Autoimmune Intervention by CD154 Blockade Prevents T Cell Retention and Effector Function in the Target Organ

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The CD40-CD154 interaction is an attractive target for therapeutic intervention in many autoimmune disorders, including multiple sclerosis. Previously, we showed that CD154 blockade both inhibited the onset of experimental autoimmune encephalomyelitis and blocked clinical disease progression (relapses) in mice with established disease. The mechanism of this protection is poorly understood. Because CD154 plays a role in Th1 development, its blockade has been thought to promote anti-inflammatory Th2 responses. However, these conclusions have primarily been based on extrapolated data from in vitro experiments, which may not accurately reflect the more complex events occurring in vivo. In this paper we determine how the immune response develops under the influence of therapeutic CD154 blockade in vivo. We demonstrate that anti-CD154 treatment does not alter the early expansion of Ag-specific T cells in secondary lymphoid organs or result in deviation to a Th2-dominant response. Interestingly, the late expansion and retention of Th1 cells in the lymph nodes were markedly reduced following immunization of Ab-treated mice, and this coincided with a recompartmentalization of these cells to the spleen. Most importantly, anti-CD154 treatment eliminated the retention/expansion of encephalitogenic Th1 cells, but not their entry into the CNS. These data indicate that a major mechanism by which CD154 blockade protects against autoimmune disease is by controlling the amplitude of acute phase Th1 responses in the draining lymph nodes and by preventing the sustained expansion of effector cells within the target organ. The Journal of Immunology, 2001, 166: 1547–1553.

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coincided with a recompartmentalization of these cells to the spleen. In addition, a major mechanism by which CD154 blockade protects against autoimmune disease is by controlling the recruitment and/or expansion of effector cells within the CNS target organ.

Materials and Methods

Mice

Five- to 6-week-old female SJL mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed under barrier conditions at the National Institutes of Health-approved Northwestern University Medical School animal facilities. All protocols were approved by the Northwestern University animal care and use committee. Paralyzed mice were afforded easier access to food and water. DO11.10 and SJL CD90.1 congenic mice were bred within the facility, and BALB/cAnNCr mice obtained from National Cancer Institute laboratories (Frederick, MD).

Peptides

Proteolipid protein (PLP)139–151 (HSLGKWLHGDPIKF) and OVA323–339 (ISQAVHAAHAEINEAGR) were synthesized by the peptide facility at University of North Carolina (Chapel Hill, NC). The amino acid compositions of these peptides were verified by mass spectrometry, and purity (97%) was confirmed by mass spectroscopy at Michigan State University Biotechnology Center (Ann Arbor, MI).

In vivo Ab treatment

Anti-CD154 (MR-1) was produced in ascites and purified by HPLC over a Biotechnology Center (Ann Arbor, MI). For actively induced R-EAE, mice were immunized s.c. with 100 μg of peptide/animal. For adoptive transfer, naive BALB/c mice were treated i.p. 1 day before OVA323–339 immunization and each day after priming until day 3 with 200 μg of control hamster Ig or MR-1 Ab.

Induction of active and adoptive R-EAE

For actively induced R-EAE, mice were immunized s.c. with 100 μg of a CFA emulsion containing 400 μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) and 50 μg of PLP139–151, distributed over three sites on the lateral hind flanks and dorsally. For OVA323–339 peptide priming, the same protocol was used with 50 μg of peptide/animal. For adoptive transfer active R-EAE, SJL mice (6–8 weeks old) were primed s.c. with 50 μg of PLP139–151, in 100 μl of CFA distributed over three dorsal sites. Draining lymph nodes (axillary and inguinal) were harvested on day 10 and cultured in vitro in 75-cm² tissue culture flasks (8 × 10⁶ cells/flask) with supplemented DMEM and 25 μg/ml PLP139–151. After 4 days in culture, the cells were harvested. Recipient female CD90.2 SJL/J mice were injected i.v. with 1.75 × 10⁷ cells in a final volume of 0.5 ml.

