The Journal of Immunology

RESEARCH ARTICLE | AUGUST 15 1996

CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. FREE

P J Perrin; ... et. al

J Immunol (1996) 157 (4): 1333–1336.
https://doi.org/10.4049/jimmunol.157.4.1333

CTLA-4 Blockade Enhances Clinical Disease and Cytokine Production During Experimental Allergic Encephalomyelitis[1,2]

Peter J. Perrin,[3]* Jairo H. Maldonado,* Tiffany A. Davis,* Carl H. June,* and Michael K. Racke†

The B7 family of cell surface molecules expressed on APC provides accessory signals to T cells via either CD28 or CTLA-4. However, while CD28 transduces a costimulatory signal that is required for an optimal immune response, CTLA-4 transmits a negative signal. These studies use an anti-CTLA-4 mAb to directly address the role of this T cell surface molecule in experimental allergic encephalomyelitis (EAE). CTLA-4 regulation of disease was assessed during initial immune cell interactions and during the effector stage of the encephalitogenic immune response. The effects of anti-CTLA-4 treatment were schedule dependent. CTLA-4 blockade during the onset of clinical symptoms markedly exacerbated disease, enhancing mortality. Disease exacerbation was associated with enhanced production of the encephalitogenic cytokines TNF-α, IFN-γ and IL-2. Hence, CTLA-4 regulates the intensity of the autoimmune response in EAE, attenuating inflammatory cytokine production and clinical disease manifestations. ***The Journal of Immunology,*** **1996, 157: 1333–1336.**

Experimental allergic encephalomyelitis (EAE)[4] is a well-characterized disorder in which activation of autoreactive T cells directed against myelin constituents results in central nervous system demyelination and subsequent paralysis (1–4). EAE is inducible in genetically susceptible animals by immunization with whole myelin, constituent proteins of the myelin sheath such as myelin basic protein (MBP) and myelin proteolipid protein, and peptides derived from these proteins (2). In addition, MBP-specific, MHC class II-restricted $CD4^+$ T cells can adoptively transfer EAE to naive, syngeneic recipients (2, 5). Although epidemiologic evidence suggests that viral infection initiates the processes leading to multiple sclerosis (MS), these early events remain largely undefined (6). However, there is increasing evidence that the pathology in MS may be mediated by autoimmune $CD4^+$ T cells and that these T cells represent principal targets for the development of therapeutics (1). Therefore, EAE is the primary animal model for the human disease MS (2–4).

While TCR ligation provides specificity to the immune response, a number of accessory receptors regulate either adhesion or costimulation (7). Although several different T cell-APC interactions can provide costimulation, the major costimulatory signal appears to be through the CD28 receptor on the T cell (8–10). Costimulation through CD28 induces specific gene transcription and enhances paracrine secretion of cytokines through post-transcriptional stabilization of a number of cytokine mRNAs, including IL-2, granulocyte-macrophage CSF, and TNF-α (9). In addition, CD28 signaling protects cells from apoptotic cell death (11).

CTLA-4, a second accessory receptor on T cells, interacts with the same counter-receptors on APC as CD28 (12–16). Although it has been shown that either CD28 or CTLA-4 can bind to both B7-1 and B7-2, increasing evidence suggests that B7-1 and B7-2 may have distinct costimulatory functions (16–20). Furthermore, recent evidence suggests T cell activation is regulated by the opposing effects of CD28 costimulation and CTLA-4 signaling (21, 22). However, the mechanism whereby CTLA-4 regulates the immune response is still largely enigmatic. While CTLA-4 cross-linking can inhibit T cell proliferation, CTLA-4 blockade enhances T cell proliferation (23). There is additional evidence that the mechanism whereby CTLA-4 signaling exerts its negative regulatory role involves apoptosis (24). CTLA-4 can mediate apoptosis of previously activated cells (24). Furthermore, a mouse genetically deficient in CTLA-4 has a fatal phenotype secondary to a lymphoproliferative disorder (25). Blockade of CTLA-4 has been successfully used to augment antitumor responses (26). The role of

*Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20889; and †Department of Neurology, School of Medicine, Washington University, St. Louis, MO 63110

Received for publication May 23, 1996. Accepted for publication June 17, 1996.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by Naval Medical Research and Development Command Grant 60223N 34 3C30 005 1413. M.K.R. is a Harry Weaver Neuroscience Scholar of the National Multiple Sclerosis Society (JF-2078-A-2) and the recipient of the Young Investigator in Multiple Sclerosis Award from the American Academy of Neurology Education and Research Foundation.

