Treatment with depleting CD4 monoclonal antibody results in a preferential loss of circulating naive T cells but does not affect IFN-gamma secreting TH1 cells in humans.

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*J Clin Invest.* 1997; 99(9):2225-2231. [https://doi.org/10.1172/JCI119396](https://doi.org/10.1172/JCI119396).

CD4(pos) TH1 T cells are considered to play a central role in a number of human autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis. Experimental treatment protocols aimed at selectively eliminating CD4(pos) T cells thus far have yielded disappointing clinical results. Here we analyzed phenotype and function of circulating T cells in multiple sclerosis patients treated with the chimeric CD4 mAb cM-T412 in a randomized, double-blind, placebo-controlled, magnetic resonance imaging-monitored phase II trial. Treatment resulted in a long-lasting depletion of CD4(pos) T cells but did not affect CD8(pos) T cell numbers. Analysis of CD4(pos) subpopulations showed that unprimed, CD45RA(pos)/R0(neg) lymphocytes were approximately three times more sensitive to the mAb than primed, CD45RA(neg)/R0(pos) T cells. Notably, within the CD45RA(pos) subset, T cells with phenotypic evidence of prior activation, i.e., expressing Fas, were relatively insensitive to cM-T412, compared with Fas(neg) cells. Remarkably, while a decrease in the number of IL-4-producing T helper 2 (TH2)-type cells in the anti-CD4 treated group was observed, numbers of IFN-gamma-producing T helper 1 (TH1)-type cells remained stable, resulting in a significant increase in the TH1/TH2 ratio. Our data show that treatment with depleting CD4 mAb does not eliminate the cells most strongly involved in the disease process, i.e., primed, IFN-gamma-producing TH1-type cells, and may therefore give an explanation for the lack of beneficial clinical effects […]

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Treatment with Depleting CD4 Monoclonal Antibody Results in a Preferential Loss of Circulating Naive T Cells but Does Not Affect IFN-γ Secreting TH1 Cells in Humans

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Abstract

CD4pos TH1 T cells are considered to play a central role in a number of human autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis. Experimental treatment protocols aimed at selectively eliminating CD4pos T cells thus far have yielded disappointing clinical results. Here we analyzed phenotype and function of circulating T cells in multiple sclerosis patients treated with the chimeric CD4 mAb cM-T412 in a randomized, double-blind, placebo-controlled, magnetic resonance imaging–monitored phase II trial. Treatment resulted in a long-lasting depletion of CD4pos T cells but did not affect CD8neg T cell numbers. Analysis of CD4pos subpopulations showed that unprimed, CD45RApos/R0pos lymphocytes were approximately three times more sensitive to the mAb than primed, CD45RAneg/R0neg T cells. Notably, within the CD45RApos subset, T cells with phenotypic evidence of prior activation, i.e., expressing Fas, were relatively insensitive to cM-T412, compared with Fasneg cells. Remarkably, while a decrease in the number of IL-4–producing TH2-type cells was observed, numbers of IFN-γ–producing T helper 1 (TH1)–type cells remained stable, resulting in a significant increase in the TH1/TH2 ratio. Our data show that treatment with depleting CD4 mAb does not eliminate the cells most strongly involved in the disease process, i.e., primed, IFN-γ–producing TH1-type cells, and may therefore give an explanation for the lack of beneficial clinical effects of depleting CD4 mAb in human chronic autoimmune disease. (J. Clin. Invest. 1997, 99:2225–2231.) Key words: CD4 antibodies, monoclonal • autoimmune diseases therapy • multiple sclerosis • T lymphocyte subsets • TH1 cells

