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CD154 Blockade Results in Transient Reduction in Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease

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Transient CD154 blockade at the onset of Theiler's murine encephalomyelitis virus-induced demyelinating disease ameliorated disease progression for 80 days, reduced immune cell infiltration, and transiently increased viral loads in the central nervous system. Peripheral antiviral and autoimmune T-cell responses were normal, and disease severity returned to control levels by day 120.

Multiple sclerosis (MS) is a T-cell-mediated autoimmune demyelinating disease of the central nervous system (CNS) (32). While much is known of the disease pathology of MS, very little is clear about its etiology (26). Epidemiological studies provide strong evidence for an environmental trigger, most likely viral (4, 16, 26). CNS pathology may result from bystander damage mediated by T cells targeting a virus that persists in the CNS and subsequent epitope spreading resulting from the release of sequestered myelin antigens secondary to virus-specific T-cell-initiated myelin damage (20, 21).

Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) is a virally induced MS model in which chronic TMEV infection of the CNS in susceptible strains of mice leads to a chronic-progressive form of paralytic disease (25, 26, 30). Inflammation is initiated by recruitment of $CD4^+$ T cells and macrophages in response to persistent low-level viral infection in the CNS (13–15, 22).

The CD154-CD40 ligand pair interaction (31) has been demonstrated in active CNS lesions of patients with multiple sclerosis (5). Members of our group and others have demonstrated that CD154 blockade is an effective long-term way to treat both the induction of and ongoing relapsing-remitting experimental autoimmune encephalomyelitis (EAE) (5, 7–9, 11). Previous studies of virally induced disease have demonstrated expression of CD40 in the CNS of TMEV-IDD mice (27) and that therapeutic blockade of CD154 can ameliorate clinical disease in the short term (3). In this paper, we address the long-term effects and mechanisms of CD154 blockade in TMEV-IDD.

CD154 blockade results in a transient amelioration of clinical TMEV-IDD and reduced immune cell infiltration into the CNS. Mice, inoculated with TMEV in the right cerebral hemisphere as previously described (17, 18), were monitored for clinical disease for approximately 140 days. Starting at the time of clinical disease onset (day 21), mice were given five treatments every other day with 200 ìg of control hamster immunoglobulin G (IgG) or blocking anti-CD154 antibody (Ab) (MR1) (9). Anti-CD154-treated mice demonstrated a significantly reduced severity of clinical disease immediately upon treatment with anti-CD154 Ab, compared to control Ab-treated mice. This amelioration continued until at least 70 days postinfection (Fig. 1, left panel). At all time points this reduction was statistically significant ($P < 0.05$), and it was most apparent by day 50 postinfection. Anti-CD154-treated mice over this period demonstrated an approximately 35 to 40% reduction in the severity of clinical disease, although the incidence of disease was 100% in both groups. Some mice were monitored for an additional 60 to 70 days (Fig. 1, right panel). By 125 days postinfection, the mean clinical disease severity for anti-CD154-treated mice was no longer significantly different from that of control-treated mice (Fig. 1, right panel).

Spinal cord sections were taken from mice 75 days postinfection, and histopathologic scores were determined as previously described (9). Sections taken from anti-CD154-treated mice at this time point, where clinical disease was reduced, demonstrated significantly less inflammatory cell infiltration than that for control Ab-treated animals and very little demyelination (Fig. 2 and Table 1). This supports the argument that reduction in disease is due to inhibition of T-cell effector function within the CNS or modulation of Th1 cell differentiation in the periphery with similar downstream effects (1, 6, 8, 10, 11, 24).

Peripheral virus- and myelin-antigen-specific Th1 responses in vivo are not affected by anti-CD154 treatment. To determine whether CD154 blockade affected T-cell differentiation in the periphery or whether this reduced infiltration in the CNS could be ascribed to effector function within the CNS alone, delayed-type hypersensitivity (DTH) responses were evaluated, as a measure of in vivo peripheral Th1-cell differentiation and effector function, 45 days after treatment (day 74 postinfection), to ensure clearance of the MR1 Ab. DTH responses to both viral antigen, $VP2_{70-86}$ peptide (WTTSQEAFSHIRIPLPH), and immunodominant myelin antigens, $PLP_{139-151}$ (HSLGKWLGHPDKF) and MBP_{84-104} (VHFFKNIVTPRTPSQGKG), were determined as previously described (9).