In vitro T cell enzyme-linked immunospot (ELISPOT) assay

Spleen and lymph node cells were obtained from mice at various stages of disease progression, and dissociated cells were cultured in 96-well microtiter ELISPOT plates (Whatman, Clifton, NJ) that had been coated overnight with capture Abs to IL-2, IL-5, and IFN-γ (BD-PharMingen, Mountain View, CA). Total cell numbers recovered were determined by use of a hemocytometer. After nonspecifically blocking the plates with 5% BSA in PBS, cells were left to stand in the dark for 8 min with gentle rocking. The plates were then incubated at 4°C overnight with biotin-conjugated Abs to IL-2, IL-5, and IFN-γ (BD-PharMingen). Finally, the wells were washed thoroughly with PBS containing 0.2% Tween 20 and alkaline phosphatase (Sigma-Aldrich) was added. Once the spots had developed, the plates were shaken clean of substrate and washed thoroughly under gently flowing distilled water. ELISPOTs were counted visually, and results were corroborated using an ELISPOT plate reader and software (Cellular Technologies, Cleveland, OH). At least four wells per sample were counted and are presented as a mean value.

Intracytoplasmic cytokine analysis and CFSE labeling of cells

CD4+ T cells from DO11.10 mice were isolated by CD4 magnetic bead separation (Miltenyi Biotech, Auburn, CA), and 5 × 10⁷ DO11.10 OVA323–339 transgenic T cells were transferred i.p. to a naive BALB/cAnNCr mouse. In some experiments before transfer the cells were labeled with CFSE (Molecular Probes, Eugene, OR). Brieﬂy, cells were resuspended at 2 × 10⁷ cells/ml in PBS and diluted 1:1 with 2.8 μg/ml CFSE in PBS. Cells were left to stand in the dark for 8 min with gentle rocking. CFSE was quenched by addition of an equal volume of FCS, mixed well, and left for 1 min. The cells were then washed twice with PBS containing 10% FCS, incubating for 5 min for each addition. Three days after transfer, the mice were primed with OVA323–339/CFA as described above. Draining lymph nodes (axillary and inguinal) and spleen cells from the primed mice were removed on day 3 after priming. For intracytoplasmic cytokine detection, cells were cultured in a 96-well microtiter plate (Nunc, Copenhagen, Denmark) at 5 × 10⁶ cells/ml and stimulated for 4 h with PMA (50 ng/ml Sigma) and ionomycin (500 ng/ml Sigma). Two hours before harvest, brefeldin A (Sigma) was added to a final concentration of 1 μg/ml to prevent cytokine secretion. Cells were then stained with KJ1-26 and APC-specific for the OVA323–339 transgenic TCR and then fixed in 2% formaldehyde. Cells were washed and resuspended in permeabilization buffer (PBS, 0.1% saponin (Sigma), and 1% FCS). Cells were stained for cytokines by incubation for 30 min with either control Abs or Abs to IL-2, IL-4, IL-5, IL-10, IFN-γ, or TNF-α (BD-PharMingen). The cells were washed twice, resuspended in PBS, and analyzed on a FACSCalibur cell analyzer (BD-PharMingen).

Identification of CNS-infiltrating T cells

Mice were anesthetized at various time points after disease transfer. Mice were perfused with 60 ml of PBS, and then the spinal cords were isolated by intrathecal hydrostatic pressure. The cords were mashed on a 100-mesh screen and resuspended in 30% Percoll and oil-alcohol over 70% Percoll. Cells were then spun for 15 min at 400 × g, and cells at the 30:70% interface were collected. CD4+ T cells were then examined by flow cytometry for expression of CD90.1 (donor) and CD90.2 (host).

