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Antigen-Specific Tolerance by Autologous Myelin Peptide–Coupled Cells: A Phase 1 Trial in Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a devastating inflammatory disease of the brain and spinal cord that is thought to result from an autoimmune attack directed against antigens in the central nervous system. The aim of this first-in-man trial was to assess the feasibility, safety, and tolerability of a tolerization regimen in MS patients that uses a single infusion of autologous peripheral blood mononuclear cells chemically coupled with seven myelin peptides (MOG_{1–20}, MOG_{35–55}, MBP_{13–32}, MBP_{83–99}, MBP_{111–129}, MBP_{146–170}, and PLP_{139–154}). An open-label, single-center, dose-escalation study was performed in seven relapsing-remitting and two secondary progressive MS patients who were off-treatment for standard therapies. All patients had to show T cell reactivity against at least one of the myelin peptides used in the trial. Neurological, magnetic resonance imaging, laboratory, and immunological examinations were performed to assess the safety, tolerability, and in vivo mechanisms of action of this regimen. Administration of antigen-coupled cells was feasible, had a favorable safety profile, and was well tolerated in MS patients. Patients receiving the higher doses ($>1 \times 10^9$) of peptide-coupled cells had a decrease in antigen-specific T cell responses after peptide-coupled cell therapy. In summary, this first-in-man clinical trial of autologous peptide-coupled cells in MS patients establishes the feasibility and indicates good tolerability and safety of this therapeutic approach.

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SUPPLEMENTARY MATERIALS

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INTRODUCTION

Approaches to induce antigen-specific tolerance in multiple sclerosis (MS) hold the promise to stop the pathogenic autoimmune response, thus preventing disease activity while avoiding the potentially severe side effects, which are associated with many of the currently used immunotherapies (1, 2). In MS, the primary target antigens are not known for certain, but it is well accepted that proteins within the myelin sheath, such as myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), and proteolipid protein (PLP), are important targets of the autoreactive immune response (3). However, the target epitopes of myelin proteins differ between MS patients, and it is likely that the myelin-specific T cell reactivity may change over time (4-6). In relapsing-remitting (RR) animal models of MS, chronic demyelination leads to the generation of new T cell responses against multiple endogenous antigens, a process called epitope spreading, and these newly generated T cells are able to induce relapses, which can be inhibited by tolerance to the spread epitope (7, 8). Therefore, it is reasonable to assume that the efficacy of antigen-specific therapies will depend not only on knowledge of the specific target antigens but also on the ability to block epitope spreading at an early stage and thereby stop diversification of T cell autoreactivity. Consequently, antigen-specific therapies should simultaneously target previously activated autoreactive T cells and also naïve autoreactive T cells specific for multiple myelin epitopes.

Antigen-coupled cell tolerance is a tolerization strategy with a long-standing and excellent track regarding efficacy and safety in several experimental models of autoimmune diseases, transplantation tolerance, and allergic disease (9, 10). Antigen-specific tolerance is induced through carrier cells, which are pulsed with antigens in the presence of the chemical cross-linker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (9, 11-15). Studies in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, have proven that a single intravenous injection of syngeneic splenocytes coupled with encephalitogenic myelin peptides/proteins is highly efficient in inducing antigen-specific tolerance in vivo (15-22). In EAE, this protocol not only prevented disease but also effectively reduced the onset and severity of all subsequent relapses when given after disease induction (17, 18, 23-25). As a major advantage of the therapy, tolerance can be simultaneously induced to multiple epitopes using a cocktail of myelin peptides (23).

With the aim to induce antigen-specific tolerance in MS, we adopted this tolerization strategy to treat patients with MS. We have established a procedure for the manufacture of antigen-coupled cells using autologous peripheral blood mononuclear cells (PBMCs) as carriers. Seven myelin peptides (MBP₁₃₋₃₂, MBP₈₃₋₉₉, MBP₁₁₁₋₁₂₉, MBP₁₄₆₋₁₇₀, MOG₁₋₂₀, MOG₃₅₋₅₅, and PLP₁₃₉₋₁₅₄), which were previously identified as important targets of autoreactive T cells in MS (26-30), were coupled to the surface of PBMCs by EDC. We assessed the feasibility, safety, and tolerability of antigen-coupled cell tolerance in a first-in-man, open-label, single-center clinical trial in RR and SP (secondary progressive) MS patients (ETIMS trial). Only patients who had an antigen-specific T cell response against at least one of the peptides used in the trial were eligible for treatment in the study, and T cell reactivity was analyzed before and after treatment.