[2] The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Navy, Department of Defense, or the United States Government.

[3] Address correspondence and reprint requests to Dr. Peter J. Perrin, Immune Cell Biology Program, Mail Stop 06, Naval Medical Research Institute, Bethesda, MD 20889-5607. E-mail address: rin0pjp@bumed30.med.navy.mil

[4] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; PT, pertussis toxin; EU, endotoxin units.

0022-1767/96/$02.00

Table I. *Minimal effect of anti-CTLA-4 blockade during Ag priming*

Group[a]	Incidence[b]	Mortality[c]	Mean Day Onset[d]	Mean Maximal Clinical score[e]
Expt. 1: CTLA-4 blockade, day 2				
Rat IgG	7/10	3	13.7	3.0
Anti-CTLA-4	10/10	5	16.1	4.3[f]
Expt. 2: CTLA-4 blockade, days 0, 2, 4, 6, 8				
Rat IgG	7/8	0	20.1	1.9
Anti-CTLA-4	6/7	0	14.5	2.7[f]

[a] Mice were immunized with MBP and treated with PT (200 ng per injection) as described in *Materials and Methods*. In Expt. 1, animals received either 100 μg of anti-CTLA-4 or 100 μg of rat IgG by i.p. injection on day 2. Animals were observed daily for clinical signs of disease for 60 days. In Expt. 2, animals received 100 μg of anti-CTLA-4 or 100 μg of rat IgG by i.p. injection on days 0, 2, 4, 6, 8. Data shown represent 45 days of observation.

[b] Proportion of animals in the experimental group that exhibited clinical signs of disease.

[c] Number of animals in the experimental group that died.

[d] Mean day that clinical disease symptoms were first observed in the individual animals of the experimental group.

[e] Mean of the maximal clinical scores obtained by the individual animals in the experimental group.

[f] Disease course is significantly more severe than that of the control group (Rat IgG recipients). Mann-Whitney sum of ranks test (29); $p < 0.05$.

CTLA-4 in the regulation of cytokine production is still uninvestigated. Therefore, both inhibition of CD28 signaling and enhancement of CTLA-4 signaling may be desirable in developing a specific therapy for EAE and MS.

These studies use an anti-CTLA-4 mAb to directly address the role of T cell surface molecule CTLA-4 in EAE induced by immunization with whole MBP. A single injection of anti-CTLA-4 on day 2 post-immunization resulted in enhanced disease. Most interestingly, when CTLA-4 blockade was delayed until after the onset of clinical symptoms, disease was markedly exacerbated. CTLA-4 blockade also increased the production of proinflammatory cytokines. This increase is clinical disease severity was associated with an increase in the production the encephalitogenic cytokines IL-2, IFN-γ, and TNF-α, after stimulation with MBP in vitro.

Materials and Methods

Mice

Female (PL × SJL)F_1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 to 8 wk of age. All mice were 10 to 12 wk of age when experiments were initiated. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, DHHS, Publication No. (NIH) 86-23 (1985).

Reagents

MBP was prepared from guinea pig spinal cords (Rockland Inc., Gilbertsville, PA) as previously described (27). Hamster anti-mouse CTLA-4 mAb UC10-4F10 (23) was generously provided by the Genetics Institute (Cambridge, MA). Rat IgG was purchased from Rockland. The endotoxin content of the UC10-4F10 used in these studies was less than 0.4 EU/mg.

Induction of EAE

PLSJLF_1/J mice were immunized s.c. with 400 μg of MBP emulsified in CFA (Difco Laboratories, Detroit, MI) above the shoulders and flanks (400 μg/mouse) and received 200 ng of PT i.v. on days 0 and 2 (1–3). Mice were evaluated daily for signs of disease and graded on the following scale (28): 0, no abnormality; 1, flaccid tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia, moribund state; and 6, death.