Results

Early in vivo Ag-specific T cell expansion is unaffected by CD154-CD40 blockade

We have previously shown that total cell recovery and in vitro Ag-specific T cell proliferation are reduced in the lymph nodes of anti-CD154-treated animals 10 days following immunization (16). One explanation for this reduction is that CD154 blockade inhibits T cell expansion within the lymph nodes. To directly determine the ability of anti-CD154 to block the proliferation of naive T cells in vivo, we examined the proliferation of CD4+ DO11.10 transgenic T cells in an adoptive transfer system (26). Naive BALB/c recipients of CFSE-labeled transgenic cells were immunized with OVA323–339/CFA, and 3 days later the lymph node and splenic cells were harvested. Ag-specific T cell expansion was evaluated by the reduction in CFSE fluorescence with each division of the labeled transgenic T cells. Immunization with PBS/CFA did not stimulate proliferation; thus, the transgenic cells retained a high level of CFSE fluorescence (Fig. 1A). Immunization with OVA323–339/CFA in the presence of control Ig (Fig. 1B) or anti-CD154 (Fig. 1D) resulted in rapid proliferation of up to seven cell divisions by 72 h. In contrast, as a positive control, treatment with CTLA-4 Ig (an antagonist of B7-CD28/CTLA-4 costimulation) at the time of immunization severely inhibited proliferation of the transgenic T cells. Therefore, blocking the CD154-CD40 interaction in vivo does not affect early T cell expansion in the lymph node similar to the recent observations by Howland et al. (24). Consistent with these data, we found that comparable total cell numbers were recovered from the lymph nodes and spleens of the control Ig and
Anti-CD154-treated mice 3 days after immunization (data not shown). Minimal T cell expansion/proliferation was observed in the spleen at this time point regardless of treatment (data not shown).

**Anti-CD154 treatment leads to recompartmentalization of the Ag-specific T cell immune response**

Because we did not observe a difference in either early T cell expansion or total cell recovery in the BALB/cAnNCr mouse 3 days after immunization, we readdressed this question in relation to the effects of anti-CD154 treatment on a prolonged immune response in both SJL and BALB/cAnNCr mice. SJL mice were immunized on day 0 with the encephalitogenic PLP139–151 peptide in CFA and the absolute numbers of Ag-specific Th0 (IL-2 secretors), Th1 (IFN-γ secretors), and Th2 (IL-5 secretors) cells in the lymph node and spleen determined by ELISPOT every other day for 14 days postimmunization (Fig. 2). Mice were treated with either control Ab or anti-CD154 blocking Ab every other day from days 0–6. As previously observed in the DO11.10 transfer system (Fig. 1), within the first 4 days there was little or no difference in the total numbers of PLP139–151-specific T cells recovered from the lymph nodes or spleens of anti-CD154-treated mice as compared with controls. In contrast, as the immune response progressed, the total numbers of both IL-2- and IFN-γ-secreting cells were significantly decreased in the lymph nodes of anti-CD154-treated mice (Fig. 2, D and E). We observed a similar reduction of Ag-specific lymph node T cells in the BALB/c-DO11.10 transfer system beyond 4 days postimmunization (data not shown), as shown recently by Howland et al. (24). The numbers of peptide-specific, IL-5-secreting Th2 cells in both spleen (Fig. 2C) and lymph node (Fig. 2F) of control mice were low, but were also reduced in the lymph nodes of anti-CD154-treated mice.

Interestingly, the absolute numbers of peptide-specific Th0 and Th1 cells in the spleen showed a pattern different from that seen in the draining lymph nodes. The total numbers of PLP139–151-specific IL-2-producing cells were comparable between anti-CD154-treated and control mice throughout the 14-day time course (Fig. 2A). However, the numbers of peptide-specific Th1 cells in the spleens of anti-CD154-treated mice, although somewhat lower than control levels on days 6 and 8 postpriming, rebounded to
levels approximately twice that of controls on days 10 and 12 postimmunization (Fig. 2B). The increase in the numbers of splenic PLP<sub>139–151</sub>-specific Th1 cells temporally correlated with the reduction in Th1 T cell numbers observed in the lymph nodes of anti-CD154-treated animals. This suggests that anti-CD154 treatment, instead of absolutely inhibiting long term differentiation Th1 cells, may reduce the retention of Th1 cells in the lymph nodes and/or transiently enhance their retention in the spleen.

Interestingly, if the numbers of IL-2- and IFN-γ-producing T cells are expressed as a frequency of total CD4<sup>+</sup> T cells over the 14-day period following immunization, a somewhat different pattern is evident (Fig. 3). The percentage of IL-2-producing cells from the spleen (Fig. 3A) and lymph nodes (Fig. 3C) of anti-CD154-treated mice did not differ significantly from that seen in controls. In contrast, there was a delay (ranging between 2 and 4 days), but not a reduction in the frequency of peptide-specific IFN-γ-producing Th1 cells in the lymph nodes of anti-CD154-treated mice (Fig. 3D). However, as seen with the absolute numbers of peptide-specific T cells (Fig. 2B), there was a transient increase in the percentage of IFN-γ-producing T cells in the spleens of anti-CD154-treated mice (Fig. 3B).