RESULTS

Preparation and infusion of antigen-coupled cells: ETIMS cell product

At the day of study drug administration, 4×10^9 to 10×10^9 PBMCs and 200 ml of autologous plasma were isolated from MS patients by leukapheresis (COBE Spectra, Terumo BCT). The manufacturing process of peptide-coupled cells was started immediately, and all steps were performed under good medicinal practice (GMP) conditions in standard blood bags while maintaining a closed system (Fig. 1C). During this process, PBMCs were

chemically coupled with seven myelin peptides (MBP_{13–32}, MBP_{83–99}, MBP_{111–129}, MBP_{146–170}, MOG_{1–20}, MOG_{35–55}, and PLP_{139–154}). A detailed description of the manufacturing process can be found in Materials and Methods. At the end of the manufacturing process, the autologous peptide-coupled cell product was resuspended in 100 ml of autologous plasma for infusion (ETIMS cell product). Before infusion, samples were analyzed to meet the following release criteria of the cellular product: residual EDC <1.9 µg/ml, endotoxin <0.5 endotoxin units/ml, viability (>70% propidium iodide–negative cells measured by flow cytometry), pH (7.2 to 7.8), and absence of aggregates (microaggregates <1/µl). ETIMS product was administered within 4 hours after the last step of preparation of study drug product. The manufacturing process was feasible in all patients, and all cell products fulfilled the release criteria.

Adverse events including changes in vital signs, blood chemistry, and blood cell counts after treatment with ETIMS cell product

To determine safety and tolerability of treatment with ETIMS cell product, we evaluated the number and severity of adverse events (AEs). Twenty-four AEs, which were not considered MS-related, were reported at different times after treatment (Table 1). From these 24 AEs, only 1 may be related to the ETIMS product. Patient 2 reported a metallic flavor during infusion of study drug. The AE was graded as mild, but relation to study drug could not be excluded. The same patient had an irritation of a punctured vein, which had been caused by the leukapheresis procedure but not the ETIMS product, because it was not administered through that vein. One SAE not related with ETIMS treatment, a diverticulitis of sigma, occurred in patient 1 six weeks after administration of study drug.

No clinically relevant changes in vital signs, blood chemistry, or differential blood cell counts, including global number of eosinophils, basophils, neutrophils, lymphocytes, monocytes, and platelets, were observed after ETIMS treatment (Fig. 2).

MS course after treatment with ETIMS cell product

As an additional outcome measure for safety and tolerability, we assessed if the ETIMS treatment led to worsening of MS, for example, occurrence of exacerbations and/or disability progression and the occurrence of new T2 lesions or contrast-enhancing lesions (CEs) by magnetic resonance imaging (MRI). The first six patients included in the trial (patients 1 to 6) were selected with low disease activity to avoid that naturally occurring disease activity confounded the assessment of tolerability and safety. None of the first six patients treated with ETIMS (patients 1 to 6) showed a relapse during the first 3 months after treatment (Fig. 3). Concerning the three more active patients (patient 7, 8, and 10), patients 7 and 8 showed one MS disease exacerbation after treatment at days 10 and 16, respectively (Fig. 3). Both patients had high disease activity before inclusion in the study, and the clinical presentation or MRI findings at the time of relapse were not uncommon with respect to previous disease history. Patient 7 presented with mild dysfunction in fine motor skills and dysaesthesia in both upper extremities. MRI revealed a CE in the cervical spinal cord. Symptoms remitted completely after corticosteroid treatment. Patient 8 presented with dysaesthesia for temperature sensation in the right leg. MRI showed contrast enhancement in the cervical spinal cord lesion, which was already present the month before treatment. At this time, the patient had a myelitis with residual paraesthesia in both legs. After corticosteroid treatment, the symptom remitted. No further relapse occurred during the trial in these two patients, and further follow-up disclosed reduced disease activity during the treatment period compared to their disease activity before enrolment and during the baseline period.

