Published in final edited form as:

J Neuroimmunol. 2009 April 30; 209(1-2): 26–32. doi:10.1016/j.jneuroim.2009.01.018.

Myelin Basic Protein-Reactive T Cells Persist in an Inactive State in the Bone Marrow of Lewis Rats that have Recovered from Autoimmune Encephalomyelitis

Taba Kheradmand, Norbert A. Wolf, and Robert H. Swanborg*

Department of Immunology and Microbiology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, Michigan 48201, USA

Abstract

Lewis rats immunized with guinea pig myelin basic protein residues 68-86 develop acute experimental autoimmune encephalomyelitis and recover. The predominant T cell receptor expressed by the encephalitogenic T cells is TCRBV8S2. They persist in bone marrow many weeks after recovery. CD3 is down-regulated, but > 90% express CD4. They fail to proliferate to GPMBP68-86 unless a nitric oxide synthase inhibitor is added to the cultures. Perhaps these are memory T cells that are maintained in a suppressed state in BM by a nitric oxide-dependent mechanism.

Keywords

EAE; Memory T cells; CD3 down-regulation; Nitric oxide

1. Introduction

The Lewis rat is highly susceptible to experimental autoimmune encephalomyelitis (EAE), developing hind limb paralysis when immunized with 10 – 20 µg of guinea pig myelin basic protein (GPMBP) emulsified in Freund's complete adjuvant (CFA) (reviewed in Swanborg, 2001). Paralysis develops within 10 – 14 days.

The dominant encephalitogenic GPMBP epitope for Lewis rats consists of residues 68-86 (YGSLPQKSQRSQDENPV (Hashim, 1977; Mannie et al., 1985), and the predominant encephalitogenic T cell population induced in response to this peptide, expresses TCRBV8S2 in the T cell receptor (Burns et al., 1989; Chluba et al., 1989; Tsuchida et al., 1993). Monoclonal antibodies (mAb) specific for TCRBV8S2 are available, and are useful for tracking the encephalitogenic T cells *in situ*. Although it is improbable that all TCRBV8S2 T cells are encephalitogenic, when used in conjunction with other tests, e.g., proliferation *in vitro* to GPMBP68-86, the finding is consistent with the conclusion that a subset of TCRBV8S2 T cells respond to the encephalitogenic antigen.

*Corresponding author: Robert H. Swanborg, Ph.D., Phone: +1 248 625 1962, Fax: +1 313 577 1155, Email: E-mail: rswanbo@med.wayne.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

By day 18 post-immunization most rats have recovered, and are resistant to subsequent attempts to re-induce EAE with MBP or GPMBP68-86 (Willenborg, 1979). However, T cells isolated from recovered Lewis rats are reactive *in vitro* with the immunizing antigen, as reflected by proliferative responses and secretion of IFN- γ when stimulated with MBP, and can also adoptively transfer EAE to Lewis recipients after short-term culture with MBP (Holda et al., 1980; Hinrichs et al., 1981). These findings indicate that recovered Lewis rats harbor T cells with the potential to induce EAE, but that these autoreactive cells are maintained in a quiescent state. We, and others, have reported that this state of remission is associated with the appearance of T regulatory (Treg) (Adda et al., 1977; Welch et al., 1980; recently reviewed in O'Connor and Anderton, 2008) and inhibitory natural killer (NK) cells (Zhang et al., 1997; Matsumoto et al., 1998).

Previous evidence from our laboratory revealed the existence of a subpopulation of bone marrow (BM)-derived NK cells that inhibits proliferation of naïve rat T cells to Con A and PMA/ionomycin (Trivedi et al., 2005; Kheradmand et al., 2008), and of MBP-primed T cells to MBP (Smeltz et al., 1999; Wolf and Swanborg, 2001). These NK cells express the NKR-P1A^{dim}CD3⁻ phenotype, and function in a contact-dependent fashion to inhibit T cell proliferation via up-regulation of the cell cycle inhibitor, p21, resulting in a G₀/G₁ cell cycle arrest (Trivedi et al., 2005). Concomitant with inhibition of T cell proliferation, these NKR-P1A^{dim} NK cells downregulate CD3 expression by the T cells. Moreover, the nitric oxide synthase inhibitor, N^G-monomethyl-arginine acetate (MMA), abrogates inhibition of T cell proliferation (Kheradmand et al., 2008). This is consistent with the report that inhibition of nitric oxide synthase in Lewis rats that have recovered from acute EAE results in chronic-relapsing disease, which suggests that NO regulates this autoimmune disease (O'Brien et al., 2001).