Cytokine ELISA

IL-2, IFN-γ, and TNF-α were assayed with specific Endogen (Cambridge, MA) ELISA kits according to the manufacturer's instructions.

Table II. *CTA-4 blockade after the onset of clinical symptoms results in increased mortality*

	Expt. 1		
Group[a]	Incidence[b]	Mortality[c]	Mean maximal clinical score[d]
Rat IgG	5/5	0	4.0
Anti-CTLA-4	5/5	4[e]	5.6[f]

[a] Mice were immunized with MBP and treated with PT as described in *Materials and Methods*. Clinical disease symptoms were first observed on day 19. On days 20, 22, and 24 animals received 100 μg of anti-CTLA-4 or 100 μg of rat IgG by i.p. injection.

[b] Proportion of animals in the experimental group that exhibited clinical signs of disease.

[c] Number of animals in the experimental group that died. The mortality following anti-CTLA-4 injections was significantly greater than the control group (Fisher's exact test, $p < 0.05$). The survival curve for this experiment is shown in Figure 1.

[d] Mean of the maximal clinical scores obtained by the individual animals in the experimental group.

[e] Mortality is significantly higher than that of the control group (Rat IgG recipients). Fischer's exact test, $p < 0.05$.

[f] Disease course is significantly more severe than that of the control group (Rat IgG recipients). Mann-Whitney sum of ranks test (29); $p < 0.05$.

Statistical methods

The effects of treatment on clinical disease were assessed by the Mann-Whitney sum of ranks test (29). Differences in mortality between experimental groups were analyzed using Fischer's exact test. ELISAs were analyzed using Student's *t* test.

Results

CTLA-4 blockade during initial immune cell interactions mildly enhances the development of EAE. To investigate the potential regulatory role of the T cell surface molecule CTLA-4 during initial Ag priming leading to the development of an encephalitogenic T cell response, mice were immunized with MBP and treated with PT on days 0 and 2. Animals were injected with either 100 μg anti-CTLA-4 or rat IgG on day 2 (Table I, Expt. 1). This schedule was attempted because we have previously shown that a single dose of CTLA4-Ig or anti-B7-1 on day 2 postimmunization resulted in the development of less severe disease (30). Conversely, a single dose of anti-B7-2 on day 2 resulted in the development of enhanced disease (30). If anti-B7-2 enhanced disease through blocking of B7-2:CTLA-4 interaction, then a single injection of anti-CTLA-4 on day 2 should also enhance disease. As shown in Table I, a single injection of anti-CTLA-4 on day 2 resulted in a mild increase in disease incidence and severity.

We also assessed whether prolonged CTLA-4 blockade would have a more dramatic effect on the development of EAE. In this experiment, we injected either 100 μg anti-CTLA-4 or rat IgG on days 0, 2, 4, 6, and 8 (Table I, Expt. 2). Similar to the results obtained with the single injection protocol, only a mild enhancement of disease severity was observed in the experimental group.

CTLA-4 blockade exacerbates established clinical disease

Because CTLA-4 blockade during initial interactions resulted in the development of enhanced disease, we next tested the ability of this reagent to alter the disease course after the onset of clinical symptoms. In these studies, anti-CTLA-4 was injected on the second day after animals initially exhibited clinical signs of EAE and every other day for the next 6 days. CTLA-4 blockade during the onset of clinical symptoms markedly exacerbated disease (Table II, Fig. 1). In one experiment, four of the five anti-CTLA-4 recipients died, and the fifth animal in this group developed a maximum disease grade of 4. In contrast, none of the five control animals in that experiment died, although they all developed severe disease

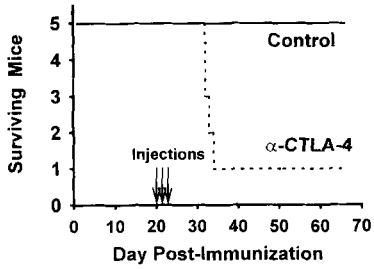

FIGURE 1. CTLA-4 injection at the onset of clinical disease results in disease mortality. Groups of five (PL × SJL)F_1 mice were immunized with MBP/CFA on day 0 and injected with PT i.v., as described in *Materials and Methods*. Mice were examined daily for clinical signs of disease. Disease symptoms were first observed on day 19. On days 20, 22, and 24, animals received 100 μg of anti-CTLA-4 or 100 μg of rat IgG. The mortality following anti-CTLA-4 injections was significantly greater than the control group mortality (Fisher's exact test, $p < 0.05$).