Neurologic function, measured by Expanded Disability Status Scale (EDSS) (31), Scripps Neurological Rating Scale (SNRS) (32), Multiple Sclerosis Impact Scale (MSIS29) (33) (Fig. 4), and Multiple Sclerosis Functional Composite (MSFC) (34), remained stable in all patients during the 6 months after treatment.

All patients completed the MRI protocol. The number of new T2 lesions as well as CELs before and at different time points after treatment for the first six patients with low disease activity is summarized in Fig. 5 (upper graphs). No increase in CEL or new T2 lesions was observed in these six patients. Regarding the second (dose-escalation) cohort, which was composed of more active patients (patients 7, 8, and 10), new CELs as well as new T2 lesions were detected after treatment (Fig. 5). In all three patients, a single new T2 lesion was detected at week 2, and even though these were small lesions that did not look different from previously observed T2 lesions in each patient, it cannot completely be excluded that these were related to the ETIMS treatment. The number of new T2 and CEL is lower compared to baseline in patient 8 and slightly elevated in patients 7 and 10.

After completion of the 3-month protocol, all patients were followed further as part of the safety analysis. During this period, one MS exacerbation was observed in patients 3 and 5, respectively (Fig. 3). Patient 3 presented in week 18 with a hypaesthesia in the left leg without motor deficits. A corresponding CEL was seen on the spinal cord MRI. The hypaesthesia remitted completely after corticosteroid treatment. Patient 5 presented in week 17 with mild paresis of the right hand, a symptom that she had experienced several times previously. Although it remained unclear if these symptoms constituted a relapse, they were counted as such. Clinical examination and routine laboratory ruled out acute infection, and brain MRI did not show any CEL or new T2 lesion. Symptoms completely resolved after corticosteroid treatment. Finally, we want to mention that patient 6 reported a transient increase in spasticity 3 months after study drug administration, which is a common symptom in MS, and induction by the treatment was unlikely at this late point.

Characterization of blood cell populations after treatment with ETIMS cell product

As a supplementary measure to determine safety and tolerability of ETIMS treatment, several relevant immune cell populations were characterized by flow cytometry before and after treatment in patients 6, 7, 8, and 10. ETIMS treatment did not induce relevant changes in the percentages of monocytes and B, T, natural killer (NK), or NK T cells (Fig. 6A). Frequencies of several functional CD4⁺ T cell subsets including T helper 1 (T_H1) [interferon- γ (IFN- γ)], T_H2 [interleukin-4 (IL-4)], T_H17 (IL-17), Tr1 (IL-10), and regulatory T cells (T_{regs}; FoxP3) were also analyzed before and after treatment (Fig. 6B; individual data of patients in the high-dose group, fig. S3). With regard to safety of the regimen, it is important to note that we did not detect increases in the frequency of T_H1 or T_H17 CD4⁺ T cell subsets 3 months after treatment. The frequency of Tr1, T_{regs}, and T_H2 cells remained stable during the course of the study. Frequencies of several functional CD8⁺ T cell subsets including “regulatory” CD8⁺ T cells (CD8⁺CD57⁺ILT2⁺ T cells) and “proinflammatory” CD8⁺ T cells (CD8⁺CD161^{high} T cells) were stable after ETIMS treatment (Fig. 6C and fig. S3).

Characterization of myelin-specific T cell responses after treatment with ETIMS product

To address whether ETIMS had an effect on the frequency of T cells specific for the antigens used in this study, we measured antigen-specific T cell responses before and 3 months after treatment. The percentage of positive wells, that is, wells with scintillation counts (CPM) higher than the mean + 3 SDs of the unstimulated wells, is summarized in Fig. 7 for each peptide before and after treatment in all patients receiving the lower dose of

ETIMS (patients 1 to 5) and in the patients receiving the higher dose (patients 6, 7, 8, and 10).