It has previously been reported that bone marrow is a major reservoir for memory CD8 cells (Becker et al., 2005; Mazo et al., 2005). Since Lewis rats that have recovered from EAE harbor T cells that can be activated to transfer disease (Holda et al., 1980; Hinrichs et al., 1981), it is conceivable that these cells may migrate to the bone marrow where they are maintained in a quiescent state. The present report describes experiments to test this hypothesis.

2. Materials and Methods

2.1. Peptide synthesis

The major encephalitogenic epitope of MBP for Lewis rats was synthesized on an Applied Biosystems Synergy model 432A peptide synthesizer (Perkin-Elmer, Foster City CA) and the structure was confirmed by mass spectrometry. The sequence of GPMBP68-86 is YGSLPQKSQRSQDENPV.

2.2. Animals and immunization

Female Lewis and DA rats were purchased from Charles River and Harlan Sprague-Dawley, respectively. Active EAE was induced in female Lewis rats by immunization with 50 μ g GPMBP68-86 in CFA, as previously described (Swanborg and Stepaniak, 1996). The rats were observed for clinical EAE and graded 0 (no disease), 1 (loss of tail tonic), 2 (hind limb weakness) or 3 (hind limb paralysis).

For adoptive transfer, spleen cells (SpC) were obtained from recovered Lewis rats, and activated for 72 hr at 37 C with 2 μ g/ml GPMBP68-86, as previously described (Ben-Nun et al., 1981; Swanborg and Stepaniak, 1996). Dead cells were removed on Ficoll, and the viable cells were washed and transferred intraperitoneally to syngeneic recipients ($3-5 \times 10^7$ /recipient).

2.3. Preparation of T cells

Bone marrow was obtained by flushing the cavities of the femur and tibia bones with cold RPMI 1640 supplemented with 5% fetal calf serum (Gibco Life Technologies), as previously described (Smeltz et al., 1999). The yield averaged $\sim 2 \times 10^8$ total cells/rat. BM T cells were isolated on T cell purification columns (Cedarlane) after depletion of adherent cells on plastic tissue culture flasks (Falcon). The yield of BM T cells averaged from $2 - 3 \times 10^7$ /rat. Splenic T cells were isolated on T cell columns after depleting erythrocytes and adherent cells.

2.4. Flow cytometry

Cells were washed with PBS containing 1% BSA and 0.02% Na-Azide and then stained with labeled antibodies. We employed PE-labeled anti-rat CD3 (clone G4.18), PE-Cy5-labeled anti-rat CD4 (clone OX35), PE-labeled anti-NKR-P1A (clone 10/78) and FITC-labeled anti-rat TCRBV8S2 (clone R78) antibodies. Cells were stained in the dark on ice for 30 min with intermittent shaking. The cells were washed and resuspended in PBS with 0.02% Na-Azide.

The expression of cell surface markers was determined by flow cytometry using the Dako Cyan-ADP flow cytometer, gating on the lymphocyte population, as previously described (Kheradmand et al., 2008). Autofluorescence and isotype controls (e.g., PE-labeled anti- KLH) were included in the assays. Approximately 20,000 cells were analyzed.

2.5. Proliferation assays

Splenic or BM Lewis rat T cells (5×10^5 /well) were cultured in RPMI 1640 containing 5% FCS with irradiated thymocytes as APCs and were stimulated at 37 C, for 72 hr with 10 and 20 μ M GPMBP68-86. DA rat T cells (5×10^5 /well) were activated for 48 hr at 37 C with Phorbol-12-myristate-13 acetate (PMA, 10 ng/ml) and ionomycin (0.4 μ g/ml). The nitric oxide inhibitor MMA was added to some culture wells at 1 – 5 mM. Previous results indicated that 3-5 mM MMA gave optimal results (Kheradmand et al., 2008). The cells were pulsed with 3 H- thymidine for the last 18-20 hr of culture time and T cell proliferation was determined using a Tomtec Harvester 96 and counted in a 1450 Microbeta Plus, liquid scintillation counter (Wallac), as previously described (Kheradmand et al., 2008). Samples were run in triplicate, and results are presented as mean cpm $\pm$ standard deviation.