Introduction

CD4pos T cells, which recognize peptide fragments that are presented in the groove of major histocompatibility complex class II molecules (1), orchestrate cellular and humoral immune responses through the secretion of immunoregulatory cytokines and via cell–cell contact (2, 3). Based on cytokine production capacity, distinct types of differentiated helper (H) CD4pos T cells are identified (4). Typically, TH1-type cells secrete IFN-γ and TNF-α and are involved in cell-mediated immunity, whereas TH2-type cells secrete IL-4 and IL-5 and exert their primary function in humoral immune reactions (5, 6). Normally, a balance exists between these two helper T cell subsets, which is in a considerable degree maintained by cross-regulatory mechanisms. IL-4 downregulates development of TH1-type cells, and vice versa IFN-γ suppresses the TH2-type response (7). Autoimmune diseases are believed to be dependent on activation of TH1-type cells which is not sufficiently counterbalanced by TH2-type cells (8, 9). Indeed, a number of experimental animal models have shown that (auto)antigen reactive CD4pos TH1-type cells are of crucial importance for disease induction and disease progression (10–12). In accordance with this notion, in vivo depletion of CD4pos T cells interferes with disease induction in experimental allergic encephalomyelitis (13–16), adjuvant arthritis (17), collagen-induced arthritis (18), experimental autoimmune glomerulonephritis (19), experimental antiphospholipid syndrome, and systemic lupus erythematosus (20).

Also in human autoimmune diseases a pivotal role for CD4pos T cells has been postulated (21, 22) and experimental trials aimed at specifically attacking these cells with depleting CD4 mAb have been initiated. Although in open studies more or less promising results have been obtained with CD4 mAb treatment of rheumatoid arthritis (RA) (23–25), systemic lupus erythematosus (26), Crohn’s disease (26a), and multiple sclerosis (MS) patients (27–29), in double-blind, placebo-controlled, phase II trials with RA patients the efficacy appeared to be absent or very low (30, 31). Recently, absence of therapeutic effect was also documented in a randomized, double-blind, placebo-controlled, magnetic resonance imaging (MRI)-monitored phase II trial in which MS patients were treated with the chimeric CD4 mAb cM-T412 (32). As was also shown in earlier studies (28, 29), in this trial, a significant and long-lasting depletion of CD4pos T cells was found in CD4 mAb–treated patients. However, no effect could be demonstrated on the primary measure of efficacy, the number of active lesions on monthly gadolinium enhanced MRI over 9 mo.

To examine the cause for therapeutic ineffectiveness of depleting CD4 mAb in humans, we here analyzed consequences of CD4 mAb treatment on phenotypical and functional characteristics of circulating T cells in MS patients. Our data show
that the absence of therapeutic success coincides with a specific inability of the antibody to delete primed, IFN-γ-producing T cells.

Methods

Study design. Detailed information on the design of the trial is described elsewhere (32, 33). In summary, a randomized, double-blind, placebo-controlled exploratory phase II trial of the CD4 antibody cM-T412 was set up using a parallel groups design. As primary measure of treatment efficacy, the cumulative number of active lesions seen on monthly brain MRI, performed over 9 mo from the start of treatment, was used.

Patients. Analyses were carried out on 28 patients treated at the Department of Neurology, Free University Hospital (Amsterdam, The Netherlands), who formed a subgroup of patients from a larger multicenter study. After randomization 13 patients received placebo, and 15 received CD4 mAb treatment. Demographic and baseline characteristics are given in Table I. In contrast to what was seen in the total group of patients (32), patients in both treatment arms in this study were also comparable with respect to relapse rate in the year preceding inclusion (mean number±SD of clinical relapses in the previous year in the placebo vs. CD4 mAb group: 1.8±1.3 vs. 1.5±1.1, P = 0.49), and with respect to the total number of active lesions at baseline (median number of active MR lesions at inclusion in the placebo vs. CD4 mAb group 0 vs. 1, P = 0.15).

Reagents. mAbs directed against CD2 (CLB-T11.1/1, CLB-T11.1/2, and Hick7), CD28 (CLB-CD28/1), CD4 (FITC-labeled) CLB-T8/4), and CD14 (FITC-labeled) CLB-mon/1) were generated at the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service. Biotinylated cytokine mAb (IL-4, IFN-γ, Tnf-[H]thymidine. 0.4 mCi/well of [H]thymidine (200 mCi/mmol; Amersham, Buckinghamshire, United Kingdom) was added 24 h before harvesting.

Membrane phenotyping. PBMC were washed twice with PBS supplemented with 0.5% BSA and Na-azide (5 × 10⁵ cells/ml). Immunofluorescence staining was performed by incubation of PBMC with saturating amounts of combinations of PerCP-, FITC-, and PE-labeled mAb against CD2 (CLB-T11.1/1, CLB-CD28/1, and Hick7); all 5 μg/ml) in the presence of CD28 mAb CLB-CD28/1 (5 μg/ml) in flat-bottom microrotter plates (Greiner, Langenthal, Switzerland). Cells cultured without stimuli served as negative controls.