Before treatment, patients were considered to have a positive T cell response against a specific peptide if they showed two or more positive wells. The ETIMS treatment was considered to have an effect when a reduction of two or more positive wells was observed 3 months after treatment. Patient 6 showed positive T cell responses before treatment to all seven myelin peptides, which were all reduced 3 months after treatment (Fig. 7). Patient 7 showed positive T cell responses against four different peptides (MOG₁₋₂₀, MOG₃₅₋₅₅, MBP₁₄₆₋₁₇₀, and PLP₁₃₉₋₁₅₄) before treatment, and these responses were all reduced after treatment (Fig. 7). Patient 8 showed positive responses before treatment against MOG₁₋₂₀, MOG₃₅₋₅₅, MBP₁₃₋₃₂, MBP₈₃₋₉₉, and MBP₁₁₁₋₁₂₉, and these responses were also reduced after treatment. Finally, patient 10 showed positive T cell responses to all seven myelin peptides, and these were all reduced after ETIMS treatment (Fig. 7).

DISCUSSION

In this first-in-man trial, we have established the feasibility of antigen-coupled cell tolerization in MS and provide evidence for the safety and tolerability of this therapeutic approach. Tolerization by ETIMS involves autologous PBMCs pulsed with seven myelin peptides in the presence of the coupling agent EDC. EDC catalyzes the formation of peptide bonds between free amino and carboxyl groups, thereby producing peptide-coated cells that function as highly tolerogenic carriers. This therapy includes the use of a set of peptides that cover the immunodominant epitopes of different myelin proteins. Six peptides of these three myelin proteins (MOG, MBP, and PLP) were chosen because they were previously shown to be targets of the high-avidity autoimmune T cell response in MS (26). MBP₈₃₋₉₉ was added because it has been shown to be immunodominant in MS patients by many previous studies and has been a target of previous tolerization trials (3, 35, 36). This tolerization strategy simultaneously targets seven peptides from three myelin proteins. Previous tolerization approaches in MS patients mainly focused on single MBP peptides or MBP protein (35-37). Recently, transdermal application of three peptides from MBP, MOG, and PLP showed efficacy in reducing myelin-specific T cell reactivity (38). Different from all other tolerization therapies, antigen-coupled cell tolerance was shown to prevent epitope spreading in animal models. Further, tolerization with antigen-coupled cells has been shown to act in part independently of the major histocompatibility complex (MHC) in mice and may thus potentially be applicable for both human leukocyte antigen (HLA)–DR15–positive and HLA–DR15–negative MS patients (39).

To translate this regimen from mice to MS patients, we have developed a manufacturing process for antigen-coupled cells, which is completely performed under GMP conditions in standard blood bags while maintaining a closed system (Fig. 1 and fig. S1). The autologous cell product can be reinfused within the same day, which renders it a feasible approach for the outpatient care setting.

For safety reasons, it was requested by the regulatory authorities that we perform a very careful dose escalation starting with 1×10^3 cells in the first patient up to the target dose of 3×10^9 antigen-coupled cells in the last patient. We did not encounter relevant safety concerns related to the study drug and have thus met the primary endpoint set for this first-in-man study. There was a single SAE during the 3-month core study period, which was considered not related to the therapy by both the investigators and the independent data and safety monitoring board (DSMB; all other AEs were graded as mild or moderate). A critical issue in antigen-specific therapies is the risk of induction of disease by the treatment as previously observed in a vaccination trial using an altered peptide ligand (35). Therefore, we

aimed to include patients who did not have highly active disease in this first-in-man trial. Because we did not observe induction of disease in the first six patients we expanded our dose-escalation regimen with three more patients who had clinically active disease. In two of these patients, we observed exacerbations and new T2 MRI lesions 10 and 16 days after therapy. In both cases, the presentation of the exacerbation was similar or identical to symptoms that the patients had experienced during recent months before treatment and, hence, different from what had been observed in a trial using an altered peptide ligand (35). In the latter trial, three of eight patients experienced exacerbations with clinical and/or MRI presentation, which were very different from the patients' previous history, and the massive increase of MBP peptide-specific T cells left little doubt that relapses had been treatment-induced (35). There was no increase in disability over the course of the study in any of the patients. As stated above, we cannot exclude completely that the disease activity (exacerbations or new MRI lesions), which was observed within the first couple of weeks in the small group of highly active patients, was related to the ETIMS therapy, although we would have expected that it occurred even earlier in the trial had this been the case. Notably, no further relapse occurred during the 6-month follow-up period in any of the highly active patients (patients 7, 8, and 10). However, the data raise the question if the dose of ETIMS cells should not be escalated even further. The two observed exacerbations in patients treated with a high dose of ETIMS are an important safety signal. Therefore, strict safety measures, both clinical and MRI, are warranted in future applications of antigen-coupled cells during clinical trials.

