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FcR Interactions Do Not Play a Major Role in Inhibition of Experimental Autoimmune Encephalomyelitis by Anti-CD154 Monoclonal Antibodies

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It has been demonstrated that anti-CD154 mAb treatment effectively inhibits the development of experimental autoimmune encephalomyelitis (EAE). However, although it appears to prevent the induction of Th1 cells and reactivation of encephalitogenic T cells within the CNS, little information is available regarding the involvement of alternative mechanisms, nor has the contribution of Fc effector mechanisms in this context been addressed. By contrast, efficacy of anti-CD154 mAbs in models of allotransplantation has been reported to involve long-term unresponsiveness, potentially via activation of T regulatory cells, and recently was reported to depend on Fc-dependent functions, such as activated T cell depletion through FcγR or complement. In this study we demonstrate that anti-CD154 mAb treatment inhibits EAE development in SJL mice without apparent long-term unresponsiveness or active suppression of disease. To address whether the mechanism of inhibition of EAE by anti-CD154 mAb depends on its Fc effector interactions, we compared an anti-CD154 mAb with its aglycosyl counterpart with severely impaired FcγR binding and reduced complement binding activity with regard to their ability to inhibit clinical signs of EAE and report that both forms of the Ab are similarly protective. This observation was largely confirmed by the extent of leukocyte infiltration of the CNS; however, mice treated with the aglycosyl form may display slightly more proteolipid protein 139–151-specific immune reactivity. It is concluded that FcR interactions do not play a major role in the protective effect of anti-CD154 mAb in the context of EAE, though they may contribute to the full abrogation of peripheral peptide-specific lymphocyte responses. ***The Journal of Immunology,*** **2004, 173: 993–999.**

The interaction between CD40 and CD154 plays a crucial role in the induction of B and T cell responses (1). It is well established that interactions between CD40 on APCs and CD154 on T cells results in the polarization of Th1 responses (2, 3). This is mainly due to the induction of IL-12 in the APC (3, 4).

Several studies have demonstrated that anti-CD154 mAbs inhibit such responses and are capable of blocking experimental diseases that are Th1-mediated. In the classical model of experimental autoimmune encephalomyelitis (EAE)[2] that can be induced in SJL mice by immunization with the dominant encephalitogenic epitope of proteolipid protein (PLP), i.e., $PLP_{139-151}$, anti-CD154 was effective in inhibiting disease when administered during the induction phase (5) and this might be attributed to inhibition of T cell priming. However, it was also demonstrated that such Abs are effective in an adoptive transfer model of EAE, which suggests that CD40-CD154 interactions may play a role in the reactivation of encephalitogenic T cells in the CNS by microglia or that anti-CD154 blocks Th1 effector functions (6, 7). This hypothesis was supported by the notion that human microglia are dependent on CD40-CD154 interactions for the induction of IL-12 (8), and by the diminished severity of disease in CD40-deficient mice as compared with wild-type mice after adoptive transfer of encephalitogenic T cells in one (9) but not another study (10). Anti-CD154 mAb treatment also has been shown to facilitate long-term graft survival in rodents (11) and non-human primates (12) and it has been suggested that this result may be due to the activation of regulatory T cells (13, 14). In addition, it is uncertain in this or other disease settings whether anti-CD154 mAb mediates part of its effect by depletion of CD154-positive targets via Fc-dependent mechanisms such as Ab-dependent cellular cytotoxicity and activation of the complement cascade. Interestingly, it was recently demonstrated that the induction of tolerance by anti-CD154 mAb in a murine model of islet cell transplantation depends on complement activation (15), and efficacy of anti-CD154 mAbs in a model of skin allotransplantation requires FcR or complement-mediated T cell depletion (16). FcRs may also enhance binding of an anti-CD154 mAb to its target by the formation of a scaffold of the mAb on the surface of FcR bearing cells or through the effects of FcR interactions on its localization in vivo.

We wished to determine whether the inhibition of EAE by anti-CD154 mAb was associated with long-term unresponsiveness or an active suppression mechanism and to what extent it was mediated by a mechanism that is dependent on Fc-dependent interactions of the Ab. To assess the latter, we used a form of anti-CD154 mAb in which Fc function is impaired by the elimination of the conserved *N*-linked glycosylation site in the CH2 domain of the Fc dimer. It is well established by in vitro studies that removal of the CH2 glycans alters the Fc structure such that Ab binding to FcRs and the complement protein C1q are significantly reduced (17–21).

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[2] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; LSD, least significant difference; H chain, heavy chain; L chain, light chain; muMR1, murine MR1; Agly, aglycosyl.

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Furthermore, in vivo studies have confirmed the reduction in effector function of the aglycosyl Abs (22–24). Importantly, it has also been shown that removal of glycans has little deleterious effect on other functional properties of Abs such as serum half-life and Ag binding activity (17, 19, 21, 25, 26).

We report that the mechanism underlying protection against EAE by anti-CD154 mAbs does not appear to involve long-term unresponsiveness or an active suppressor mechanism in this particular model. We also show that FcR interactions of the mAb do not play a major role with respect to clinical efficacy of anti-CD154 in EAE.

Materials and Methods

Preparation of mAbs

The variable domains of the heavy (H) chain and light (L) chain of the hamster anti-mouse CD154 mAb (MR1) were cloned by RT-PCR from total RNA from the hybridoma. Expression vectors for hamster/mouse chimeric mAb were constructed by engineering murine IgG2a or murine κ constant region cDNAs (derived from full-length cDNA clones of the H chain and L chain from the anti-human CD154 mAb 5c8) onto the variable domains of the H chain or L chain, respectively, using standard recombinant DNA techniques. Transiently expressed chimeric MR1 mAb, designated muMR1, was demonstrated to recapitulate the CD154 binding properties of the hamster mAb by flow cytometry and immunoprecipitation. The aglycosyl chimeric MR1, designated agly muMR1, was constructed by site-directed mutagenesis of the H chain to change the asparagine residue N297 (Kabat nomenclature (38)) in the Fc *N*-linked glycosylation site to a glutamine residue. Stable expression vectors containing CMV-immediate early (IE) promoter-driven tandem transcription cassettes for the Ig L chain and H chain and a glutamine synthetase gene as a selectable marker were constructed for both murine MR1 (muMR1) and agly muMR1 IgG2a and κ mAbs. The expression vectors were transfected into NSO cells and stable clones were isolated by selection in glutamine-free medium.

