Immune functions in untreated and treated multiple sclerosis patients
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Chapter 7

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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Depleting CD4 mAb does not affect IFN-γ-secreting TH1 cells in humans

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

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Abstract

CD4⁺ 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⁺ 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⁺⁺ T cells but did not affect CD8⁺⁺ T cell numbers. Analysis of CD4⁺⁺ subpopulations showed that unprimed, CD45RA⁺/R0⁺⁺ lymphocytes were approximately three times more sensitive to the mAb than primed, CD45RA⁺/R0⁺⁺ T cells. Notably, within the CD45RA⁺⁺ subset, T cells with phenotypic evidence of prior activation, i.e., expressing Fas, were relatively insensitive to CM-T412, compared with Fas⁺⁺ cells. Remarkably, while a decrease in the number of IL-4-producing 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

CD4⁺⁺ 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 reactions through the secretion of immunoregulatory cytokines and via cell–cell contact (2, 3). Based on cytokine production capacity, distinct types of differentiated helper (H) CD4⁺⁺ 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 CD4⁺⁺ TH1-type cells are of crucial importance for disease induction and disease progression (10–12). In accordance with this notion, in vivo depletion of CD4⁺⁺ T cells interferes with disease induction in experimental allergic encephalomyelitis (13–15), 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 CD4⁺⁺ 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 CD4⁺⁺ 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

1. Abbreviations used in this paper: MRI, magnetic resonance imaging; MS, multiple sclerosis; PE, phycoerythrin.

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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 the treatment, after baseline correction, 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 multi-center 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-T11.2/1, and Hik27), CD28 (CLB-CD28/1), CD8 (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. PerCP-labeled CD3, CD4, and CD6, mAb, phycoerythrin (PE)-labeled CD4 mAb, and FITC-labeled CD14 mAb were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). PE-labeled CD45RA mAb (2H4-RD1) was obtained from Coulter Corp. (Miami, FL). FITC-labeled CD45RO mAb (UCHL-1) and biotinylated IgGl control mAb were from Dako (Glostrup, Denmark), and FITC-labeled CD95 (Fas) mAb was purchased from Immunotech S.A. (Marseille, France). Streptavidin-PE was obtained from Molecular Probes (Leiden, The Netherlands). Biotinylated αIFN-γ mAb was purchased from Dr. P. van der Meide (Biomedical Primate Research Centre, Rijswijk, The Netherlands) and biotinylated αIL-4 mAb was kindly provided by Dr. T.C.T.M. van der Pouw-Kraa (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

**PMA** (CML Cancer Research, Katonah, NY) and ionomycin (Calbiochem, La Jolla, CA) were prepared as stock solutions in DMSO, stored at −20°C and diluted properly before use.

**Blood samples and cell separation.** At each visit venous blood was collected in evacuated blood collection tubes (Vacutainer, Becton Dickinson, Meylan, France) containing sodium heparin (143 USP units). The samples were kept at room temperature and processed within 24 h.

PBMC were isolated from heparinized blood by ficoll-isopaque density gradient centrifugation and were cryopreserved immediately. To minimize interassay variability, samples from all time points from individual patients were analyzed in one experiment. Viability of the cells was > 95%, as indicated by trypan blue exclusion.

**Standard culture technique.** PBMC (2 × 10^6 cells/ml) were cultured in IMDM, supplemented with 10% FCS, penicillin, streptomycin, and β-mercaptoethanol, in a final volume of 200 μl and stimulated in triplicate cultures with a triplet of CD2 mAb (CLB-T11.1/1, CLB-T11.2/1, and Hik27; all 5 μg/ml) in the presence of CD28 mAb (CLB-CD28/1) (5 μg/ml) (34) in flat-bottom microtiter 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 [3H]thymidine. 0.4 μCi/well of [3H]thymidine (200 μCi/mmol, Amersham, Buckinghamshire, United Kingdom) was added 24 h before harvesting.

**Membrane phenotype.** PBMC were washed twice with PBS supplemented with 0.5% BSA and Na-azide (5 μg/ml). Immunofluorescence staining was performed by incubation of PBMC with saturating amounts of combinations of PerCP, FITC, and PE-labeled mAb in PBS/BSA. Stained cells were washed twice and 10^6 viable lymphocytes were analyzed using a fluorescence activated cell sorter (FACS², Becton Dickinson, Sunnyvale, CA). Results are given as the absolute number of cells per mm³ blood. For the different T cell subsets these numbers were calculated using the total number of either CD3⁺ or CD4⁺ T cells per mm³ blood.