Although the primary aim of the study was safety and tolerability, another important objective was to gather information on its immunologic effects. Thus, we treated only patients in whom T cell responses toward the myelin peptides used in the trial could be measured at baseline. Myelin peptide-specific T cell responses at baseline were higher in those patients with ongoing inflammatory disease activity. Patients treated in the high-dose group (patients 6, 7, 8, and 10) showed a uniform reduction in myelin-specific T cell response, although a few positive wells were measured in patients 6, 7, and 10 after treatment (Fig. 7). Therefore, together with the above clinical/MRI data, we conclude with respect to dose finding that minimally a dose of 3×10^9 peptide-pulsed cells or even more should be used. Given the good tolerability of the regimen, further dose escalation is not expected to pose problems. Three patients (1, 7, and 8) were HLA-DR15-positive; thus, a definite conclusion on the influence of the HLA cannot be drawn. In a mouse EAE model, it has been shown that whereas antigen-coupled cells can induce tolerance independently of the MHC, tolerance induction with antigen-coupled allogeneic cells required the administration of higher cell numbers than syngeneic cells or repeated injections (14, 39). Thus, beside a further dose escalation, as already mentioned, future trials should consider to give repeated injections of ETIMS product.

The exact mechanism of action of the tolerization regimen is not yet fully understood, but there is evidence that several distinct mechanisms are involved. It has been shown that antigen-specific T cells encountering their cognate antigen/MHC complexes on EDC-treated cells are anergized as a result of failure to receive adequate CD28-mediated costimulation (40). However, in vivo, another mechanism might be more important, which is based on the fact that EDC efficiently induces apoptosis in treated cells. Experiments in animal models suggest that apoptotic EDC-treated cells are phagocytosed in the spleen within a few hours by antigen-presenting cells residing in this organ (immature dendritic cells or monocytes/macrophages), which leads to the production of IL-10 and expression of PD-L1 on macrophages as important factors for the induction of tolerance. Additionally, the induction of regulatory T cells plays a central role in the long-term maintenance of tolerance induced by this procedure (39, 41). In our patients, we observed only a slight increase in the overall frequency of regulatory T cell subsets in peripheral blood; however, a large number of

patients, who are treated with a homogeneous cell number, are needed to analyze this further.

A few limitations of the study have to be considered in the interpretation of the data. The baseline to treatment crossover study design is considered ideal for proof-of-concept studies with low patient numbers. However, the dose escalation performed in the current trial anticipates comparability between patients and consequently limits the statistical analysis. Further, the included study population was highly heterogeneous with respect to disease activity when comparing patients in the low-dose group and patients in the high-dose group. Finally, immunological analysis of antigen-specific T cells is based on proliferation, measured by thymidine incorporation, as a readout, which does not reflect changes in phenotypes as can be measured by flow cytometry-based assays.

Contingent on future studies confirming the antigen-specific therapeutic effects of the regimen, antigen-coupled cell tolerance has the potential for wide applicability in different autoimmune diseases, transplantation tolerance, and allergy. The feasibility and easy applicability without the need for long-term cell culture or expansion of cells *ex vivo* are major advantages of this approach. The clinical and MRI data as well as the incomplete response with respect to reducing the frequency of myelin peptide-specific T cells in all of the small group of patients with highly active disease indicate that further dose escalation should be explored and that a better understanding of the mechanism/s of action in humans needs to be gained, and it is likely that this will be achieved in the phase 2a trial with a more homogeneous group of patients, who will all be treated with the same dose of cells. Despite these caveats, we believe that tolerization by ETIMS has distinct advantages over other approaches, which include the possibility for blocking epitope spreading, and that only a single or few treatment courses are needed. Given the fact that experimental studies demonstrated efficacy and safety of this approach in different T cell-driven autoimmune diseases with defined antigens, allergy, and transplantation, antigen-coupled cell tolerance could, in principle, be applied in several immunopathological conditions in humans; however, further data are clearly needed, and it is open at this point if the effects of this treatment are equally broad in humans.