muMR1 and agly muMR1 were affinity-purified from bioreactor cell supernatants on protein A-Sepharose followed by size exclusion chromatography on Sephacryl 300 to remove aggregates. Chromatography resins were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The mAbs were shown to be >95% pure by SDS-PAGE, and endotoxin analysis ensured safeness of these reagents for in vivo use. The murine IgG2a isotype control mAb, P1.17 (American Type Culture Collection no. TIB-10) was protein A-purified from ascites at Protos Immunoresearch (Burlingame, CA) under contract by Biogen (Cambridge, MA).

Characterization of chimeric mAbs

As summarized in Table I, separate studies demonstrated that muMR1 and agly muMR1 have the same relative affinity for cell surface murine CD154 based on a competitive binding assay in vitro and the same pharmacokinetic half-life in vivo. Agly muMR1 does not bind to murine FcγR$^+$ cells at concentrations as high as 400 μg/ml whereas the EC_{50} for muMR1 was 10 μg/ml in this assay, and agly muMR1 had a 2-fold decreased binding capacity for human C1q.[3] Although murine C1q was not available for comparable binding studies in our hands, this reduction in C1q binding activity in an aglycosylated mouse IgG2a mAb is consistent with that previously reported (18).

Table I. *Characterization of chimeric MR1 mAbs*

	muMR1	Agly muMR1
CD154 binding[a] (IC_{50})	0.77 μg/ml	0.87 μg/ml
Half-life[b]	8.5 d	8.5 d
FcγR binding[c] (EC_{50})	10 μg/ml	No binding at 400 μg/ml
C1q binding[d] (relative)	1	0.5

[a] The chimeric mAbs compete with hamster MR1 for binding to murine CD154$^+$ cells in FACS assay.

[b] The chimeric mAbs have the same half-life in BALB/c mice after a single 100 μg dose.

[c] Glycosylated muMR1 binds to murine FcR$^+$ cells whereas the aglycosyl form does not.

[d] Agly muMR1 has decreased human C1q binding ability compared with the glycosylated form.

Induction of EAE and treatment with anti-CD154

Female SJL mice (10–12 wk of age; Harlan Breeders, Gannat, France) were immunized s.c. with 50 μg of $PLP_{139-151}$ emulsified in IFA supplemented with 1 mg/ml *Mycobacterium tuberculosis* H37 Ra (Difco, Detroit, MI). Three days later, mice were injected i.v. with 10^9 heat-killed *Bordetella pertussis* organisms (Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands). Development of EAE was monitored by daily assessment of bodyweight and a disability score. This score ranges from 0: no symptoms, 0.5: partial loss of tail tonus, 1: complete loss of tail tonus, 2: limb weakness, 2.5: partial paresis, 3: complete paralysis of hind limbs, 3.5: complete paralysis from diaphragm and hind limbs, incontinence, 4: moribund, to 5: death due to EAE.

Mice were treated (i.p.) on day 0, 2, and 4 with 200 μg (or less where indicated) of muMR1, agly muMR1, or the murine IgG2a isotype control P1.17. Where indicated mice were re-immunized on day 80 with 50 μg of $PLP_{139-151}$ emulsified in complete H37 Ra adjuvant.

In one experiment spleen cells were collected 7 days after treatment with these three dosages of 200 μg of the Abs (two mice per Ab), in conjunction with immunization with 50 μg of $PLP_{139-151}$ emulsified in complete H37 Ra adjuvant (no *B. pertussis*). After lysing the erythrocytes spleen cells were injected i.v. (20×10^6 cells/recipient), 1 day before active EAE induction with 50 μg of $PLP_{139-151}$ emulsified in complete H37 Ra adjuvant, followed by i.v. injection of *B. pertussis* 3 days later.

All of these studies were performed with the approval of the Animal Ethical Committee and in compliance with Dutch governmental regulations on Animal Experimentation. This approval has been filed in the protocol numbers DEC 803 and DEC 1257.

Histology

Brain tissue and spinal cord of each individual mouse was fixated in 10% formalin and embedded in paraffin. From each individual mouse three spinal cord sections (4 μm) and six sections of cerebellum separated by 100 μm were stained with hematoxylin. The area of each section was measured by morphometry and the number of infiltrating mononuclear cells per section was counted. For each individual mouse the mean number of infiltrating cells per square millimeter tissue was determined. Results are expressed as group means.

Lymphocyte culture

Lymph node cells were isolated from inguinal, axillary and bracchial lymph nodes. Cells were suspended in RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 2 mM L-glutamine, and 5% FCS (Life Technologies, Gaithersburg, MD). Lymph node cells (3×10^5 per well) were cultured (four replicate-wells per culture condition) in 200 μl in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) and stimulated with 0, 3, 10, or 30 μg/ml $PLP_{139-151}$. Replicate plates were incubated to enable kinetics of proliferation. Cells were labeled on day 3, 4, and 5 with 0.5 μCi [^{3}H]-labeled TdR (2 Ci/mmol; Radiochemical Center, Amersham, Buckinghamshire, U.K.) for 6 h and harvested onto glass fiber filters. Incorporated label was counted using a Wallac Trilux 1450 Microbeta liquid scintillation counter (PerkinElmer Life Science, Turku, Finland).

Antibodies

Sera of individual mice were collected on day 58. $PLP_{139-151}$-specific IgG1, IgG2a, and IgG2b were measured as previously described (27). Pooled serum from mice with EAE was used as an external standard, defined as containing 10,000 arbitrary units of peptide-specific IgG subclass Ab per milliliter. Results are expressed as arbitrary units per milliliter. Ab levels in sera of unimmunized mice are below the detection limit of the assay.

