFN-γ (ng/ml)	27.4 ± 0.1	0.2 ± 0.0	16.3 ± 0.2 (p < 0.001)
Passive EAE*	20 x 10^6 T cells		20 x 10^6 T cells
Incidence	6/6	ND	1/3 (p = 0.08)
Maximum score	5.5 ± 0.2		1±1 (p = 0.001)
10-day CDI	36 ± 6		7±7 (p = 0.018)

FIGURE 7. Inhibition of MBP-specific T cells cocultured in vitro with BV8S2-specific T cells. T cells specific for MBP-NAc_{1-11} (1 or 2 × 10^4/well) were cultured alone or cocultured with 2 × 10^4 LN BV8S2-specific T cells at a 1:2 or 1:1 ratio in the presence of NAc_{1-11} or Mo-BP Ag and APC. Soluble BV8S2 protein was not added to any of these culture wells. BV8S2-specific T cells (2 × 10^4) cultured alone in the presence of NAc_{1-11} peptide did not show any proliferation above background (NR, no response). Background cpm ranged from 200 to 300. In a separate experiment, BV8S2-specific T cells did not demonstrate any proliferation to Mo-BP (not shown). ND, not done. In additional experiments conducted in transwells, MBP-NAc_{1-11}-specific T cells were cultured in the bottom well with or without Rt-BV8S2-specific T cells in the top well. Both top and bottom wells contained APC, MBP-NAc_{1-11} peptide, and Rt-BV8S2 protein, and were separated by a semipermeable membrane. At the end of the 3-day culture period, supernatants were saved and tested for release of IFN-γ, and MBP-NAc_{1-11}-specific T cells from the bottom well were transferred into naive recipient mice to evaluate encephalitogenic activity. Details of the culture conditions are described in *Materials and Methods*. * Indicates that the recipient mice received two injections of pertussigen. Note significantly reduced severity of passive EAE induced by cocultured T cells.

T cells by soluble secreted factors. Vaccination with heterologous Rt-BV8S2 protein induced strong cross-reactivity to Mo-BV8S2 determinants and appeared to be more effective than Mo-BV8S2 in inducing protection against EAE. This result suggests that there is some degree of tolerance to self BV8S2 determinants, a finding consistent with that of Falcioni et al. (23).

Protection against EAE was associated with a striking decrease in IFN-γ production by LN T cells specific for the encephalitogenic MBP-NAc_{1-11} determinant, an increased production of IL-10 by BV8S2-reactive T cells, a decrease in CNS lesions and infiltrating activated inflammatory cells, and an elevated Ab response to BV8S2 proteins. Vaccination with BV8S2 protein induced specific T cells that directly inhibited the proliferation response, IFN-γ secretion, and encephalitogenic activity of MBP-NAc_{1-11}-reactive T cells in vitro and resulted in the skewed production of Th2 cytokines by an MBP-NAc_{1-11}-specific T cell line. However, the BV8S2-specific T cells were not self-regulated, even though they predominantly expressed the BV8S2 transgene. Taken together, these results suggest that activated regulatory T cells specific for BV8S2 proteins, through a nondeletional mechanism involving skewed production of soluble cytokines, prevented pre-effector T cells specific for MBP-NAc_{1-11} from developing into Th1 effector cells capable of inducing EAE. Although anti-BV8S2 Abs were also present and may have contributed to protection against EAE, selective regulatory effects of these Abs on Th1 cells have not as yet been demonstrated. However, there was no evidence of deletion of $BV8S2^+$ T cells, unlike the study of Haqqi et al. (24), in which immunization of BUB mice with a BV10 peptide induced deletion of most of the $BV10^+$ T cells.

The expression of the rearranged BV8S2 transgene specific for MBP-NAc_{1-11} clearly favored T cell recognition of this encephalitogenic determinant in naive mice not previously exposed to exogenous MBP. Of importance, however, the relatively strong 5-18X T cell proliferation response to MBP-NAc_{1-11} in these mice was accompanied by a low to absent release of all lymphokines tested, strongly suggesting a lack of maturation to effector cells that might otherwise cause clinical EAE. The functional lack of development of encephalitogenic effector cells specific for MBP-NAc_{1-11} may well have been influenced by naturally induced T cells specific for Mo-BV8S2 determinants. Upon stimulation with Mo-BV8S2 protein, naive splenocytes produced significantly higher levels of both IFN-γ and IL-10 than cells stimulated with MBP-NAc_{1-11}. Moreover, elevated levels of secreted IFN-γ and IL-10 were also induced in naive splenic T cells stimulated with the heterologous Rt-BV8S2 protein.