(each developed a maximum disease grade of 4 during the 60 days of observation).

CTLA-4 exacerbation of disease is associated with enhanced secretion of TNF-α, IFN-γ, and IL-2

Clinical EAE has been associated with the production of various Th1 cytokines, including IL-2, TNF-α, and IFN-γ (31). In addition, costimulatory and other accessory receptors, such as CD28, may regulate the production of these inflammatory cytokines (8). ELISA was used to assess in vitro cytokine secretion by cells derived from the draining lymph nodes of mice, which underwent the same protocol as in the experiments shown in Figure 1. Cells were derived from animals treated with anti-CTLA-4 or control rat IgG after the onset of clinical symptoms. Ab was injected on the second day after animals initially exhibited clinical signs of EAE and every other day for the next 6 days. Animals were killed 2 days after the last injection, and cytokine production in response to MBP stimulation was assessed. As shown in Figure 2, cells derived from animals that received anti-CTLA-4 in vivo produced markedly enhanced levels of IL-2, TNF-α, and IFN-γ. The increase in cytokine production following CTLA-4 blockade in response to MBP varied from 3-fold (IL-2) to 10-fold (TNF-α). IL-10 did not accumulate to levels that permitted meaningful comparisons between these cultures (data not shown).

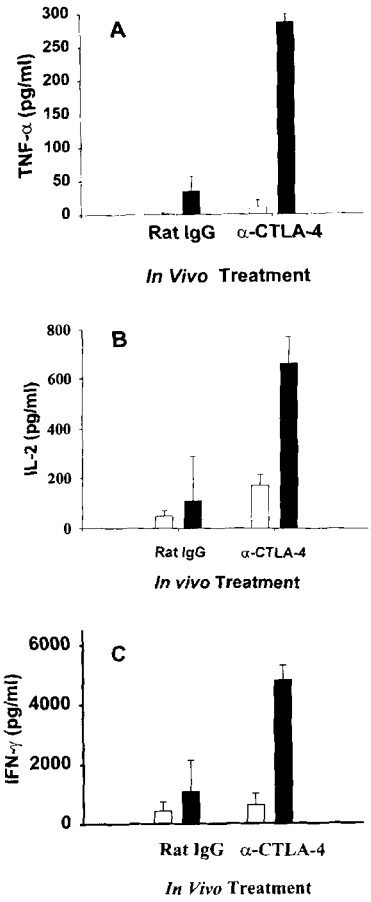

FIGURE 2. CTLA-4 injection at the onset of clinical disease results in enhanced production of proinflammatory cytokines. (PL × SJL)F_1 mice were immunized with MBP/CFA on day 0 and injected with PT i.v., as described in *Materials and Methods*. Animals received 100 μg anti-CTLA-4 or 100 μg rat IgG every other day starting on the second day that clinical signs were first observed. Each animal received a total of three injections ($n \geq 3$). Three days later, the draining lymph nodes were obtained and single cell suspensions were prepared. Cells from individual animals were cultured in the presence of medium (open bars) or 25 μg/ml MBP (filled bars). Supernatants were harvested 24 h later, and TNF-α (*A*), IL-2 (*B*), and IFN-γ (*C*) levels were assessed by ELISA as described in *Materials and Methods*. Data are presented as mean ± SD of the individual mice within each group.