Statistical analysis

Results of multiple group comparisons were analyzed by one-way ANOVA, followed by posthoc analysis using the Fisher least significant difference (LSD) test. p values <0.05 were regarded significant. Where indicated the Mann-Whitney U test was applied to compare muMR1 and agly muMR1 with the isotype control P1.17 at a 3×200 μg dosage.

[3] J. Ferrant, C. Benjamin, A. Cutler, S. Kalled, Y.-M. Hsu, E. Garber, D. Hess, R. Shapiro, N. Kenyon, D. Harlan, et al. The contribution of Fc effector mechanisms in the efficacy of anti-CD154 immunotherapy depends on the nature of the immune challenge. *Submitted for publication.*

Results

Influence of glycosylation of anti-CD154 on its ability to inhibit EAE

Previous studies have demonstrated that anti-CD154 is very effective in the suppression of EAE. However, it is as yet unclear to what extent this inhibitory effect is mediated by mechanisms that are dependent on its Fc region, such as depletion of target cells via complement activation or Ab-dependent cell-mediated cytotoxicity. Because Fc effector functions are highly dependent on glycosylation of the Ab CH2 domain (22–24) an aglycosyl form of anti-CD154 mAb was engineered to address this issue. In a separate study it is shown that the aglycosyl form of a murinized anti-CD154 Ab (agly muMR1) has CD154 binding activity in vitro and a pharmacokinetic profile in vivo that are comparable with that of its glycosylated counterpart.[3] However, agly muMR1 is heavily impaired in terms of FcR binding and its ability to bind human C1q is 2-fold decreased (summarized in Table I). We compared muMR1 with its aglycosyl form with respect to their ability to inhibit the development of EAE in SJL mice induced by immunization (day 0) with 50 μg of $PLP_{139-151}$ emulsified in complete H37 Ra adjuvant. On days 0, 2, and 4 mice were treated with muMR1, agly muMR1, or isotype control Ab P1.17.

As shown in Fig. 1, both forms of anti-CD154 mAb were effective in inhibiting clinical signs of EAE during the entire follow-up period, when administered as three dosages of 200 μg. This result was evident from the mean cumulative EAE score (as indicated by p values in Fig. 1) and from the mean maximal EAE score ($p < 0.0005$ for both groups as compared with P1.17-treated mice; data not shown). In this respect the Abs were comparable with hamster MR1 (data not shown).

Administration of lower dosages of these Abs resulted in less inhibition of disease as measured by the cumulative EAE score (Fig. 1). Although muMR1 appears to be slightly more effective than agly muMR1 at three dosages of 75 μg, all groups had a significantly lower maximal EAE score (all groups $p < 0.0005$ as compared with P1.17 with the exception of 3×25 μg muMR1, $p < 0.05$; data not shown). Therefore, these experiments did not reveal major differences between the Abs with respect to their ability to inhibit EAE.

To assess whether the Abs differed in inhibiting the development of inflammatory infiltrates within the CNS, mice treated with different amounts of Ab were sacrificed on day 16, i.e., at the peak of disease activity in mice treated with the isotype control Ab. Cerebellum and spinal cord representing the major sites of inflammation were analyzed with regard to the number of infiltrating mononuclear cells. Our historical data show that mononuclear cell infiltrates are not detectable in the CNS 18 or 60 days after immunization with a non-encephalitogenic peptide (data not shown).

The results are shown in Fig. 2. We observed a dose-dependent decrease in infiltrates in mice treated with muMR1 or agly muMR1 and sacrificed on day 16, although simultaneous analysis of all groups by ANOVA did not indicate significant differences at this time point. However, a less stringent analysis comparing only the 3×200 μg groups by the Mann-Whitney U test revealed significantly less infiltrates in the cerebellum and the spinal cord for the highest dosage of either form of anti-CD154 as compared with P1.17, indicating that at this dosage both Abs inhibit infiltration. No significant differences were observed between the two anti-CD154 Abs with regard to their ability to suppress the development of inflammatory infiltrates within the CNS.

In addition, we evaluated the CNS tissues from 6 (low and intermediate dosages) to 14 mice (high dosage and controls) per group at the endpoint of this study (day 58). The majority of mice treated with P1.17 still had high numbers of inflammatory cells in cerebellum, whereas these numbers were 15-fold lower in spinal cord as compared with day 16. Mice treated with muMR1 or agly muMR1 revealed significantly less infiltrates in their cerebellum than P1.17 treated mice; the only difference between the two forms of anti-CD154 was found at a dosage of 3×25 μg in which muMR1 was not effective.

Thus, anti-CD154 has a slightly inhibitory effect on the infiltration of the CNS by mononuclear cells at day 16, apparently sufficient to inhibit clinical symptoms, and this is independent of glycosylation of its Fc part. Anti-CD154 inhibits mononuclear