**Anti-CD154 treatment does not lead to a Th2 skewed response**

Because SJL mice are poor Th2 responders, we readdressed the possibility that anti-CD154 treatment may preferentially lead to a Th2 response using the BALB/c DO11.10 transfer system. Recipients of DO11.10 T cells were immunized with OVA<sub>323–337</sub>/CFA, and frequencies of peptide-specific Th0 (IL-2), Th1 (IFN-γ and TNF-α), and Th2 (IL-4, IL-5, and IL-10) cells were determined by intracytoplasmic staining for the individual cytokines 3 days postpriming. The frequency of IL-2-secreting cells was significantly reduced in the lymph node, but was comparable in the spleens of anti-CD154-treated vs control Ig-treated animals. As observed previously in SJL mice (Fig. 3), the frequency of OVA<sub>323–339</sub>-specific Th1 cells was reduced in the lymph nodes (Fig. 4A), but was enhanced in the spleens (Fig. 4B) of anti-CD154-treated BALB/c recipients. As seen previously in the PLP<sub>139–151</sub> system (Fig. 2), very few Th2 (IL-4- or IL-5-producing) cells were detectable regardless of previous Ab treatment.

**FIGURE 3.** Anti-CD154 treatment delays the differentiation, but not the frequency, of Th1 cells in the draining lymph nodes. Frequencies of Th0 (IL-2) and Th1 (IFN-γ) cells were determined by ELISPOT assay as described in Materials and Methods. Th0 frequencies were largely unchanged in draining lymph node and splenic compartments in both control Ig- and anti-CD154-treated animals (A and C). Th1 differentiation was delayed in the draining lymph nodes of anti-CD154-treated animals, while the peak frequency was comparable to that in controls (D). In contrast, Th1 cells in the spleen were delayed, but appeared transiently in higher frequencies (B). The data shown are representative of three separate experiments.

**FIGURE 4.** Anti-CD154 treatment does not lead to a Th2 skewed response. DO11.10 transgenic OVA<sub>323–339</sub>-specific T cells (5 × 10<sup>6</sup>) were transferred to groups of naive recipient BALB/cAnNCr mice. The mice were primed with OVA<sub>323–339</sub>/CFA. Peptide-primed mice were treated daily starting 1 day before priming with either hamster control Ig or anti-CD154. Three days after priming, draining lymph nodes (A) and spleens (B) were pooled from three mice per group. Cells were stimulated with PMA and ionomycin for 6 h and with Brefeldin A for the last 4 h as described in Materials and Methods. CD4<sup>+</sup> KJ1-26<sup>+</sup> cells were then analyzed for intracytoplasmic cytokine production by flow cytometry. The data shown are representative of three separate experiments.
indicating that immune deviation is not responsible for anti-CD154-induced protection from clinical autoimmune disease.