2.6. NK cells

NK cells were isolated from the BM of DA rats, enriched on Percoll, and co-cultured with DA rat SpC in proliferation assays and flow cytometry, as previously described (Smeltz et al., 1999).

2.7. Statistical analysis

All experiments were repeated at least two times with similar results. Representative results are presented as mean $\pm$ standard deviation. Statistical significance was calculated using Student's *t* test.

3. Results

3.1. TCRBV8S2 T cells persist in BM after recovery from active EAE

Lewis rats develop acute paralytic EAE when immunized with GPMBP68-86, and spontaneously recover by about day 18. Subsequently, they are resistant to further attempts to induce active disease. TCRBV8S2 T cells are the predominant encephalitogenic cells elicited after immunization with GPMBP68-86, and can be monitored by flow cytometry using anti-TCRBV8S2 mAb. The immunized Lewis rats were monitored for at least two months after recovery for the presence of TCRBV8S2⁺ T cells in both spleen and bone marrow.

The yield of BM cells from each rat was approximately 2×10^8 . After purification, $2 - 3 \times 10^7$ T cells were obtained. Thus, our studies were limited by the low yield of T cells from rat bone marrow. This precluded conducting experiments with purified TCRBV8S2 T cells.

As shown in Fig. 1, relatively few ($\sim 2-3\%$) TCRBV8S2⁺ T cells were present in BM and spleens of naïve Lewis rats, but the percentages increased significantly during the paralytic phase of EAE (day 10) and remained elevated on day 24, after recovery from disease. This was most apparent in BM, and notably these BM TCRBV8S2 T cells were deficient with respect to CD3 expression (Fig. 1, top panels). This was not true for splenic TCRBV8S2 T cells, which express normal levels of CD3 (Fig. 1, bottom panels). Even 82 days post-immunization, TCRBV8S2⁺ T cells could be found in BM and SpC (Fig. 1), although at this time, some TCRBV8S2⁺CD3⁺ cells were also detectable.

Although the BM T cells were deficient in CD3, the majority ($\sim 90\%$) were CD4 positive (data not shown).

3.2 TCRBV8S2 BM T cells do not proliferate in response to immunizing antigen but the nitric oxide synthase inhibitor N^G-monomethyl-arginine acetate can abrogate suppression of proliferation

Since we previously reported that NKR-P1A^{dim}CD3⁻ BM NK cells from DA rats down-regulate CD3 expression on DA rat T cells and inhibit T cell proliferation in response to PMA + ionomycin (Kheradmand et al., 2008), it was of interest to determine whether the Lewis rat TCRBV8S2 BM T cells that are also deficient in CD3 proliferate in response to GPMBP68-86. As shown in Fig. 2, BM T cells from Lewis rats immunized with GPMBP68-86 did not respond to the immunizing peptide. Data from paralyzed rats (day 10 post-immunization) and recovered rats (day 24) are presented (Figs. 2A and 2B, respectively). However, when the nitric oxide synthase inhibitor N^G-monomethyl-arginine acetate (MMA) was added to the cultures, the ability of the BM T cells to proliferate to the peptide was restored. This is consistent with our earlier finding that MMA abrogated BM NK cell-mediated suppression of proliferation of DA rat T cells to PMA + ionomycin (Kheradmand et al., 2008). In contrast, splenic T cells responded to GPMBP68-86, and the addition of MMA did not increase proliferation (Fig. 3). Indeed, MMA slightly decreased the proliferative response. O'Brien et al. (2001) presented evidence that NO plays a dual role in EAE in the Lewis rat and that MMA can either inhibit or enhance the autoimmune response to MBP. Perhaps this provides an explanation for the different responses of BM and splenic T cells in the present investigation.

3.3. Activated donor TCRBV8S2 T cells with down-regulated CD3 migrate to the BM of recipient rats after recovery from adoptive EAE

To determine whether adoptively transferred encephalitogenic T cells traffic to the BM of recipient rats, we activated spleen cells from GPMBP68-86-immunized donors with EAE *in vitro* with GPMBP68-86 (2 $\mu\text{g}/\text{ml}$) for 72 hr, and transferred them to naïve recipients ($3 - 5 \times 10^7$). After the recipients had recovered from adoptive EAE, we evaluated splenic and BM cells for CD3 and TCRBV8S2 by flow cytometry. As shown in Fig. 4, TCRBV8S2⁺ T cells deficient in CD3 (9%) were detected in the BM, whereas TCRBV8S2⁺ splenic T cells expressed normal levels of CD3 ($\sim 5\%$). Thus, it appears that donor encephalitogenic cells traffic to the bone marrow of naïve rats and down-regulate CD3. Yields of activated T cells from BM of recovered donor rats were insufficient to permit reliable analyses. BM from naïve Lewis rats contained $< 3\%$ TCRBV8S2 cells (Fig. 1).