Anti-CD154 Ab results in reduced DTH to immunizing an-

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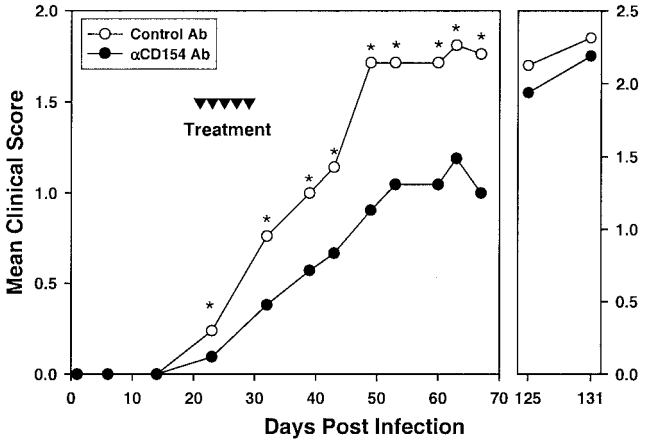

FIG. 1. Short-term anti-CD154 treatment at disease onset results in transient reduction of severity of TMEV-induced demyelinating disease. Mice were infected intracerebrally with 9×10^7 PFU of TMEV on day zero and administered a total of five treatments with either control hamster IgG Ab (○) or anti-CD154 Ab (●) at the indicated time points (▼). Clinical disease was evaluated on an incremental six-point severity scale until day 68 for a total of 21 mice from three separate experiments (left panel). An additional 14 mice from two of these experiments were monitored for clinical disease until day 135 (right panel). Statistically significant difference as determined by a Mann-Whitney nonparametric test between control- and anti-CD154-treated mice is denoted by an asterisk above the day on which differences were observed ($P < 0.05$).

tigens, provided treatment is given at the time of immunization (8, 9, 11). DTH responses to $VP2_{70-86}$ were comparable between control- and anti-CD154-treated mice (Fig. 3), reflecting the lack of effect of delayed Ab treatment (day 21 postinfection) on antiviral responses. Curiously, CD154 blockade did not affect development of DTH responses to the myelin autoantigen $PLP_{139-151}$ or MBP_{84-104} (Fig. 3). This indicates that although the response to myelin antigens cannot be detected by DTH until over 50 days postinoculation (22), epitope spreading has probably already begun by day 21 when CNS damage begins.

Diminished clinical severity in TMEV-IDD is associated with a transient increased viral load in the CNS. The data clearly demonstrate that while clinical disease severity (Fig. 1) and histopathology scores (Fig. 2 and Table 1) were significantly reduced in anti-CD154 versus control Ab-treated mice, Th1 activation (Fig. 3) is not affected in the long term by transient CD154 blockade at the onset of disease. This suggests that perhaps Th1 effector function, but not the continued development of peripheral Th1 responses, is blocked within the CNS, similar to what we have previously reported in the EAE model (10). Since the TMEV-IDD model is dependent on persistent infection of macrophages infiltrating into the CNS (12, 19, 29), we determined the effect of anti-CD154 treatment on viral activity in the CNS using a viral plaque assay (23). Anti-CD154 treatment suppressed antiviral effector immune responses in the CNS as illustrated by the finding that at 75 days postinfection, a time point where inflammation, demyelination, and clinical disease were suppressed by anti-CD154 treatment, the viral load present in both the brains (40-fold increased) and spinal cords (12-fold increased) of treated mice