**Ab to CD154 inhibits T cell retention/expansion in the CNS**

EAE is a complex, multistep immunopathologic process that requires activation and differentiation of encephalitogenic Th1 cells, migration of the T cells to the CNS, and local production of proinflammatory cytokines and chemokines that mediate the inflammatory demyelination. The data to this point indicate that although anti-CD154 treatment has a profound effect on inhibiting induction and progression of EAE (16), it does not significantly affect very early T cell expansion and only marginally delays Th1 differentiation without significantly skewing the response to a Th2 pattern. Phenotypic analysis of DO11.10 T cells 3 days after OVA323–339/CFA immunization showed that anti-CD154 treatment did not significantly affect the expression of homing receptors (e.g., VLA-4, CD44, and ICAM-1) demonstrated to be involved in trafficking of T cells to the CNS (data not shown). Based on these findings and our previous observation that anti-CD154 treatment impaired the expression of clinical disease in adoptive recipients of encephalitogenic T cells (16), we asked whether anti-CD154 therapy affected CNS T cell recruitment and/or retention. SJL CD90.1 congenic mice were immunized with PLP139–151/CFA. Ten days following immunization, lymph node cells were harvested and cultured with peptide for 4 days before transfer. SJL (CD90.2) recipient mice received four treatments with 200 μg of control Ig or anti-CD154 beginning immediately after cell transfer. At varying times post-transfer, spinal cords were harvested, and infiltrating cells were isolated, stained for CD4 and CD90.1, and analyzed by flow cytometry. Due to the transfer of only a limited number (1.75 × 10⁶) of T cell blasts, detection of significant numbers of donor CD90.1 T cells (0.6%) was first demonstrable in the spinal cord on day 16 posttransfer (Fig. 5B). The numbers of both donor (CD90.1⁺) and recipient (CD90.1⁻) T cells increased dramatically by day 18 (Fig. 5C), which corresponded to the day of disease onset in the control Ig-treated mice (data not shown). A relatively comparable number of donor CD90.1⁺ T cells was found in the CNS of anti-CD154-treated mice on day 16 (Fig. 5E), suggesting that the Ab treatment did not prevent early T cell entry. However, unlike the control mice, the percentages of donor and recipient T cells decreased in the CNS of anti-CD154-treated mice on day 18 (Fig. 5F), indicating that anti-CD154 inhibited the retention/expansion of these cells in the target organ. Coincident with the absence of persistent numbers of donor T cells in the CNS of anti-CD154-treated mice, these animals did not develop clinical EAE (data not shown).

**Discussion**

The CD154-CD40 interaction is critical for the induction and progression of EAE (14–16) and many other autoimmune diseases. Because of the role of the CD154-CD40 interaction in regulating IL-12 production, it has been proposed that the protection from autoimmunity is due to a deviation of T cell response from a proinflammatory Th1 response to an anti-inflammatory Th2 response (23, 24). However, CD40-CD154 interactions are critical in regulating many other immune cell functions (4). Here we address the specific mechanism(s) by which clinical autoimmune disease may be prevented by CD40 ligand blockade. The actual mechanism appears to have little or nothing to do with Th1 to Th2 cell deviation, but instead is associated with significantly reduced Ag-specific T cell responses in the lymph nodes and the prevention of CD4 T cell effector expansion/function within the autoimmune target organ.

Unlike therapies that target the B7-CD28/CD152 costimulatory pathways, interference with the CD40-CD154 interaction did not inhibit the early activation and proliferation of naive T cells in vivo (Fig. 1). Although early T cell expansion within the lymph node is not affected by CD154 blockade, the later progression of the immune response is affected. Up to 4 days postimmunization the expansion of Ag-specific T cells appeared normal (Figs. 2 and 3). However, after that time there was a dramatic reduction, but not ablation, in the continued development of the primary immune response in the draining lymph nodes of anti-CD154-treated mice.

Engagement of CD40 is known to be a critical survival signal for B cells (4). In the absence of CD40 ligation, B cells activated via their B cell receptor undergo apoptosis (4). Similarly, CD40 ligation is also a survival signal for Ag-presenting dendritic cells (6). In the absence of this event, dendritic cells also undergo apoptosis (6). Thus, it is likely that after 4 days of the immune response in vivo, dendritic cell death could explain this sudden reduction in the normal progression of the T cell immune response. However, it is notable that this reduction in the T cell response is not complete and that some continued increase in the development of the T cell response is observed in anti-CD154-treated mice. This may be due to the fact that the Ab doses employed to treat clinical disease do not completely prevent CD40-CD154 interactions and that some dendritic cells survive. However, as discussed below, long term expression of clinical disease is dramatically inhibited.
by a brief treatment with anti-CD154 (16), implying that CD154 blockade may act at multiple levels.