3.4. CD3 is downregulated in T cells and re-expressed by addition of nitric oxide synthase inhibitor MMA

For these experiments we employed PMA + ionomycin activated splenic T cells and BMNK cells from DA rats, as reported in our recent paper (Kheradmand et al., 2008), to avoid adding APCs to the system. As shown in Fig. 5, in the presence of BMNK cells, PMA + ionomycin-stimulated splenic T cells failed to respond *in vitro*, but proliferative responses were restored in the presence of the nitric oxide synthase inhibitor, MMA. This confirms our recent finding that NKR-P1A^{dim} BM NK cells inhibit T cell proliferation, and that inhibition is abrogated by MMA.

Moreover, flow cytometry revealed that CD3 expression on PMA + ionomycin-cultured splenic T cells was significantly reduced in the presence of BMNK cells (Fig. 6, green histogram), but was restored to normal levels (Fig. 6, red histogram) in the presence of MMA (Fig. 6, blue histogram). Taken together with the results presented in Fig. 5, these findings support a direct correlation between CD3 expression and the ability of T cells to proliferate when stimulated *in vitro*.

4. Discussion

Although Lewis rats that have recovered from EAE are resistant to further attempts to induce the disease (Willenborg, 1979), they harbor T cells that can be activated to transfer EAE (Holda et al., 1980; Hinrichs et al., 1981; Conant and Swanborg, 2004). The present study was carried out to test the hypothesis that these potentially encephalitogenic T cells migrate to the bone marrow where they are maintained in a non-responsive state by regulatory cells. Since the predominant encephalitogenic T cells elicited by immunization with GPMBP68-86 express TCRBV8S2 (Burns et al., 1989; Chluba et al., 1989), we monitored these cells in BM and spleen by flow cytometry using mAb specific for TCRBV8S2 during the course of the disease. We were able to detect TCRBV8S2-positive T cells in both BM and spleen during the paralytic phase of the disease and for at least two months following spontaneous recovery. Of interest was the finding that CD3 was significantly downregulated in BM-derived TCRBV8S2 T cells. These T cells responded to GPMBP68-86 in proliferative assays, but only in the presence of MMA, confirming that the BM population contained myelin-reactive T cells. The CD3 complex is an important component of the T cell receptor and is essential for initiation of the signaling cascade which leads to T cell activation (Huang and Wange, 2004). Therefore, down-regulation of CD3 can decrease the activation status of T cells and promote a non-responsive state.

We also detected TCRBV8S2-positive T cells in the spleens of recovered rats. These T cells expressed normal levels of CD3. Since these rats do not develop relapses of EAE it is possible that regulatory T cells inhibit pathogenic function (Welch et al., 1980; reviewed in O'Connor and Anderton, 2008).

We previously reported that rat BM contains a subset of NK cells with the NKR-P1A^{dim}CD3⁻ phenotype that inhibit T cell proliferation to Con-A and to PMA + ionomycin, and observed that suppression of proliferation could be abrogated by MMA (Kheradmand et al., 2008). We confirmed this finding in the present report (Fig. 5), and also demonstrated that MMA restored expression of CD3 to splenic T cells stimulated with PMA + ionomycin in the presence of BM-derived NK cells (Fig. 6). This is consistent with the possibility that nitric oxide secreted by NK cells might be responsible for down-regulation of CD3 and impaired T cell function. Low BM cell yields precluded conducting the same co-culture experiments with GPMBP68-86-primed BM T cells and BM-derived NK cells.

It has been reported that granulocytes down-regulate CD3 in activated T cells from synovial fluid of rheumatoid arthritis patients, resulting in suppression of T cell proliferation and reduction of IFN- γ production (Berg et al., 2000). In another study, Upham et al. (1995) reported that rat and human alveolar macrophages inhibit the proliferation of activated T cells through a nitric oxide-dependent mechanism. IL-2 receptor expression was up-regulated and IL-2 levels were increased, although the T cells were unable to utilize this cytokine (Upham et al., 1995). This is compatible with our previous report that bone marrow NK cells inhibit T cell proliferation in a contact-dependent manner that is reversible, and does not involve inhibition of IL-2 secretion or IL-2 receptor up-regulation (Trivedi et al., 2005). We also observed that the NK cells down-regulate CD3 expression in T cells (Kheradmand et al., 2008). Therefore down-regulation of CD3 in T cells could be a mechanism to prevent T cell activation to self-antigens. So it is conceivable the several cell types can exert this regulatory function. Hence, NK cells might use this very mechanism in order to maintain homeostasis in the bone marrow.