**Flow cytometric measurement of intracellular cytokine production.** Measurement of cytokine production was performed as previously described (35, 36). Briefly, 0.5 × 10⁶ 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-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 μg/ml). 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 CD4⁺ T cells. Treatment with the CD4 mAb resulted in a significant reduction in absolute numbers of circulating CD3⁺ T cells (Fig. 1, *P = 0.004*). Within the total CD3⁺ T cell population the mAb induced a significant and progressive decline
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CD4⁺ T cell numbers remained low, 18 mo after cessation of treatment CD4⁺⁺ 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 CD4⁺⁺ 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 CD4⁺⁺ T cells for depleting CD4 mAb. Next, to more precisely define the target cells of the CD4 mAb we investigated if different subsets of CD4⁺⁺ T cells are equally affected by CD4 mAb treatment. Triple-color immunofluorescence analysis revealed that in placebo-treated patients, numbers of unprimed CD4⁺⁺ CD45RA⁺⁺⁺⁺ R⁺ and primed CD4⁺⁺⁺⁺⁺⁺ CD45RA⁺⁺⁺⁺ R⁺⁺⁺⁺ 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 CD4⁺⁺ T cells (Fig. 3b; NS).

in CD4⁺⁺ 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,
Figure 3. Preferential deletion of unprimed CD4⁺ T cells while primed CD4⁺ T cells are relatively resistant. Numbers of both CD4⁺CD45RA⁺/R0⁰ (open squares) and CD4⁺CD45RA⁻/R0⁺ (closed diamonds) T cells remain relatively stable in the placebo-treated group (a), while in the CD4 mAb-treated group (b) CD4⁺CD45RA⁺/R0⁰ are more depleted than CD4⁺CD45RA⁻/R0⁺ T cells. Differences between groups were significant for both parameters (CD4⁺CD45RA⁺/R0⁰ T cells: P < 0.001, CD4⁺CD45RA⁻/R0⁺ T cells: P = 0.002). Within the CD4⁺CD45RA⁺/R0⁺ subset no changes were found in both Fasneg (open triangles) and Fas⁺ (closed squares) T cells in the placebo-treated group (c). In the CD4 mAb-treated group a far more vigorous depletion of CD4⁺CD45RA⁺/Fas⁺ T cells occurs as compared with the CD4⁺CD45RA⁻/Fas⁺ T cells (d). Differences between groups were significant for CD4⁺CD45RA⁺/Fas⁺ T cells (P = 0.002), but not for CD4⁺CD45RA⁻/Fas⁺ T cells.

Remarkably, although absolute numbers of both subsets declined as a result of CD4 mAb treatment, the decline was much more pronounced in the CD4⁺CD45RA⁺/R0⁰ subset (at month 4 to 18% of the pretreatment level, P < 0.001) as compared with the CD4⁺CD45RA⁻/R0⁺ subset (at month 4 to 59% of the pretreatment level, P = 0.002). As a consequence, the ratio CD45RA⁺/CD45RA⁻ CD4⁺ T cells was reversed, which remained so until the end of the study period. After termination of treatment, numbers of both CD45RA⁺ and CD45RA⁻ CD4⁺ T cells increased in parallel.

When unprimed, naive CD4⁺ T cells are activated, the Fas antigen is rapidly upregulated (41). Consequently, a further subdivision can be made within the CD4⁺CD45RA⁺/R0⁰ subset based on Fas expression. Fas⁺ cells may be regarded as truly naive CD4⁺ 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⁺CD45RA⁺/Fas⁺ T cells (Fig. 3 c, P = 0.002), while the decline in the number of CD4⁺CD45RA⁺/Fas⁺ T cells was much less pronounced (Fig. 3 d, NS).

IFN-γ-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⁺ T cell subset (36, 42). Since, as we have already mentioned, autoimmune diseases are believed to be dependent on the activation of IFN-γ-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-γ- and IL-4-producing T cells using flow cytometry (35, 36). IFN-γ- 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⁺ T cells showed an almost 50% decline in the CD4 mAb-treated group (Fig. 4 b, NS), which is comparable with the decline seen in the CD4⁺CD45RA⁺/R0⁰ T cells. Remarkably, however, numbers of IFN-γ-producing CD3⁺ T cells remained relatively stable over time in the CD4 mAb-treated group (Fig. 4 a, NS). As a result, treatment with CD4 mAb resulted in a significant increase in the TH1/TH2 ratio (Fig. 4 c, 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⁺ compartment (Fig. 1 and reference 32). Repopulation studies in mice and in patients undergoing intensive chemotherapy (43, 44) have shown that CD4⁺ 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-
Our data demonstrate a differential sensitivity of unprimed CD45RA<sup>+</sup> versus primed CD45RA<sup>−</sup>CD45RO<sup>+</sup> T cells for depletion by the mAb anti-CD4. The fact that within the CD4<sup>+</sup>CD45RA<sup>−</sup>CD45RO<sup>+</sup> subpopulation Fas<sup>+</sup> T cells are more sensitive to treatment than Fas<sup>−</sup> T cells shows that the depletion is not simply related to expression of CD45RA, but that actually CD4<sup>+</sup> T cells which have not yet been activated in vivo are most sensitive to the depletion effect of the mAb. Also within the CD4<sup>+</sup>CD45RA<sup>−</sup>CD45RO<sup>+</sup> 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<sup>+</sup> TH1 cells in the peripheral blood.

The mechanism of CD4<sup>+</sup> 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 depletion process has been suggested by the observation that mice lacking a functional Fas receptor do not show a decrease of CD4<sup>+</sup> T cells after treatment with depleting CD4 mAb (48). In agreement with this, engagement of the CD4 molecule by CD4 mAb independent of the T cell antigen receptor leads to the loss in CD4<sup>+</sup> T cells (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 apoptosis. This observation suggests that T cells with membrane characteristics of activation (including Fas expression) are relatively resistant to depleting CD4 mAb (43, 53).
Chapter 7

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 antibody 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.

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