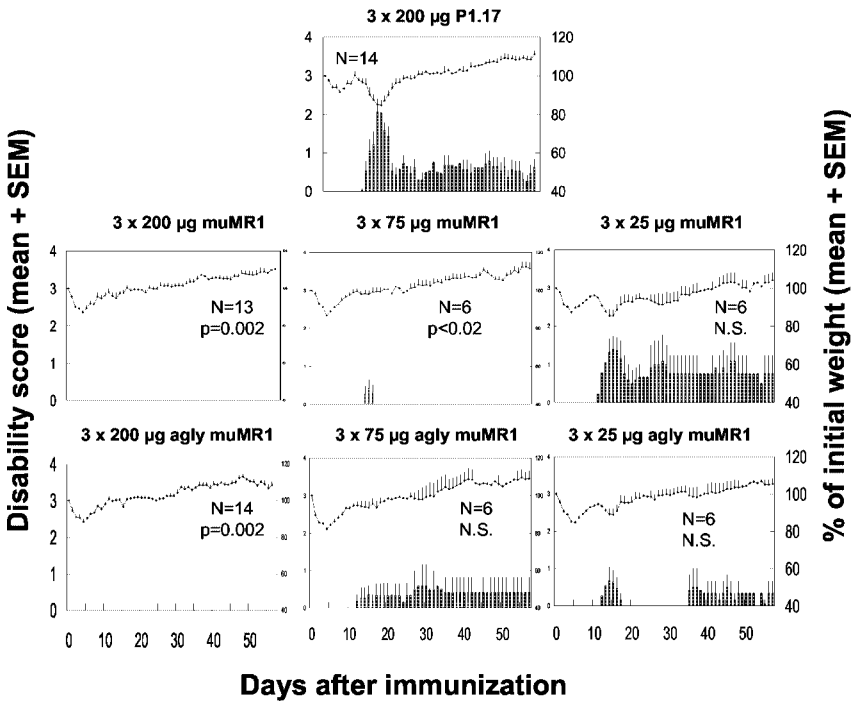

FIGURE 1. Glycosylation is not essential for the inhibitory effect of anti-CD154 in EAE. Female SJL mice were subjected to EAE induction by immunization (s.c.) with 50 μg of $PLP_{139-151}$ in complete H37 Ra adjuvant (day 0). Mice received three injections (i.p.) of isotype control Ab P1.17, muMR1, or agly muMR1. Group size (N) and amount of Ab are indicated. Results show the development of disease in time in terms of mean disability and mean percentage of initial bodyweight. The cumulative scores (area under the curve) of each individual mouse was determined and included in statistical evaluation (ANOVA and posthoc LSD) of the data as compared with P1.17 treated mice. Values of p for the cumulative score are indicated. N.S., Not significant.

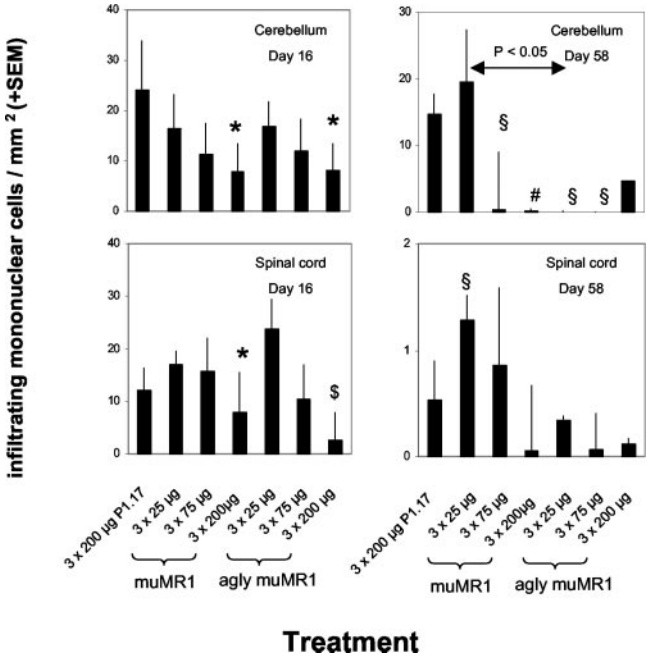

FIGURE 2. Glycosylation of anti-CD154 is not required for inhibition of CNS infiltration in EAE. Mice subjected to EAE induction with or without Ab treatment were sacrificed on day 16 ($n = 11$, *left panels*) or day 58 ($n = 6$–11, *right panels*). Cerebellum and spinal cord were evaluated with regard to the number of inflammatory cells, and statistical analysis was performed as described in *Materials and Methods*. Mann-Whitney *U* test: ∗, $p < 0.05$ and \$, $p < 0.01$ as compared with P1.17-treated mice at day 16. ANOVA and posthoc LSD: §, $p = 0.05$–0.1 and #, $p < 0.05$, as compared with P1.17-treated mice at day 58. The arrow indicates a significant difference between muMR1 and agly muMR1 at a dosage of 3×25 μg.

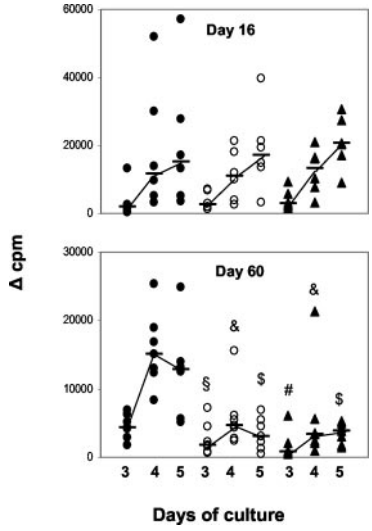

FIGURE 3. Effect of anti-CD154 on $PLP_{139-151}$ specific T cell proliferation. Lymph node cells from mice subjected to EAE induction and anti-CD154 treatment were collected 16 or 60 days after immunization. Cells derived from mice treated with 3×200 μg control Ab P1.17 (●), muMR1 (○), or agly muMR1 (▲) were stimulated with 30 μg/ml $PLP_{139-151}$ as described in *Materials and Methods*. Proliferation was assessed after 3, 4, and 5 days of culture by [^{3}H]thymidine incorporation. Symbols represent the response of individual mice; results are expressed as Δcpm obtained by subtraction of background proliferation. Statistical analysis was performed by ANOVA, followed by posthoc LSD: §, $p < 0.05$; #, $p < 0.005$; &, $p < 0.0001$; \$, $p < 0.000001$.

infiltration significantly at day 58, and this effect is also independent of glycosylation of its Fc part. The significant, albeit weak effect of anti-CD154 mAbs at the early time point and significant effect at the later time point is consistent with previously published studies (7) showing that anti-CD154 does not eliminate early T cell entry into the CNS but rather inhibits retention/expansion of these cells within the target organ.

Altogether we conclude that FcR-mediated mechanisms do not play a major role in the inhibition of EAE by anti-CD154, although we cannot rule out a role for residual C1q binding.