We also investigated whether anti-CD154 treatment led to immune deviation, resulting in skewing of the response from Th0 to Th2 instead of Th1 cells. We observed reduced overall numbers of Th1 cells in the lymph nodes employing two different systems, PLP<sub>139–151</sub> immunization of SJL mice and OVA<sub>223–339</sub> immunization using the DO11.10 TCR transgenic adoptive transfer system (Figs. 2–4). However, in neither case did we observe any significant deviation toward Th2-type cells in either the lymph node or the spleen. In fact, in both systems even fewer Th2 cells were observed in the draining lymph nodes of anti-CD154 compared with control Ig-treated mice. This suggests that the mechanism of action of CD154 blockade in preventing the induction/progression of EAE is not due to Th1 to Th2 deviation. This is supported by observations in the CD40 knockout mouse, in which both Th1- and Th2-type responses are significantly diminished or absent (4). Previous studies supporting a role for immune deviation in anti-CD154-treated or CD40 knockout mice (23, 24) showed only moderate short term increases in IL-4 secretion, as measured by ELISA in vitro. This is not supported by the current observation that fewer Th2 cells are found in Ab-treated mice. Those previous observations may be due to reduced IFN-γ secretion, resulting in less functional inhibition of IL-4 secretion by a small number of Th2 cells in the in vitro culture systems employed. Because there are very few Ag-specific Th2 cells in anti-CD154-treated mice, and Th2 cells demonstrate a reduced ability to migrate to target tissues and organs, it is unlikely that immune deviation is a major mechanism in anti-CD154 therapy of autoimmune disease.

We found that Th1 differentiation was only delayed, reaching its peak 2–4 days after that of control treated animals. This indicated that even in the presence of CD154 blockade that effectively prevented EAE, Th1 differentiation was not ablated as dramatically as previously thought (16, 23, 24). At the same time, higher absolute numbers and frequencies of peptide-specific Th1 cells were observed in the spleens of Ab-treated mice compared with controls, while Th0 cell numbers appeared equivalent. This suggests that those cells that had differentiated within the lymph node recompartmentalized to the spleen.

We previously showed that anti-CD154 treatment of only recipient mice in a disease transfer model could effectively inhibit the effector phase of EAE (16). One possible explanation for this was that T cells may not gain access to the CNS in the absence of CD40 ligation. However, because murine cerebrovascular endothelial cells that line the blood-brain barrier do not express CD40, and T cells from anti-CD154-treated mice express control levels of homing molecules involved in CNS trafficking (our unpublished observations), it was puzzling how this could occur. Here we show that the initial entry of activated encephalitogenic T cells is not inhibited, but their continued retention/expansion within the CNS is blocked. Prevention of T cell recruitment of other immune effector cells to that target organ via CD40/CD154 regulation of chemokine gradients and/or the expansion of the effector T cells within the target organ appears to be a major mechanism by which CD154 blockade prevents the induction/expression of EAE.

Once disease-initiating encephalitogenic T cells get into the CNS, they may require CD154 expression to up-regulate MHC class II on the resident CNS microglial APCs or infiltrating macrophages, which have been shown to play an effector role in EAE (27). CD40 ligation has been shown to up-regulate class II as well as costimulatory molecule CD80/CD86 expression by macrophages (6), and we speculate that without CD40 ligation within the target organ, Ag-specific T cell expansion cannot occur, as we failed to observe the large increase in T cell numbers observed in control mice (Fig. 5). Furthermore, astrocytes, microglia, and CNS-infiltrating macrophages have been implicated in the secretion of chemokines necessary for T cell recruitment to the CNS (28). Because CD40 ligation has been shown to induce chemokine secretion (29, 30), it is possible that this is another factor in disease prevention. Finally, proinflammatory cytokines, reactive oxygen species, NO, as well as some matrix metalloproteinases are induced by CD40 engagement on macrophages (31, 32). Thus, these effector molecules may also be inhibited by CD154 blockade. We are currently investigating how these events are affected by CD154 blockade within the CNS during ongoing EAE.

For the first time, we quantitate how the overall T cell immune response develops in varying lymphoid compartments during Ab treatment of the CD154-CD40 ligand pair interaction, which is an effective treatment in many autoimmune disease models. Therapeutic intervention apparently has little to do with early Th1 to Th2 deviation or overall long term reduction in Th1 responses, although the amplitude of the Th1 response leading up to the acute phase of disease is diminished. Whether Th modulation is associated with long term prevention of disease by CD154 blockade is currently under investigation. Rather, CD40/CD154 interactions appear to be critical for recruitment to and/or expansion of Ag-specific T cells within the target organ. Thus, although activated encephalitogenic T cells can apparently initially access the CNS, their retention/expansion and their ability to recruit additional inflammatory cells are blocked.

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