Discussion

Recent evidence suggests that T cell activation is regulated by the opposing effects of CD28 costimulation and CTLA-4 signaling (21). The B7 family of cell surface molecules expressed on APC provides accessory signals to T cells via either CD28 or CTLA-4. However, while CD28 transduces a costimulatory signal that is required for an optimal immune response, CTLA-4 transmits a negative signal. We have previously demonstrated a central role for the B7 ligands in EAE induced by active immunization with MBP (20, 30) and by adoptive transfer of MBP-specific lymph node T cells (20, 32). Blockade of the B7 receptors (both B7-1 and B7-2) with the fusion protein CTLA4-Ig can result in either suppression or enhancement of clinical disease (20).

The roles of the individual B7 molecules, B7-1 and B7-2, during the initiation of EAE have been investigated by us (20, 30) and others (16, 33). It appears that the effects of anti-B7-1 and anti-B7-2 reagents in vitro and in vivo depend upon the model of EAE studied. However, it is clear that anti-B7-1 treatment ameliorates subsequent disease development, while anti-B7-2 treatment tends to exacerbate disease. The mechanism of differential regulation of EAE by the B7 molecules involves differential regulation of cytokines. Kuchroo et al. have attributed the effects of repeated injections of Ab to differential regulation of Th1/Th2 differentiation (16). The effects of a single injection of these Abs may also regulate the encephalitogenic cytokine TNF-α (30). Blockade of B7-1 during ongoing EAE prevents epitope spreading and subsequent relapse (33).

Given the complexity of B7:CD28/CTLA-4 interactions, studies with B7-specific reagents do not distinguish between effects upon CD28-mediated costimulation from CTLA-4 regulation. These studies directly address the role of CTLA-4 on both the initiation and effector stages of the immune response during EAE by blocking CTLA-4:B7 interactions specifically.

We assessed the effect of CTLA-4 blockade at the time of immunization in an active model of EAE. A single injection of anti-CTLA-4 mAb on day 2 mildly exacerbated the development of

EAE. We have previously shown that a single injection of anti-B7-2 on day 2 postimmunization resulted in an exacerbated disease, while a single injection of anti-B7-1 resulted in ameliorated disease (30). Therefore, these new findings complement our previous findings and suggest that B7-2 blockade may have prevented down regulation of the immune response through CTLA-4.

CTLA-4 primarily regulates the effector phase of established EAE. When anti-CTLA-4 was injected after the onset of clinical symptoms, disease was markedly exacerbated. These results predict that CTLA4-Ig therapy could prevent B7:CTLA-4 interaction and result in enhanced disease. Direct manipulation of CD28 signaling may be a better approach to autoimmune disease with fewer confounding factors.

Disease exacerbation was accompanied by the increased production of proinflammatory cytokines in response to MBP stimulation in vitro. IL-2, TNF-α, and IFN-γ production were all enhanced significantly. Most dramatically, TNF-α production in response to MBP stimulation was increased 10-fold (Fig. 2*A*). Thus, CTLA-4 regulation of immunity involves the regulation of proinflammatory cytokines.

In summary, the T cell surface CTLA-4 plays a regulatory role during both the priming and the effector stages of EAE. Thus, CTLA-4 represents a potential target for manipulation in the treatment of autoimmune disorders such as EAE. These results further demonstrate that costimulation by the B7 family of molecules participates in events that both amplify and subsequently down-regulate an immune response.