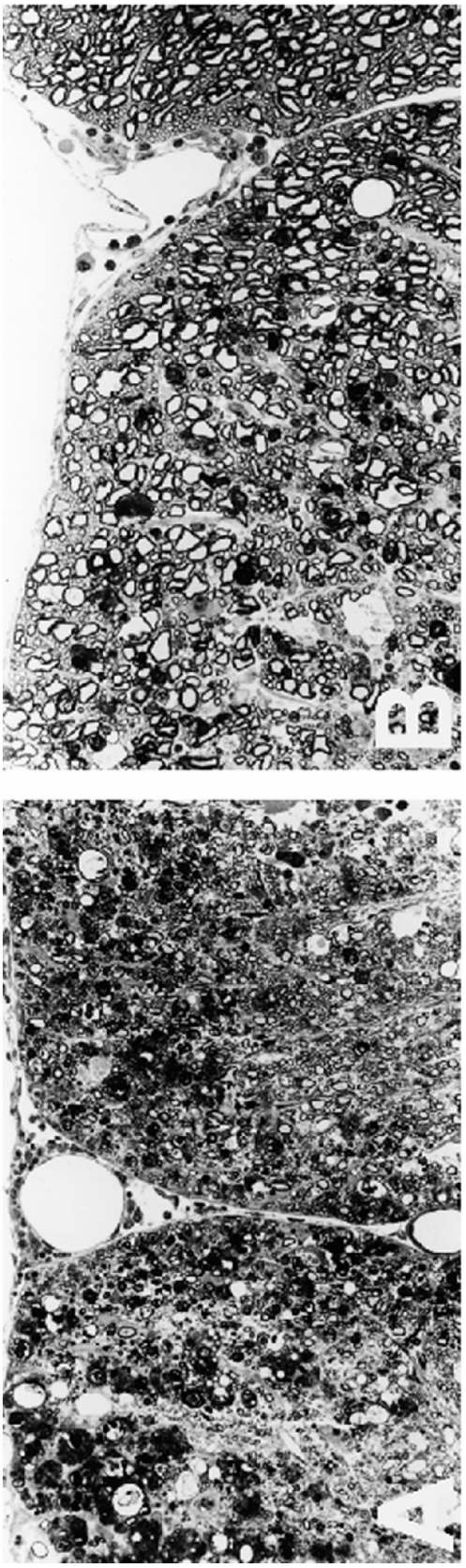

FIG. 2. Anti-CD154-treated mice demonstrate reduced immune cell infiltration and demyelination. Lumbar spinal cord sections from representative animals of the treatment groups described in Fig. 1 were examined for CNS histopathology. (A) Section of spinal cord from a mouse treated with hamster control IgG from days 21 to 29 and sacrificed on day 75. Inflammatory cell infiltrates are identified by dark filled spots throughout the spinal cord section, while extensive demyelination was seen by the absence of myelinated axons (ring structures with dark circumferences). (B) Section of spinal cord from a mouse treated with anti-CD154. Details of the degree of inflammation and demyelination are given in Table 1. One-micrometer-thick Epon-embedded sections stained with toluidine blue. Original magnification, ×220. Images are taken from two mice in the same experiment and are representative of a total of six mice from each group over two experiments, as noted in Table 1.

TABLE 1. Summary of histology of spinal cord sections from mice treated from day 21 post-TMEV inoculation

Experiment No.[a]	Hamster Ig controls		Anti-CD154 treated	
	Disease score[b]	Histopathology score[c]	Disease score	Histopathology score
1A	4	+++	1	++
1B	3	+++	1	+
1C	3	+++	2	++
2A	2	+++	1	++
2B	2	+++	1	+
2C	2	+++	2	+++

[a] Lumbar spinal cord sections from mice treated from days 21 to 29, every other day for five treatments, with either hamster Ig control or anti-CD154 MR1 antibody, were assessed for CNS histopathological changes 75 days after inoculation with TMEV.

[b] Peak disease score severity at time of spinal cord removal.

[c] Ten sections were examined for each mouse and scored for disease on the following scale: 0, no disease; ±, meningitis; +, focal infiltration and demyelination; ++, multiple infiltrates and demyelination; +++, confluent infiltrates and demyelination.

was markedly higher than that of controls (Fig. 4a). However, at a later time point, 134 days after inoculation (105 days after the end of Ab therapy), viral loads in both the brains and spinal cords of anti-CD154 treated mice had returned to control levels (Fig. 4b). This may be explained by the fact that as the therapeutic anti-CD154 Ab is cleared, Th1 cells may then resume their effector activity in the CNS and both help to control viral replication (Fig. 4b) and contribute to demyelination as a consequence of their inflammatory activity (Fig. 1).