The proliferative response (cpm) was measured after 4 d of culture by means of incorporation of [H]thymidine. 0.4 mCi/well of [H]thymidine (200 mCi/mmol; Amersham, Buckinghamshire, United Kingdom) was added 24 h before harvesting.

Flow cytometric measurement of intracellular cytokine production. Measurement of cytokine production was performed as previously described (35, 36). Briefly, 0.5 × 10⁵ cells/ml were stimulated for 4 h with PMA (1 ng/ml) and ionomycin (1 μM) in the presence of the protein-secretion inhibitor monensin (1 μM). All subsequent steps were performed at 4°C. After cell surface staining with CD3-PE or CD4-FITC, cells were washed twice with PBS and fixed with PBS/4% paraformaldehyde (5 min). Fixation was followed by permeabilization with PBS/0.1% saponin (Sigma)/10% human pooled serum (10 min). For all subsequent washing and incubation steps, PBS/0.1% saponin/0.5% BSA was used. Staining of the cytoplasm with biotinylated cytokine mAb (IL-4, IFN-γ; both 5 μg/ml) was followed by incubation with streptavidin-PE (20 min). Analysis was performed as described for the measurement of membrane markers.

Statistical analysis. Differences between groups at baseline were analyzed using Student’s t test, Wilcoxon’s test, or χ² test. Repeated measurements MANOVA with age as a covariate was used to evaluate treatment effects. We considered P values < 0.05 to be statistically significant.

Results

CD4 mAb cM-T412 induces a long-lasting specific depletion of CD3⁺CD4⁺ T cells. Treatment with the CD4 mAb resulted in a significant reduction in absolute numbers of circulating CD3⁺ T cells (Fig. 1a, P = 0.004). Within the total CD3⁺ T cell population the mAb induced a significant and progressive decline

Table I. Demographic and Baseline Characteristics of Patients in Both Treatment Arms

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>Anti-CD4</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>9</td>
<td>0.13</td>
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<tr>
<td>Disease type</td>
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<tr>
<td>Relapsing remitting</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Secondary progressive</td>
<td>5</td>
<td>8</td>
<td>0.44</td>
</tr>
<tr>
<td>Age (yr)¹</td>
<td>36.6±8.5</td>
<td>33.7±6.8</td>
<td>0.34</td>
</tr>
<tr>
<td>Disease duration (yr)</td>
<td>5.8±7.1</td>
<td>3.2±3.0</td>
<td>0.23</td>
</tr>
<tr>
<td>Number of clinical relapses</td>
<td>1.8±1.3</td>
<td>1.5±1.1</td>
<td>0.49</td>
</tr>
<tr>
<td>Proportion of EDSS progression</td>
<td>1.0</td>
<td>1.5</td>
<td>0.33</td>
</tr>
<tr>
<td>EDSS at inclusion⁰</td>
<td>5.5</td>
<td>5.5</td>
<td>0.85</td>
</tr>
<tr>
<td>CD4 cell numbers/mm³ at inclusion*</td>
<td>985±300</td>
<td>810±237</td>
<td>0.10</td>
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</table>

Presence of active MR lesions at inclusion

<table>
<thead>
<tr>
<th>Present</th>
<th>Absent</th>
<th>Number of active MR lesions at inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Mean±SD; ¹EDSS, Expanded Disability Status Scale; ⁰median; ⁰inclusion: month 0.
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in CD4pos T cell numbers which was most prominent until month 4 (to 30% of the pretreatment level, Fig. 1b, P < 0.001), at which time point CD4 mAb treatment for the majority of the patients was interrupted or discontinued (32). A remarkable finding was that, long after termination of treatment, CD4pos T cell numbers remained low. 18 mo after cessation of treatment CD4pos T cell numbers were still reduced to ~39% of the pretreatment level. CD8 numbers showed a slight increase in the CD4 mAb–treated group (to 128% of the pretreatment level at month 18, Fig. 1c), but this was not significantly different from the placebo-treated group.