Effect of anti-CD154 treatment on peptide-specific T cell and B cell responses

To further investigate whether there was subclinical activity in mice treated with muMR1 and agly muMR1 in the absence of signs of EAE, the activation state of their T cells was evaluated. Sixteen days after immunization, i.e., shortly after treatment with the Abs, we did not observe an effect of Ab treatment on T cell proliferation. As shown in Fig. 3*A*, lymph node cells from mice treated with 3×200 μg of muMR1 or agly muMR1 were comparable with lymph node cells from P1.17-treated mice regarding their ability to proliferate in response to $PLP_{139-151}$. Also lower dosages of the Abs did not reveal significant differences (data not shown). When T cell proliferation was studied 60 days after immunization lymph node cells isolated from muMR1 or agly muMR1 treated mice showed significantly decreased T cell responses as compared with cells from P1.17 treated mice (Fig. 3*B*). The two forms of anti-CD154 did, however, not differ in their ability to inhibit peptide-specific T cell proliferation. In two other independent experiments, using group-wise pooled lymph node cells collected on day 58, one indicated that agly muMR1 may be less effective in suppression of T cell proliferation; these experiments also showed that the decreased ability of lymphocytes from anti-CD154 treated mice to proliferate was reflected by a decreased secretion of IFN-γ, without evidence for an up-regulation of IL-4 or IL-10 (data not shown).

Our observations are in line with previous studies by Howard et al. (6, 7) who found that CD40-CD154 blockade does not affect early expansion of T cells but rather the development of Th1 effector cells, without the preferential expansion of Th2 cells.

This was further substantiated by the assessment of the subclass of $PLP_{139-151}$-specific Abs in serum (Fig. 4). $PLP_{139-151}$-specific Abs were undetectable in sera of nonimmunized mice (data not shown). Mice that had been treated with three dosages of 200 μg muMR1 or agly muMR1 did not differ from P1.17 treated mice with regard to peptide-specific IgG1 Abs. However, both forms of anti-CD154 mAb resulted in lower levels of peptide-specific IgG2b. Whereas muMR1 treated mice showed a decrease in IgG2a Abs ($p < 0.0001$), mice treated with three dosages of 200 μg agly muMR1 showed only a trend toward a significant decrease ($p = 0.098$) The lower dosages (3×75 μg or 3×25 μg) of either muMR1 or agly muMR1 were ineffective in the inhibition of these Ab responses (data not shown). These data further support the hypothesis that anti-CD154 mAb treatment suppresses the development of Th1 responses without the concomitant up-regulation of a Th2 response and indicate that possibly the aglycosyl form of the mAb is slightly less effective in inhibiting immune reactivity than the glycosylated anti-CD154 in EAE.

No demonstrable long-term unresponsiveness or active suppression by anti-CD154 mAb treatment in EAE

The efficacy of agly muMR1 in EAE demonstrates that FcR interactions do not play a major role in the inhibition by anti-CD154 in

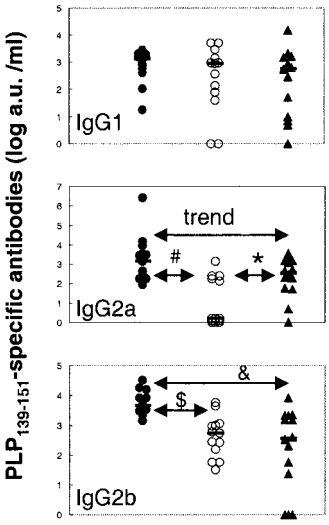

FIGURE 4. Anti-CD154 treatment inhibits the development of $PLP_{139-151}$-specific IgG2a and IgG2b Abs. Mice received three injections (i.p.) of 200 μg isotype control Ab P1.17 (●), muMR1 (○), or agly muMR1 (▲). $PLP_{139-151}$-specific Abs present in sera collected on day 58 were assayed as described in *Materials and Methods*. Results are expressed as arbitrary units per milliliter. ANOVA was followed by posthoc LSD test: *, $p < 0.01$; $, $p < 0.001$; #, $p < 0.0001$; and &, $p < 0.00001$

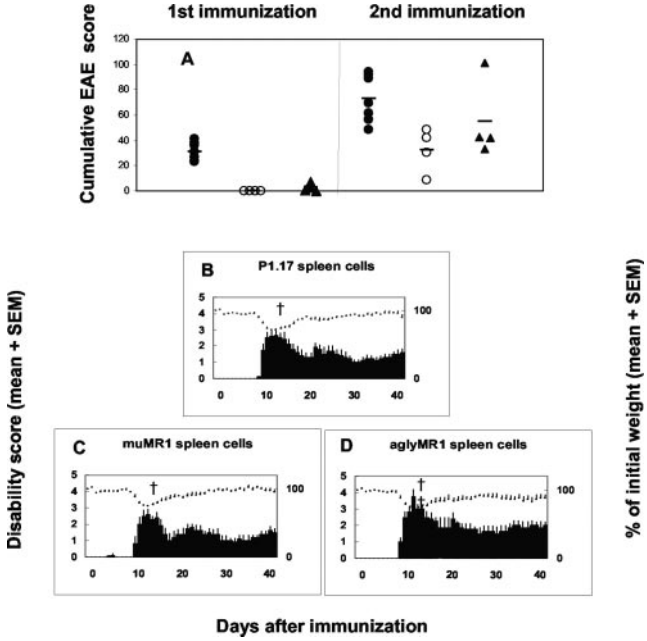

FIGURE 5. No demonstrable long-term unresponsiveness or active suppression of EAE by anti-CD154 mAb. *A*, No long-term protection to EAE by anti-CD154 treatment. Female SJL mice were subjected to EAE induction by immunization (s.c.) with 50 μg of $PLP_{139-151}$ in complete H37 Ra adjuvant (day 0). Mice received three injections (i.p.) of 200 μg isotype control Ab P1.17 (●, $n = 8$), muMR1 (○, $n = 4$), or agly muMR1 (▲, $n = 4$). On day 80 EAE induction was repeated. The cumulative EAE score of each individual mouse, assessed during the first 31 days after each immunization is shown. *B–D*, Spleen cells from anti-CD154-treated mice do not transfer protection to EAE. Recipient mice ($n = 6$ per group) were treated (i.v.) with 20×10^6 spleen cells from donor mice, 1 day before active EAE induction. Donor spleen cells were isolated on day 7 from mice ($n = 2$ per group) that had received three injections (i.p.) of 200 μg isotype control Ab P1.17 (*B*), muMR1 (*C*), or agly muMR1 (*D*) on days 0, 2, and 4 after immunization with 50 μg of $PLP_{139-151}$ in complete H37 Ra adjuvant (day 0). Each dagger marks a mouse that died as a consequence of EAE.