Immunization of the Tg mice with MBP-NAc_{1-11} in CFA was a powerful stimulus for activating Th1 effector cells that were quite clearly capable of inducing EAE, even in the presence of the naturally induced BV8S2-specific T cell population. The activation process of MBP-NAc_{1-11}-specific T cells produced a 150-fold increase in IFN-γ, with a negligible increase in IL-10, compared with naive LN cells (Table I). Surprisingly, MBP-NAc_{1-11}-specific LN cells from immunized Tg mice had an even lower proliferation index than LN cells from naive Tg mice, indicating that immunization in CFA caused T cell maturation rather than simply an expansion of these T cells.

Immunization with MBP-NAc_{1-11} in CFA also induced profound changes in T cells specific for Mo- and Rt-BV8S2 proteins. Although proliferation responses (Fig. 5) were even less than in unimmunized Tg mice (Fig. 1), activation of LN cells from MBP-NAc_{1-11}/CFA-immunized mice with Rt- or Mo-BV8S2 proteins induced >10-fold increases of IFN-γ (Table I), due probably to the strongly inflammatory environment imposed by CFA. However, without further exposure to exogenous BV8S2 proteins, these BV8S2-specific T cells were unable to prevent the onset of EAE after immunization with MBP-NAc_{1-11}. Yet, neonatal tolerization of these naturally induced BV8S2-specific T cells with BV8S2 protein resulted in more severe EAE, clearly demonstrating their regulatory activity in vivo (13).

In contrast to the strong influence of CFA in directing a Th1 response, prior vaccination with BV8S2 proteins in IFA induced significant increases in both IFN-γ and IL-10 (Table I). It is noteworthy that in mice, IFN-γ can inhibit EAE if applied intraventricularly 7 to 8 days after disease induction (25), and anti-IFN-γ Ab can enhance the severity of EAE in susceptible as well as resistant strains (26, 27). Additionally, IL-10 has been shown to directly inhibit Th1 responses (28), including those specific for MBP-NAc_{1-11}. Thus, the increased secretion of both of these cytokines by BV8S2-specific T cells was associated with and probably contributed to the inhibition of MBP-NAc_{1-11}-specific T cells in coculture and transwell experiments and in EAE-protected mice, as well as the unusual maturation of MBP-NAc_{1-11}-specific T cell line responses toward the Th2 subtype (Table I). The production of soluble suppressive cytokines by BV8S2-specific T cells in mice is consistent with our previous studies of TCR peptide-specific T cells in both rats (29) and humans (30). However, the finding that vaccination with BV8S2 proteins can redirect the maturation of

pre-effector T cells specific for MBP-NAc_{1-11} toward a Th2 cell type is novel, although a similar outcome has been reported using altered encephalitogenic peptide ligands (31) or IL-4 (32). In addition to these direct regulatory effects, the BV8S2-specific T cells potentially could activate a third subset of $CD8^+$ effector T cells that have been proposed by Kumar and Sercarz (33) to interact with naturally processed, MHC I-associated TCR determinants thought to be expressed by the target MBP-specific Th1 cells.

Taken together, these experiments reveal the emergence of a powerful regulatory mechanism specific for BV8S2 determinants that is enhanced rather than muted in Tg mice overexpressing the BV8S2 gene specific for MBP-NAc_{1-11}. There are two major findings from this study that may have important therapeutic implications for humans, including: 1) the apparent enhanced protective activity of heterologous TCR sequences that are cross-reactive with homologous TCR proteins; and 2) the selective regulation of inflammatory Th1 cells through a cytokine-driven mechanism that could inhibit bystander as well as target T cells. These findings, which have parallels in human studies using TCR peptides to induce regulatory Th2 cells for treatment of multiple sclerosis (30), provide an instructive foundation for regulation of human autoimmune diseases.

Acknowledgments

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