MATERIALS AND METHODS

Ethics statement

The protocol was reviewed and approved by the Ethics Committee of the Hamburg Chamber of Physicians. An independent DSMB oversaw the study. All procedures were done following the rules of the Declaration of Helsinki Guidelines, and all regulatory steps were performed under guidance of the German regulatory authority for biologics/cell therapies, the Paul-Ehrlich Institute. The trial is registered under the EudraCT#2008-004408-29. All patients signed a written informed consent before inclusion in the study.

Trial design and outcome measures

Ten patients with RRMS ($n = 8$) or SPMS ($n = 2$) were included in this single-center, open-label, phase 1 trial. Patient 9 withdrew from the study at month -2 before the treatment for personal reasons. The clinical and demographic data of patients treated with ETIMS cell product are shown in Table 2. Inclusion criteria included the following: ages between 18 and 55 years, RRMS or SPMS disease course, disability score (EDSS) between 1 and 5.5 (31), patients had to be able to provide written informed consent before any testing under the protocol, and patients had to have a specific T cell response against at least one of the peptides used in the trial (see “Antigen-specific T cell responses” section in Materials and Methods). All patients included in the trial fulfilled this criterion by showing a T cell

response against at least one of the peptides used in the trial. Exclusion criteria included the following: primary progressive MS; pregnancy and breast-feeding; history or actual signs of immunodeficiency; concurrent clinically relevant cardiac, immunological, pulmonary, neurological, renal, or other major disease; splenectomy; and cognitive impairment.

The primary outcome of the study was safety and tolerability and was determined by the (i) number and severity of AEs, including (ii) relevant changes in vital signs, (iii) changes in blood chemistry and differential blood counts, and (iv) aggravation of the disease at month +3.

The first cohort of six patients was followed for 1 month before study drug administration with two consecutive clinical MRI and general physical examinations as well as clinical and laboratory assessments (Fig. 1A). A careful dose escalation was performed in these patients to ensure safety and reduce the risk for individuals (for details on the rationale for the lowest and highest dose, see the Supplementary Methods). Each patient received a single infusion of antigen-coupled cells. Patient 1 received 1×10^3 , patient 2 received 1×10^5 , patient 3 received 1×10^7 , patient 4 received 1×10^8 , patient 5 received 5×10^8 , and patient 6 received 1×10^9 antigen-coupled cells. The last three patients (patients 7, 8, and 10) were included in the trial as an amendment to increase dose escalation, and because safety was already documented, more active disease patients were included. Patient 7 received 1×10^9 , patient 8 received 2.5×10^9 , and patient 9 received 3×10^9 antigen-coupled cells. In these three patients, the observation period before study drug administration was extended to 3 months (Fig. 1A). At the day of study drug administration, all patients were monitored in a phase 1 inpatient unit for 24 hours (Fig. 1B). The trial design stipulated measurement of the primary outcome at month 3 and additional safety follow-up until month 6 with consecutive clinical, MRI, and general physical examinations as well as clinical and laboratory analyses to assess AEs and monitor MS disease activity (Fig. 1A). Hematological analyses including differential blood counts and clinical chemistry were performed in the Institute of Clinical Chemistry, University Medical Center Hamburg-Eppendorf. MS disease course was evaluated by neurological examination and MRI assessing the presence of new T2 lesions or new CELs on MRI, occurrence of exacerbations, and disability progression. Neurologic function was scored with EDSS (31), SNRS (32), and MSFC (34). MSIS29 was used as a patient-based outcome measures of disability (33).