this context. Anti-CD154 mAb treatment has been suggested to inhibit the rejection of an allogeneic transplant by the induction of regulatory T cells (13, 14) and at least one study indicated that CD154 blockade was sufficient (28). To investigate whether anti-CD154 inhibits EAE via the induction of an active suppressor mechanism, we assessed whether mice treated with muMR1 or agly muMR1 on days 0, 2, and 4 after EAE induction display a long-lasting resistance to disease. To ensure that levels of circulating Ab would be low enough and not further capable of mediating a direct inhibitory effect, mice were not re-immunized until 80 days after the first immunization and Ab treatments.

As can be concluded from Fig. 5, mice treated with muMR1 or agly muMR1 did not develop symptoms of disease after primary peptide immunization in contrast with mice treated with the isotype control P1.17. The muMR1 or agly muMR1 treated animals also did not develop clinical symptoms during the follow-up period of 80 days (data not shown). When mice were re-immunized with $PLP_{139-151}$ emulsified in complete H37 Ra adjuvant there was an increased severity of clinical symptoms in P1.17 treated animals. Mice that had been treated on days 0, 2, and 4 with either of the anti-CD154 forms developed EAE after rechallenge with a severity that was less than observed after reimmunization of P1.17 treated mice, though comparable with the first phase of EAE in P1.17-treated mice and to that normally found after EAE induction in untreated mice (data not shown). We therefore conclude that anti-CD154 mAb treatment did not result in complete or long-lasting unresponsiveness in this model system.

We also studied whether the transfer of 20×10^6 spleen cells collected from mice, 1 wk after EAE induction and treatment with muMR1 or agly muMR1, would render naive recipient mice resistant to subsequent active EAE induction. As shown in Fig. 5, neither spleen cells from muMR1-treated mice nor spleen cells from agly muMR1-treated mice were capable of transferring resistance against EAE. Therefore, we did not obtain evidence for an active suppression mechanism in this particular experimental setting.

Discussion

Previous studies have demonstrated that treatment with anti-CD154 mAb inhibits induction (5) and progression (6) of EAE and pointed to reduced T cell priming, Th1 differentiation or reactivation of previously activated T cells, preventing the development of Th1 effector cells (6, 7). However, it is unclear whether the inhibitory activities of the mAb in this disease context are dependent on Fc effector mechanisms, i.e., complement-mediated lysis, or FcR-dependent mechanisms such as Ab-dependent cytotoxicity, Fc/FcR-dependent distribution of the Ab to critical sites of immune activation, or scaffolding of the mAb by FcR thereby potentially increasing its avidity for CD154. In this report, we used a novel tool, an aglycosyl version of anti-CD154 mAb, to address the contribution of Fc function to the inhibitory activity of anti-CD154 mAb in murine EAE. We demonstrate for the first time that the mechanism of protection against clinical EAE is not dependent on FcR-mediated functions of the mAb, although anti-CD154-mediated effects on subclinical immune activity may be partly dependent on Fc interactions.

The role of the CD154 pathway in EAE, has previously been independently demonstrated by resistance of CD154 knockout and CD40 knockout mice to disease (29). These studies indicate that the lack of CD154-CD40 interactions is sufficient for protection, and suggest that mAb blocking of CD154-CD40 may be adequate for its therapeutic effects. However, the mechanism(s) whereby

Abs mediate therapeutic effects may be highly complex and involve secondary Fc-dependent mechanisms. For example, in transplant settings, partial or complete engraftment has been obtained in CD154 and CD40 knockout mice (30–34). However, a dependence on complement for mAb efficacy was recently demonstrated in an islet transplant model (15). In addition, there are other reports showing that treatment with an anti-CD154 mAb results in a better reduction in immune response than that found in the knockout mice (35, 36). In these instances, there must be additional activity of the mAb beyond CD154/CD40 blockade.

To obtain insight into the role of such mechanisms in the inhibitory effect of anti-CD154 on EAE we compared the efficacy of a murinized form of MR1 with its agly counterpart. As shown in this study these Abs did not show major differences with regard to their efficacy to inhibit clinical signs of EAE. However, we obtained evidence that the aglycosyl form may be less able to suppress $PLP_{139-151}$-related immune reactivity in this model system. Although T cell PLP-reactivity at day 58 was comparably inhibited by muMR1 and agly muMR1 in two independent experiments, the inhibitory activity of agly muMR1 was somewhat reduced in one other; in addition, agly muMR1 was less effective in suppressing the development of $PLP_{139-151}$-specific IgG2a Ab. In contrast, the 3×25 μg dosage agly muMR1 was actually more effective than muMR1 in limiting cerebellar inflammation at day 58. Thus, the glycosylated form may have an additional mechanism of action that enables it to limit the generation of peripheral T cell responses. Because our data also show that the anti-CD154 mAb treated mice are not protected from disease upon rechallenge, the glycosylated form of the mAb does not appear to significantly deplete peptide-specific T cells in this system. We speculate that Fc/FcR interactions of the mAb contribute to its blocking activity by altering its distribution or increasing its avidity for CD154 in vivo.

Interestingly, a study using an agly mutant of a humanized anti-CD154 mAb in non-human primates showed that it could inhibit the humoral immune response and was comparable in this respect with the glycosyl form, but had decreased efficacy in allograft rejection in cynomolgus monkeys.[3] Thus the contribution of Fc interactions in anti-CD154-mediated efficacy depends on the nature or magnitude of the immune response. Possibly, the use of CFA, which is required for the induction of EAE and the concomitant production of IL-12, is responsible for the somewhat diminished efficacy of agly muMR1 in the EAE setting. Thus our data substantiate the notion that the importance of Fc effector function for immune inhibition is dependent on the type of immune challenge.