The finding that NK cell depletion with mAb causes exacerbations of EAE in Lewis rats (Matsumoto et al., 1998) and mice (Zhang et al., 1997) provide support for a role for NK cells in the regulation of this autoimmune disease. Furthermore, treatment of multiple sclerosis (MS) patients with humanized mAb specific for the IL-2 receptor reduces brain inflammation concomitant with the expansion of a subset of NK cells (Bielekova et al., 2006). It has also been reported that relapses of MS are associated with decreased NK cell activity and function (Munschauer et al., 1995; Kastrukoff et al., 2003). Others have reported that SJL/J mouse NK cells exert a cytotoxic effect on encephalitogenic T cells (Xu et al., 2005). We cannot exclude a cytotoxic mechanism in the present study, although our previous findings indicate that T cell proliferation is restored when the NK cells are removed from the cultures (Trivedi et al., 2005).

Adoptive transfer experiments confirmed that TCRBV8S2 T cells indeed traffic to the BM (Fig. 4). Memory T cells develop after encounter with an antigen, and it has been reported that CD8 central memory cells traffic to the BM (Becker et al., 2005; Mazo et al., 2005). We postulate that some CD4⁺ GPMBP68-86-specific T cells home to the bone marrow where they persist as memory cells in an inactive state. Other GPMBP68-86-primed T cells localize in other organs (e.g., spleen), where Treg cells may suppress their encephalitogenic activity.

In conclusion, we have observed that myelin-specific T cells persist in the BM of Lewis rats that have recovered from EAE. Although EAE in the Lewis rat is an acute disease, it is conceivable that pathogenic memory cells could also persist in the BM of patients with relapsing-remitting MS. Were these cells to become activated, disease relapses could occur. This hypothesis is consistent with the observation of Cavanagh and colleagues (Cavanagh et al., 2005), who reported that antigen-bearing dendritic cells migrate to the BM where they trigger central memory T cell responses in the bone marrow. This possibility merits investigation.

Acknowledgments

This work was supported by research grant 5R01 NS048070-04 from the National Institutes of Health.

References

- Adda DH, Beraud E, Depieds R. Evidence for suppressor cells in Lewis rats' experimental allergic encephalomyelitis. *Eur J Immunol* 1977;7:620–623. [PubMed: 923633]
- Becker TC, Coley SM, Wherry EJ, Ahmed R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* 2005;174:1269–1273. [PubMed: 15661882]