Because monocytes also express the CD4 antigen, albeit at lower levels than T cells, we investigated whether these cells were affected by treatment with the CD4 antibody. However, at none of the time points were differences seen between the CD4 mAb–treated group and the placebo-treated group in the number of monocytes per mm3 blood (data not shown).

CD4 mAb treatment does not affect T cell reactivity in a quantitative fashion. In HIV-1 infection numeric depletion of the CD4pos T cell compartment coincides with a diminished ability of T cells to proliferate in vitro (37, 38). To investigate whether treatment with CD4 mAb had a similar effect on T cell reactivity, T cell proliferation in vitro was analyzed. These assays were performed in standard cultures, using isolated PBMC, under optimal stimulation conditions, in order to measure the response per standard amount of PBMC (39). There were no differences in proliferative capacity of circulating T cells between the two groups as a result of treatment (Fig. 2). Therefore, we conclude that CD4 mAb treatment does not affect reactivity of the remaining circulating T cells in a quantitative way.

Distinct sensitivities of unprimed and primed CD4pos T cells for depleting CD4 mAb. Next, to more precisely define the target cells of the CD4 mAb we investigated if different subsets of CD4pos T cells are equally affected by CD4 mAb treatment. Triple-color immunofluorescence analysis revealed that in placebo-treated patients, numbers of unprimed CD4pos CD45RApos/R0neg and primed CD4pos CD45RAneg/R0pos T cells (40) remained constant during the study period (Fig. 3a). At baseline the CD4 mAb–treated group had relatively more unprimed than primed circulating CD4pos T cells (Fig. 3b; NS).

Figure 1. Depletion of CD4pos T cells in peripheral blood of CD4 mAb–treated MS patients. Mean numbers (±SD) of T cells per mm3 blood are given for the placebo-treated group (n = 13; open circles) and the CD4 mAb–treated group (n = 15; closed triangles). (a) Mean CD3 numbers in the CD4 mAb–treated group are decreased to ~53% of the baseline level at month 4, and to 68% at month 18. The difference with the placebo-treated group is statistically significant. (b) A significant decline in CD4pos T cell numbers in the CD4 mAb–treated group in comparison with the placebo-treated group. At month 4 mean CD4pos T cell numbers are decreased to ~30% of the baseline level. Strikingly, at month 18, long after termination of treatment, mean CD4pos T cell numbers are still reduced to ~39% of the pretreatment level. (c) CD8pos T cell numbers are not affected by the treatment.

Figure 2. Mean proliferative capacity of circulating T cells in standard cultures (per standard amount of PBMC) is not decreased in the CD4 mAb–treated group (closed triangles) and is not different from that of the placebo-treated group (open circles). T cells were stimulated with a triplet of CD2 mAb in combination with CD28 mAb.
Remarkably, although absolute numbers of both subsets declined as a result of CD4 mAb treatment, the decline was much more pronounced in the CD4<sup>pos</sup>CD45RA<sup>neg</sup>/R0<sup>pos</sup> subset (at month 4 to 18% of the pretreatment level, \( P < 0.001 \)) as compared with the CD4<sup>pos</sup>CD45RA<sup>pos</sup>/R0<sup>neg</sup> subset (at month 4 to 59% of the pretreatment level, \( P = 0.002 \)). As a consequence, the ratio CD45RA<sup>pos</sup>/CD45RA<sup>neg</sup> CD4<sup>pos</sup> T cells was reversed, which remained so until the end of the study period. After termination of treatment, numbers of both CD45RA<sup>neg</sup> and CD45RA<sup>pos</sup> CD4<sup>pos</sup> T cells increased in parallel.

When unprimed, naive CD4<sup>pos</sup> T cells are activated, the Fas antigen is rapidly upregulated (41). Consequently, a further subdivision can be made within the CD4<sup>pos</sup>CD45RA<sup>pos</sup>/R0<sup>pos</sup> subset based on Fas expression. Fas<sup>pos</sup> cells may be regarded as truly naive CD4<sup>pos</sup> T cells, whereas Fas-expressing T cells are likely to have been recently activated in vivo. This type of analysis showed that CD4 mAb treatment resulted in a sharp decline in the number of CD4<sup>pos</sup>CD45RA<sup>pos</sup>Fas<sup>neg</sup> T cells (Fig. 3c, \( P = 0.002 \)), while the decline in the number of CD4<sup>pos</sup>CD45RA<sup>pos</sup>Fas<sup>pos</sup> T cells was much less pronounced (Fig. 3d, NS).