Apart from the inhibition of T cell reactivity, anti-CD154 treatment might result in the development of a tolerizing or active suppressive mechanism. Although anti-CD154 treatment was associated with prolonged graft survival we did not obtain evidence that anti-CD154 treatment during EAE-induction resulted into long-term protection to EAE. In anti-CD154 treated mice the severity of EAE after re-immunization was comparable with the disease activity during first phase in P1.17-treated mice (or untreated mice, data not shown) and the increase in disease activity during the second phase in P1.17-treated mice. Also spleen cells isolated on day 7 after anti-CD154 treatment and EAE induction did not protect naive recipients from subsequent EAE induction. In a study by Howard et al. (37), T cells from anti-CD154-treated animals were shown to have retained their encephalitogenic capacity inasmuch as they aggravated EAE when cotransferred with suboptimal numbers of encephalitogenic Th1 blasts. These and our data do not provide support for the possibility that anti-CD154 treatment of EAE is associated with an active suppressor mechanism. At this stage the reason for the discrepancy between observations in the transplantation models wherein anti-CD154 induces long-term graft acceptance, possibly through T regulatory activity, and in the EAE model in which it did not induce long-term unresponsiveness or active suppression, are uncertain. In the transplantation models alloantigen is present for a prolonged period of time and when these are recognized when other inflammatory signals have subsided, lack of appropriate costimulation may favor expansion of regulatory T cells and active suppression. In the EAE model described, it is likely that the encephalitogenic peptide will eventually disappear and with it a stimulus for regulatory T cells. Conversely, it should be taken into account that EAE induction occurred by immunization of the peptide in an emulsion with complete adjuvant, containing *M. tuberculosis* and by additional administration of heat-killed *B. pertussis* bacteria. Indeed, we have demonstrated that *B. pertussis* or pertussis toxin can abrogate tolerance induction in a setting in which tolerance to EAE (i.e., resistance to active induction of disease) is induced by immunization with a mannosylated form of the encephalitogenic peptide.[4] Most likely, in the classical EAE model T cell recognition occurs in the presence of costimulatory signals and in the context of ligation of Toll-like receptors. Therefore, additional studies are required to address the role of regulatory T cells in anti-CD154-mediated suppression of experimental autoimmune models that do not require strong adjuvants.

Anti-CD154 mAb is a promising tool in the treatment of a variety of immunological disorders. Understanding their mechanism of action is key to the development and optimization of safe and effective therapeutic candidates. Thromboembolic complications that occurred during the course of anti-CD154 human 5c8 clinical studies remain unexplained but could be related to FcR interactions. Our studies show that mice treated with agly MR1 may develop some subclinical immune reactivity; however, this reactivity is insufficient to allow for the development of EAE. Thus the efficacy profile of the agly form of anti-CD154 mAb may be beneficial for dampening the T cell reactivity that is heightened in immune disorders without completely abrogating protective responses of the host to infectious agents.

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References

1. Grewal, I.S., and R. A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol. 16:111*.
2. Stuber, E., W. Strober, and M. Neurath. 1996. Blocking the CD154-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. *J. Exp. Med. 183:693*.
3. Howland, K. C., L. J. Ausubel, C. A. London, and A. K. Abbas. 2000. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J. Immunol. 164:4465*.
4. Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heufler, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med. 184:741*.
5. Gerritse, K., J. D. Laman, R. J. Noelle, A. Aruffo, J. A. Ledbetter, W. J. Boersma, and E. Claassen. 1996. CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. *Proc. Natl. Acad. Sci. USA 93:2499*.
6. Howard, L. M., A. J. Miga, C. L. Vanderlugt, M. C. Dal Canto, J. D. Laman, R. J. Noelle, and S. D. Miller. 1999. Mechanisms of immunotherapeutic intervention by anti-CD154 (CD154) antibody in an animal model of multiple sclerosis. *J. Clin. Invest. 103:281*.
7. Howard, L. M., and S. D. Miller. 2001. Autoimmune intervention by CD154 blockade prevents T cell retention and effector function in the target organ. *J. Immunol. 166:1547*.

[4] M. Luca, J. Kel, W. van Rijs, J.-W. Drijfhout, F. Koning and L. Nagelkerken. Mannosylated self-peptide induces tolerance to experimental autoimmune encephalomyelitis. *Submitted for publication*.