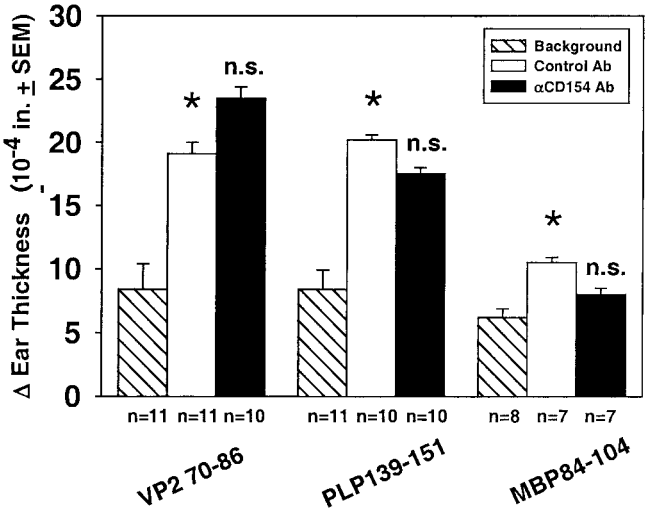

FIG. 3. Day 75 DTH responses to viral and myelin antigens are not affected by short-term anti-CD154 treatment. DTH responses to viral ($VP2_{70-86}$) and myelin ($PLP_{139-151}$ and MBP_{84-104}) protein epitopes were determined in hamster control-treated and anti-CD154 Ab-treated mice 75 days after intracerebral infection with TMEV. Hatched bars represent naïve background responses from uninfected, untreated mice; clear bars represent responses from hamster control IgG-treated, TMEV-infected mice; and filled bars represent responses from anti-CD154-treated, TMEV-infected mice. Data represent pooled individual results from three separate grouped experiments. Numbers of mice in each group are identified below the *x* axis. Asterisks denote a significant DTH response in control-treated inoculated mice versus naïve mice ($P < 0.05$), while n.s. denotes no significant difference between anti-CD154 and control Ab-treated, TMEV-infected mice.

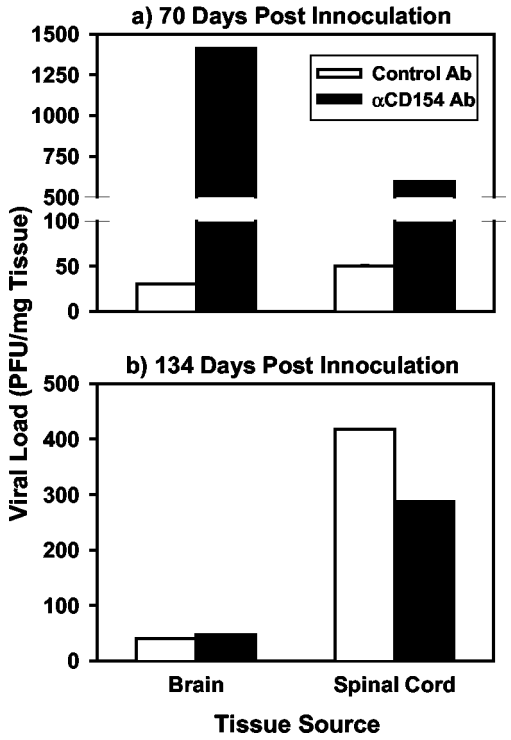

FIG. 4. Short-term anti-CD154 treatment results in a transient increase in TMEV viral loads in the CNS. Mice from Fig. 1 were sacrificed on either day 70 (a) or day 134 (b), and viral titers in the CNS were determined from spinal cords and brains. Clear bars represent control Ab-treated, TMEV-infected mice, while filled bars represent anti-CD154-treated mice. Three organs per group per experiment were collected, pooled, and used for viral titration assays, which were done in triplicate. Plaque counts were then adjusted to the gram weight of each tissue harvested and were expressed in PFU/milligram of harvested tissue. Data are representative of two separate experiments.

Progress of clinical disease is sustained in anti-CD154-treated mice, albeit at a significantly slower rate than that of control-treated mice (Fig. 1). Direct viral cytopathic effects on oligodendrocytes, as reflected by the high CNS viral titers, were most probably the cause of low-level demyelination observed in the CNS of mice recently treated with anti-CD154, since little or no effector Th1 cells are present at this time (Fig. 2). We and others have demonstrated the critical nature of the CD154-CD40 ligand pair interaction in the initiation of both CD4- and CD8-T-cell effector function in the target organ (1, 2, 10). Thus, inhibition of T-cell function within the CNS would allow viral replication to continue unchecked, as demonstrated in a lymphocytic choriomeningitis virus model (28). In contrast, since viral loads are significantly lower in control Ab-treated mice, with higher levels of clinical disease, it is likely that the T-cell-mediated inflammatory response to both viral and myelin antigens within the CNS is the major cause for demyelination and disease.

In summary, we demonstrate that CD154 blockade transiently inhibits T-cell effector function in the CNS of mice with ongoing TMEV-IDD. However, due to the significant long-term effect of anti-CD154 Ab treatment on suppression of

antiviral immune responses in the CNS, these data suggest caution in the use of this immunotherapeutic regimen for the treatment of chronic-progressive forms of MS, which may be associated with a persistent CNS virus infection, and perhaps of relapsing forms where disease exacerbation is triggered by flaring of a viral infection.

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