- Ben-Nun A, Wekerle H, Cohen IR. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* 1981;11:195–199. [PubMed: 6165588]
- Berg L, Ronnelid J, Klareskog L, Bucht A. Down-regulation of the T cell receptor CD3 zeta chain in rheumatoid arthritis (RA) and its influence on T cell responsiveness. *Clin Exp Immunol* 2000;120:174–182. [PubMed: 10759780]
- Bielekova B, Calfamo M, Reichert-Schriener S, Packer S, Cerna M, Waldmann TA, McFarland H, Henkart PA, Martin R. Regulatory CD56^{bright} natural killer cells mediate immunomodulatory effects of IL-2R α -targeted therapy (daclizumab) in multiple sclerosis. *Proc Natl Acad Sci USA* 2006;103:5941–5946. [PubMed: 16585503]
- Burns FR, Li XL, Shen N, Offner H, Chou YK, Vandenbark AA. Both rat and mouse T cell receptors specific for the encephalitogenic determinant of myelin basic protein use similar V α and V β chain genes even though the major histocompatibility complex and encephalitogenic determinants being recognized are different. *J Exp Med* 1989;169:27–39. [PubMed: 2462609]
- Cavanagh LL, Bonasio R, Mazo IB, Halin C, Cheng G, van der Velden AW, Cariappa A, Chase C, Russell P, Starnbach MN, Koni PA, Piliai S, Weninger W, von Andrian UM. Activation of bone marrow resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat Immunol* 2005;6:1029–1037. [PubMed: 16155571]
- Chluba J, Steeg D, Becker A, Wekerle H, Epplen JT. T cell receptor β chain usage in myelin basic protein specific rat T lymphocytes. *Eur J Immunol* 1989;19:279–284. [PubMed: 2467813]
- Conant SB, Swanborg RH. Autoreactive T cells persist in rats protected against experimental autoimmune encephalomyelitis and can be activated through stimulation of innate immunity. *J Immunol* 2004;172:5322–5328. [PubMed: 15100271]
- Hashim GA. Experimental allergic encephalomyelitis in Lewis rats: chemical synthesis of disease-inducing determinant. *Science* 1977;196:1219–1221. [PubMed: 67639]
- Hinrichs DJ, Roberts CM, Waxman FJ. Regulation of paralytic experimental allergic encephalomyelitis in rats. Susceptibility to active and passive disease reinduction. *J Immunol* 1981;126:1857–1862. [PubMed: 6163820]
- Holda JH, Welch AM, Swanborg RH. Autoimmune effector cells. I. Transfer of experimental encephalomyelitis with lymphoid cells cultured with antigen. *Eur J Immunol* 1980;10:657–659. [PubMed: 6967418]
- Huang Y, Wange RL. T cell receptor signaling: beyond complex complexes. *J Biol Chem* 2004;279:28827–28830. [PubMed: 15084594]
- Kastrukoff LF, Lau A, Wee R, Zecchini D, White R, Paty DW. Clinical relapses of multiple sclerosis are associated with “novel” valleys in natural killer cell functional activity. *J Neuroimmunol* 2003;145:103–114. [PubMed: 14644036]
- Kheradmand T, Trivedi PP, Wolf NA, Roberts PC, Swanborg RH. Characterization of a subset of bone marrow-derived natural killer cells that regulates T cell activation in rats. *J Leukocyte Biol* 2008;83:1128–1135. [PubMed: 18272589]
- Mannie MD, Paterson PY, U'Prichard DC, Flouret G. Induction of experimental allergic encephalomyelitis in Lewis rats with purified synthetic peptides: delineation of antigenic determinants for encephalitogenicity, in vitro activation of cellular transfer, and proliferation of lymphocytes. *Proc Natl Acad Sci USA* 1985;82:5515–5519. [PubMed: 3875098]
- Matsumoto Y, Kohyama K, Aikawa Y, Shin T, Kawazoe Y, Suzuki Y, Tanuma N. Role of natural killer cells and TCR $\gamma\delta$ T cells in acute autoimmune encephalomyelitis. *Eur J Immunol* 1998;28:1681–1688. [PubMed: 9603475]
- Mazo IB, Honczarenko M, Leung H, Cavanaugh LL, Bonasio R, Weninger W, Engelke K, Xia L, EmEver RP, Koni PA, Silberstein LE, von Andrian UH. Bone marrow is a major reservoir and site of recruitment for central memory CD8⁺ T cells. *Immunity* 2005;22:259–270. [PubMed: 15723813]
- Munschauer FE, Hartrich LA, Stewart CC, Jacobs L. Circulating natural killer cells but not cytotoxic T lymphocytes are reduced in patients with active relapsing multiple sclerosis and little clinical disability as compared to controls. *J Neuroimmunol* 1995;62:177–181. [PubMed: 7499506]