8. Becher, B., M. Blain, and J. P. Antel. 2000. CD40 engagement stimulates IL-12 p70 production by human microglial cells: basis for Th1 polarization in the CNS. *J. Neuroimmunol. 102:44*.
9. Becher, B., B. G. Durell, A. V. Miga, W. F. Hickey, and R. J. Noelle. 2001. The clinical course of experimental autoimmune encephalomyelitis and inflammation is controlled by the expression of CD40 within the central nervous system. *J. Exp. Med. 193:967*.
10. Mendel, I., and E. M. Shevach. 2002. Differentiated Th1 autoreactive effector cells can induce experimental autoimmune encephalomyelitis in the absence of IL-12 and CD40/CD154 interactions. *J. Neuroimmunol. 122:65*.
11. Honey, K., S. P. Cobbold, and H. Waldmann. 1999. CD40 ligand blockade induces $CD4^+$ T cell tolerance and linked suppression. *J. Immunol. 163:4805*.
12. Kirk, A. D., L. C. Burkly, D. S. Batty, R. E. Baumgartner, J. D. Berning, K. Buchanan, J. H. Fechner, R. L Germond, R. L. Kampen, N. B. Patterson, et al. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat. Med. 5:686*.
13. Taylor, P. A., T. M. Friedman, R. Korngold, R. J. Noelle, and B. R. Blazar. 2002. Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40: CD154 costimulatory pathway results in the generation of a potent immune regulatory cell. *Blood 99:4601*.
14. Graca, L., K. Honey, E. Adams, S. P. Cobbold, and H. Waldmann. 2000. Anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J. Immunol. 165:4783*.
15. Sanchez-Fueyo, A., C. Domenig, T. B. Strom, and X. X. Zheng. 2002. The complement dependent cytotoxicity (CDC) immune effector mechanism contributes to anti-CD154 induced immunosuppression. *Transplantation 74:898*.
16. Monk, N. J., R. E. G. Hargreaves, J. E. Marsh, C. A. Farrar, S. H. Sacks, M. Millrain, E. Simpson, J. Dyson, and S. Jurcevic. 2003. Fc-dependent depletion of activated T cells occurs through CD40L-specific antibody rather than costimulation blockade. *Nat. Med. 9:1275*.
17. Nose, M., and H. Wigzell. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA 80:6632*.
18. Leatherbarrow, R. J., T. W. Rademacher, R. A. Dwek, J. M. Woof, A. Clark, D. R. Burton, N. Richardson, and A. Feinstein. 1985. Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement component C1 and interaction with human monocyte Fc receptor. *Mol. Immunol. 22:407*.
19. Tao, M. H., and S. L. Morrison. 1989. Studies of aglycosylated chimeric mouse-human IgG: role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol. 143:2595*.
20. Lund, J., T. Tanaka, N. Takahashi, G. Sarmay, Y. Arata, and R. Jefferis. 1990. A protein structural change in aglycosylated IgG3 correlates with loss of huFcγ R1 and huFcγ R111 binding and/or activation. *Mol. Immunol. 27:1145*.
21. Dorai, H., B. M. Mueller, R. A. Reisfeld, and S. D. Gillies. 1991. Aglycosylated chimeric mouse/human IgG1 antibody retains some effector function. *Hybridoma 10:211*.
22. Isaacs, J. D., M. R. Clark, J. Greenwood, and H. Waldmann. 1992. Therapy with monoclonal antibodies: an in vivo model for the assessment of therapeutic potential. *J. Immunol. 148:3062*.
23. Friend, P. J., G. Hale, L. Chatenoud, P. Rebello, J. Bradley, S. Thiru, J. M. Phillips, and H. Waldmann. 1999. Phase I study of an engineered aglycosylated humanized CD3 antibody in renal transplant rejection. *Transplantation 68:1632*.
24. Boyd, P. N., A. C. Lines, and A. K. Patel. 1995. The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. *Mol. Immunol. 32:1311*.
25. Hand, P. H., B. Calvo, D. Milenic, T. Yokota, M. Finch, P. Snoy, K. Garmestani, O. Gansow, J. Schlom, and S. V. Kashmiri. 1992. Comparative biological properties of a recombinant chimeric anti-carcinoma mAb and a recombinant aglycosylated variant. *Cancer Immunol. Immunother. 35:165*.
26. Hobbs, S. M., L. E. Jackson, J. Hoadley. 1992. Interaction of aglycosyl immunoglobulins with the IgG Fc transport receptor from neonatal rat gut: comparison of deglycosylation by tunicamycin treatment and genetic engineering. *Mol. Immunol. 29:949*.
27. Nagelkerken, L., B. Blauw, and B. M. Tielemans. 1997. IL-4 abrogates the inhibitory effect of IL-10 on the development of experimental allergic encephalomyelitis in SJL mice. *Int. Immunol. 9:1243*.
28. Jarvinen, L. Z., B. R. Blazar, O. A. Adeyi, T. B. Strom, and R. J. Noelle. 2003. CD154 on the surface of $CD4^+CD25^+$ regulatory T cells contributes to skin transplant tolerance. *Transplantation 76:1375*.
29. Grewal, I. S., H. G. Foellmer, K. D. Grewal, J. Xu, F. Hardardottir, J. L. Baron, C. A. Janeway, and R. A. Flavell. 1996. Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science 273:1864*.
30. Shimizu, K., U. Schonbeck, F. Mach, P. Libby, and R. N. Mitchell. 2000. Host CD40 ligand deficiency induces long-term allograft survival and donor-specific tolerance in mouse cardiac transplantation but does not prevent graft arteriosclerosis. *J. Immunol. 165:3506*.
31. Kurtz, J., H. Ito, T. Wekerle, J. Shaffer, and M. Sykes. 2001. Mechanisms involved in the establishment of tolerance through costimulatory blockade and BMT: lack of requirement for CD40L-mediated signaling for tolerance or deletion of donor-reactive $CD4^+$ cells. *Am. J. Transplant. 1:339*.
32. Bingaman, A. W., J. Ha, M. M. Durham, S. Y. Waitze, C. Tucker-Burden, S. R. Cowan, T. C. Pearson, and C. P. Larsen. 2001. Analysis of the CD40 and CD28 pathways on alloimmune responses by $CD4^+$ T cells in vivo. *Transplantation 72:1286*.
33. Larsson, L. C., M. Corbascio, H. Widner, T. C. Pearson, C. P. Larsen, and H. Ekberg. 2002. Simultaneous inhibition of B7 and LFA-1 signaling prevents rejection of discordant neural xenografts in mice lacking CD154. *Xenotransplantation 9:68*.
34. Zhai, Y., X. D. Shen, F. Gao, A. J. Coito, B. A. Wasowska, A. Salama, I. Schmitt, R. W. Busuttil, M. H. Sayegh, and J. W. Kupiec-Weglinski. 2002. The CD154-CD40 T cell costimulation pathway is required for host sensitization of $CD8^+$ T cells by skin grafts via direct antigen presentation. *J. Immunol. 169:1270*.
35. Gorbachev, A. V., P. S. Heeger, and R. L. Fairchild. 2001. $CD4^+$ and $CD8^+$ T cell priming for contact hypersensitivity occurs independently of CD40-CD154 interactions. *J. Immunol. 166:2323*.
36. Taylor, P. A., C. J. Lees, H. Waldmann, R. J. Noelle, and B. R. Blazar. 2001. Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. *Blood* 98:467.
37. Howard, L. M., S. Ostrovidov, C. E. Smith, C. M. Dal Canto, and S. D. Miller. 2002. Normal Th1 development following long-term therapeutic blockade of CD154-CD40 in experimental autoimmune encephalomyelitis. *J. Clin. Invest. 109:233*.
38. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequence of Proteins of Immunological Interest*, 3rd Ed., Vol. 1. National Institutes of Health, Bethesda.