References

1. Zamvil, S. S., and L. Steinman. 1990. *Annu. Rev. Immunol. 8:579.*
2. Martin, R., H. F. McFarland, and D. E. McFarlin. 1992. *Annu. Rev. Immunol. 10:153.*
3. Raine, C. S. 1984. *Lab. Invest. 50:608.*
4. Arnason, B. G. 1983. *Neurol. Clin. 1:765.*
5. Mokhtarian, F., D. E. McFarlin, and C. S. Raine. 1984. *Nature 248:356.*
6. Waksman, B. H. 1995. *Nature 377:105.*
7. Mondino, A., and M. K. Jenkins. 1994. *J. Leukocyte Biol. 55:805.*
8. June, C. H., J. A. Ledbetter, P. S. Linsley, and C. B. Thompson. 1990. *Immunol. Today 11:211.*
9. Thompson, C. B., T. Lindsten, J. A. Ledbetter, S. L. Kunkel, H. A. Young, S. G. Emerson, J. M. Leiden, and C. H. June. 1989. *Proc. Natl. Acad. Sci. USA 86: 1333.*
10. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. *Science 261:609.*
11. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. *Immunity 3:87.*
12. Freeman, G. J., F. Borriello, R. J. Hodes, H. Reiser, J. G. Gribben, J. W. Ng, J. Kim, J. M. Goldberg, K. Hathcock, G. Laszlo, L. A. Lombard, S. Wang, and G. S. Gray. 1993. *J. Exp. Med. 78:2185.*
13. Freeman, G. J., F. Borriello, R. J. Hodes, H. Reiser, K. S. Hathcock, G. Laszlo, A. J. McKnight, J. Kim, L. Du, D. B. Lombard, G. S. Gray, L. M. Nadler, and A. H. Sharpe. 1993. *Science 262:907.*
14. Caux, C., B. Vanbervliet, C. Massacrier, M. Azuma, K. Okumura, L. L. Lanier, and J. Banchereau. 1994. *J. Exp. Med. 180:1841.*
15. Hathcock, K. S., G. Laszlo, C. Pucillo, P. Linsley, and R. J. Hodes. 1994. *J. Exp. Med. 180:631*
16. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1995. *Cell 80:707.*
17. Azuma, M., D. Ito, H. Yagita, K. Okumura, J. H. Phillips, L. L. Lanier, and C. Somoza. 1993. *Nature 366:76.*
18. Larsen, C. P., S. C. Ritchie, R. Hendrix, P. S. Linsley, K. S. Hathcock, R. J. Hodes, R. P. Lowry, and T. C. Pearson. 1994. *J. Immunol. 152:5208.*
19. Lenschow, D. J., S. C. Ho, H. Sattar, L. Rhee, G. Gray, N. Nabavi, K. C. Herold, and J. A. Bluestone. 1995. *J. Exp. Med. 181:1145.*
20. Racke, M. K., D. E. Scott, L. Quigley, G. S. Gray, R. Abe, C. H. June, and P. J. Perrin. 1995. *J. Clin. Invest. 96:2195.*
21. Krummel, M. F., and J. P. Allison. 1995. *J. Exp. Med. 182:459.*
22. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. *J. Immunol. 155:1032.*
23. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. *Immunity 1:405.*
24. Gribben, J. G., G. J. Freeman, V. A. Boussiotis, P. Rennert, C. L. Jellis, E. Greenfield, M. Barber, V. A. Restivo, Jr., X. Ke, G. S. Gray, and L. M. Nadler. 1995. *Proc. Natl. Acad. Sci. USA 92:811.*
25. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. *Science 270:985.*
26. Leach, D. R., M. F. Krummel, and J. P. Allison. 1996. *Science 271:1734.*
27. Deibler, G. E., R. E. Martenson, and M. W. Kies. 1972. *Prep. Biochem. 2:139.*
28. Pettinelli, C. B., R. B. Fritz, C. H. Chou, and D. E. McFarlin. 1982. *J. Immunol. 129:1209.*
29. Glantz, S. A. 1992. In *Primer of Biostatistics*, 3rd Ed. S. A. Glantz, ed., McGraw-Hill, Inc., New York, pp. 324–330.
30. Perrin, P. J., D. Scott, T. A. Davis, G. S. Gray, M. J. Doggett, R. Abe, C. H. June, and M. K. Racke. 1996. *J. Neuroimmunol. 65:31.*
31. Ando, D. G., J. Clayton, D. Kono, J. L. Urban, and E. E. Sercarz. 1989. *Cell. Immunol. 124:132.*
32. Perrin, P. J., D. Scott, L. Quigley, P. S. Albert, O. Feder, G. S. Gray, R. Abe, C. H. June, and M. K. Racke. 1995. *J. Immunol. 154:1481.*
33. Miller, S. D., C. L. Vanderlugt, D. J. Lenschow, J. G. Pope, N. J. Karandikar, M. C. Dal Canto, and J. A. Bluestone. 1995. *Immunity 6:739.*