- O'Brien NC, Charlton B, Cowden WB, Willenborg DO. Inhibition of nitric oxide synthase initiates relapsing remitting experimental autoimmune encephalomyelitis in rats, yet nitric oxide appears to be essential for clinical expression of disease. *J Immunol* 2001;167:5904–5912. [PubMed: 11698467]
- O'Connor RA, Anderton SM. Foxp3⁺ regulatory T cells in the control of experimental CNS autoimmune disease. *J Neuroimmunol* 2008;193:1–11. [PubMed: 18077005]
- Smeltz RB, Wolf NA, Swanborg RH. Inhibition of autoimmune T cell responses in the DA rat by bone marrow-derived natural killer cells in vitro: implications for autoimmunity. *J Immunol* 1999;163:1390–1398. [PubMed: 10415039]
- Swanborg RH. Experimental autoimmune encephalomyelitis in the rat: lessons in T cell immunology and autoreactivity. *Immunol Reviews* 2001;184:129–135.
- Swanborg, RH.; Stepaniak, JA. Experimental autoimmune encephalomyelitis in the rat. In: Coligan, JE.; Kruisbeek, AM.; Margulies, DH.; Shevach, EM.; Strober, W., editors. *Current Protocols in Immunology*. Vol. 3. New York: Wiley; 1996. p. 15.2.1–15.2.14.
- Trivedi PP, Roberts PC, Wolf NA, Swanborg RH. NK cells inhibit T cell proliferation via p21-mediated cell cycle arrest. *J Immunol* 2005;174:4590–4597. [PubMed: 15814681]
- Tsuchida M, Matsumoto Y, Hirahara H, Tomiyama K, Abo T. Preferential distribution of Vβ8.2-positive T cells in the central nervous system of rats with myelin basic protein-induced autoimmune encephalomyelitis. *Eur J Immunol* 1993;23:2399–2406. [PubMed: 7691605]
- Upham JW, Strickland DH, Bilyk N, Robinson BW, Holt PG. Alveolar macrophages from humans and rodents inhibit T-cell proliferation but permit T-cell activation and cytokine secretion. *Immunology* 1995;84:147–147.
- Welch AM, Holda JH, Swanborg RH. Regulation of experimental allergic encephalomyelitis. II. Appearance of suppressor cells during the remission phase of the disease. *J Immunol* 1980;125:186–189. [PubMed: 6155396]
- Willenborg DO. Experimental allergic encephalomyelitis in the Lewis rat: Studies on the mechanism of recovery from disease and acquired resistance to reinduction. *J Immunol* 1979;123:1145–1150. [PubMed: 313947]
- Wolf NA, Swanborg RH. DA rat NK+CD3- cells inhibit autoreactive T cell responses. *J Neuroimmunol* 2001;119:81–87. [PubMed: 11525803]
- Xu W, Fazekas G, Hara H, Tabira T. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2005;163:24–30. [PubMed: 15885305]
- Zhang, Bn; Yamamura, T.; Kondo, T.; Fujiwara, M.; Tabira, T. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* 1997;186:1677–1687. [PubMed: 9362528]

Fig. 1.

Flow cytometry analysis of TCRVB8S2 T cells in bone marrow (upper panels) and spleen (lower panels) during the course of EAE.

Upper panels: TCRVB8S2 T cells in BM of naïve rat: 3% (CD3 downregulated); day 10 post-immunization (paralytic phase of EAE: 22% (CD3 downregulated); day 24 (6 days post-recovery): 30% (CD3 downregulated); day 82 (2 months post-recovery): 9% (CD3 downregulated), 3% CD3 expressed.

Lower panels: TCRVB8S2 T cells in spleen of naïve rat: < 3%; day 10: 5.6 %; day 24 7%; day 82 6.4%. CD3 expression in spleen is normal in all cases.

A **B** **Fig. 2.**

A. Proliferative response of BM T cells to GPMBP68-86 on day 10 (paralytic phase of EAE). T cells do not respond to priming peptide unless MMA is added to the cultures.

B. Proliferative response of BM T cells on day 24 (post-recovery phase). T cells do not respond to GPMBP68-86 unless MMA is added to the cultures; MMA alone does not induce proliferative response.

Fig. 3. Proliferative response of splenic T cells to GPMBP68-86 on day 24 (post-recovery). T cells respond to priming peptide in the absence of MMA; MMA slightly reduces proliferative response (O'Brien et al., 2001).

Fig. 4.

Spleen cells from recovered Lewis rats were cultured *in vitro* with GPMBP68-86 as described in Materials and Methods and transferred intraperitoneally to naïve recipient. After the recipient recovered, TCRVB8S2 T cells deficient in CD3 were detected in BM (9%), whereas TCRVB8S2 T cells that expressed CD3 were present in spleen (4.8%).

Fig. 5.
Splenic T cells co-cultured with BMNK cells (5:1 = T:NK) do not proliferate in response to PMA + ionomycin unless MMA is included in the cultures. MMA alone does not stimulate proliferation.

Fig. 6.

Flow cytometry analysis of aliquots of the same cells shown in Fig. 5. PMA + ionomycin stimulated splenic T cells express CD3 (red histogram, positive control). The addition of NK cells T:NK = 5:1) to PMA + ionomycin stimulated splenic T cells reduces CD3 expression on the T cells (green histogram). If MMA is included in the co-cultures, CD3 is again expressed at comparable levels to the positive control (blue histogram). Brown histogram = autofluorescence.