IFN-\(\gamma\)-producing T cells are not affected by CD4 mAb treatment. Secretion of TH1 and TH2 cytokines, a qualitative feature of T cell function, is largely restricted to the primed CD4<sup>pos</sup> T cell subset (36, 42). Since, as we have already mentioned, autoimmune diseases are believed to be dependent on the activation of IFN-\(\gamma\)-producing TH1-type cells, we investigated, taking into account the above documented relatively low sensitivity of primed T cells to therapy, whether CD4 mAb treatment would differentially affect TH1- and TH2-type cells. For this we measured absolute numbers of IFN-\(\gamma\) and IL-4-producing T cells using flow cytometry (35, 36). IFN-\(\gamma\)- and IL-4–producing T cells remained constant in the placebo group over time (Fig. 4, a and b). Numbers of IL-4–producing CD3<sup>pos</sup> T cells showed an almost 50% decline in the CD4 mAb–treated group (Fig. 4b, NS), which is comparable with the decline seen in the CD4<sup>pos</sup>CD45RA<sup>neg</sup>/R0<sup>pos</sup> T cells. Remarkably, however, numbers of IFN-\(\gamma\)-producing CD3<sup>pos</sup> T cells remained relatively stable over time in the CD4 mAb–treated group (Fig. 4a, NS). As a result, treatment with CD4 mAb resulted in a significant increase in the TH1/TH2 ratio (Fig. 4c, \( P = 0.035 \)).

**Discussion**

Analysis of the T cell compartment in MS patients treated with the chimeric monoclonal antibody cM-T412 shows that the CD4 mAb induces a long-lasting selective depletion of the circulating CD4<sup>pos</sup> compartment (Fig. 1 and reference 32). Repopulation studies in mice and in patients undergoing intensive chemotherapy (43, 44) have shown that CD4<sup>pos</sup> T cell repopulation is inversely correlated with age, and that rapid reconstitution of this pool requires residual thymic function. Because even young adults already have relative deficiencies in this thymus-dependent pathway, it is therefore not surpris-
CD4 pos/CD45RA pos/R0 neg T cells, rather than from the division of CD4 pos/CD45R0 pos T cells.

The blind homeostasis model predicts that in a given individual a constant number of circulating CD3 pos lymphocytes is maintained irrespective of CD4 pos or CD8 pos phenotype (45, 46) and implies that in case of CD4 pos T cell loss, such as in HIV-1 infection, both CD4 pos and CD8 pos T cells will be produced in increasing amounts until the CD3 pos T cell steady state is restored. Because of the effect of HIV-1 on CD4 pos, but not on CD8 pos T cells, this would then result in CD8 pos T cell lymphocytosis in the circulation. However, the data from the CD4 mAb-treated patients in this study show that although the loss in CD4 pos T cell numbers is pronounced, this is not compensated by an increase in CD8 pos T cell numbers, not even in the long term. Consequently, numbers of CD3 pos T cells are significantly reduced after treatment. It is likely that in HIV-1 infection other factors, such as continuous antigenic pressure and bystander activation through regulatory cytokines, contribute to the CD8 pos lymphocytosis (47). Based on the data presented here we feel that the blind homeostasis model needs reconsideration.

Our data demonstrate a differential sensitivity of unprimed CD45RA pos versus primed CD45RA neg/CD4 pos T cells for depletion by the cM-T412 mAb. The fact that within the CD4 pos/CD45RA pos subpopulation Fas neg T cells are more sensitive to treatment than Fas pos T cells shows that the depletion is not simply related to expression of CD45RA, but that actually CD4 pos T cells which have not yet been activated in vivo are most sensitive to the depleting effect of the mAb. Also within the CD4 pos/CD45RA neg T cell subset, a distinction in sensitivity for the CD4 mAb could be made on the basis of qualitative features of T cell function. While numbers of circulating T cells producing IFN-γ were not affected, IL-4–producing T cells were selectively depleted. Therefore, treatment results in a relative increase in R0 pos TH1 cells in the peripheral blood.

The mechanism of CD4 pos T cell depletion after treatment with CD4 mAb is unclear and consequently the basis for the distinct sensitivities of T cell subpopulations to the CD4 mAb is hard to define. A role for Fas in the CD4 mAb–induced deletion process has been suggested by the observation that mice lacking a functional Fas receptor do not show a decrease of CD4 pos T cells after treatment with depleting CD4 mAb (48). In agreement with this, engagement of the CD4 molecule by anti-CD4 mAb activates a small but rapid increase in cell surface Fas expression and Fas antigen–dependent apoptosis of T cells (49). Interestingly, when T cells are activated by CD3 antibodies, either in vitro or in vivo, they become resistant to CD4 mAb–mediated deletion (50, 51). At first glance it seems paradoxical that activation of T cells with CD3 mAb, which induces membrane expression of Fas, renders them insensitive to a deletion process that involves Fas-mediated apoptosis. However, it should be noted that when unprimed T cells are activated with CD3 mAb, they are induced to express Fas but remain insensitive to Fas-mediated cell death (52). These observations suggest that T cells can be rescued from CD4 mAb–induced Fas-mediated deletion by signals, generated via TCR/CD3, which normally protect antigen-stimulated T cells from apoptosis. If this notion is valid it could explain the finding that murine and human T cells with membrane characteristics of activation (including Fas expression) are relatively resistant to depleting CD4 mAb (43, 53).
Treatment with the cM-T412 mAb did not have any effect on disease activity in our group of MS patients as measured by the number of active lesions on monthly gadolinium-enhanced MRI over 9 mo (32). Although phase I clinical trials seemed to be promising (24, 27–29) also in other chronic human autoimmune diseases, little or no clinical effect of treatment with CD4 antibodies could be demonstrated in placebo-controlled phase II clinical trials (31, 32, 54). The absence of clinical benefit differs markedly from the encouraging results that have been obtained in experimental model systems for autoimmunity. An important point in the explanation for this discrepancy seems to be the timing of the administration of the CD4 antibody in relation to the stage of the disease process. In most of the animal models where this kind of treatment is successful, the antibody was given during the period of disease induction (13, 19, 55). In these models, development of disease is probably dependent on the recruitment of naive T cells upon antigenic stimulation. Therefore, it is conceivable that treatment is effective, considering the fact that naive cells are preferentially deleted by the antibody. In agreement with the recent observations in various patient groups, in animal models administration of CD4 mAb has been unsuccessful in the treatment of already established disease (18, 56–58). Apparently, ongoing immune responses in vivo are not effectively influenced by the administration of depleting CD4 mAb. In agreement with this, our results show that depleting CD4 mAb does not affect the cell type most strongly implicated in the pathogenesis of human autoimmune disease, i.e., IFN-γ-producing TH1-type cells. Future therapies for chronic inflammatory T cell–mediated diseases have to take into account that intervention in ongoing immune responses must be directed at the primed TH1 T cell subset.

Acknowledgments

The authors wish to thank Drs. S.M.A. Lens, W.L. v.d. Graaf, R.O. Hintzen, M.R. Klein, P.T.A. Schellekens, and F. Medema for critically reviewing the manuscript, and people working in the lab of Marjike Roos (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and Guus Westra from the lab of Peter Huygens (Haematology, Free University Hospital, Amsterdam, The Netherlands) for technical assistance. This study was supported by grant 94-192 from the Dutch Society for Support of Research on Multiple Sclerosis.

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18. Bottomly, K. 1989. Treatment of already established disease (18, 56–58). Apparently, ongoing immune responses in vivo are not effectively influenced by the administration of depleting CD4 mAb. In agreement with this, our results show that depleting CD4 mAb does not affect the cell type most strongly implicated in the pathogenesis of human autoimmune disease, i.e., IFN-γ-producing TH1-type cells. Future therapies for chronic inflammatory T cell–mediated diseases have to take into account that intervention in ongoing immune responses must be directed at the primed TH1 T cell subset.
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