Preparation of antigen-coupled cells

PBMCs (4×10^9 to 10×10^9) and 200 ml of autologous plasma were isolated from MS patients by leukapheresis (COBE Spectra, Terumo BCT). Immediately after collection of cells, the manufacturing process was started under GMP conditions in the clean room at the Institute of Transfusion Medicine, Center for Diagnostics, University Medical Center Hamburg-Eppendorf, Germany. Briefly, red blood cells were lysed by 15-min incubation in 200 ml of ACK lysis buffer [consisting of ammonium chloride, PhEur (Merck), potassium hydrogen carbonate, PhEur (Merck), and water for injection PhEur (Baxter)] at room temperature. Subsequently, PBMCs were washed twice in 200 ml of saline (Baxter) containing citrate-phosphate-dextrose buffer (CPD, Fresenius). After cell counting, 1.5×10^9 to 4×10^9 PBMCs were re-suspended in 10 to 20 ml of saline, and 1 ml containing 0.5 mg of each GMP peptide was added (MBP_{13–32}, MBP_{83–99}, MBP_{111–129}, MBP_{146–170}, MOG_{1–20}, MOG_{35–55}, and PLP_{139–154}; Bachem AG; the final concentration of each peptide in the coupling reaction was 0.05 mg/ml). The coupling reaction was initiated by the addition of 100 to 200 mg of freshly prepared water-soluble EDC (AppliChem). After 1-hour shaking incubation at 4°C, the cells were washed two times with 100 ml of CPD-saline and resuspended in 100 ml of autologous plasma for injection.

Coupling of the peptides on the surface of PBMCs was verified during validation of manufacturing process and at regular intervals during the trial. Briefly, PLP_{139–154} was replaced by a biotinylated PLP_{139–154} peptide. At the end of the manufacturing process, coupling of biotin PLP_{139–154} was visualized by fluorescence-activated cell sorting (FACS) analysis with streptavidin conjugated with allophycocyanin (APC). The respective drug product was not used for patient injection because the use of biotin-conjugated peptides is not licensed for use in humans. The binding efficiency was not tested individually for all peptides used in the trial; however, from preclinical experience as well as the chemical properties of the peptides, it is expected that the coupling is efficient for all peptides.

Magnetic resonance imaging

The MRI examinations were performed on a 1.5-T MRI scanner MAGNETOM Sonata (Siemens) with a standard head coil at the Department of Neuroradiology, University Medical Center Hamburg-Eppendorf. The following sequences were obtained: T1 pre- and post-gadolinium (0.1 mM/kg, Gd-BOPTA), PD/T2-weighted images, fluid-attenuated inversion recovery, and diffusion-weighted imaging sequences. All brain MRIs were analyzed by experienced neuroradiologists blinded for the clinical findings.

Flow cytometric analysis

Frequency of different cell subsets was analyzed in whole blood (EDTA tubes) by flow cytometry with the following antibody panels: for immune cell subsets (granulocytes, eosinophils, monocytes, and B, T, NK, and NK T cells)—anti-CD45 (PE-Cy7, eBioscience), anti-CD16 (APC-Cy7, BioLegend), anti-CD19 [fluorescein isothiocyanate (FITC), BD], anti-CD14 (V450, BD), anti-CD3 [peridinin chlorophyll protein (PerCP), BD], and anti-CD56 [phycoerythrin (PE), eBioscience] (gating strategy is shown in fig. S2); for T cell subsets including CD4⁺, FoxP3⁺ T_{regs}, regulatory CD8⁺CD57⁺ILT2⁺, and proinflammatory CD8⁺CD161^{high} T cells—anti-CD3 (PE-Cy7, eBioscience), anti-CD4 (APC, eBioscience), anti-CD8 [Pacific Blue (PB), Dako-Biozol], anti-FoxP3 (PE, Miltenyi), anti-CD25 (APC, eBioscience), anti-CD57 (FITC, BD), anti-ILT2 (PE, Beckman), and anti-CD161 (APC, Miltenyi). The corresponding isotype controls were included in all stainings. Cells were analyzed with an LSR-II flow cytometer (BD) and FACSDiva Software (BD).

PBMCs were isolated by Ficoll density gradient centrifugation (PAA), and functional phenotype of T cells was evaluated by intracellular cytokine staining as follows: 5×10^5 freshly isolated PBMCs were incubated overnight in 200 μ l of X-VIVO 15 (Lonza) in a sterile FACS tube. The next day, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml, Sigma) and ionomycin (1 μ g/ml, Sigma) in the presence of brefeldin A (10 μ g/ml, eBioscience) for 5 hours. After washing with phosphate-buffered saline, cells were stained with LiveDead kit (AmCyan, Invitrogen), fixed, permeabilized, and stained with different antibodies: anti-IL-17 (Alexa Fluor 647; eBioscience), anti-IL-4 (PE-Cy7, BioLegend), anti-IFN- γ (FITC, BioLegend), anti-IL-10 (PE; BioLegend), anti-CD3 (PE, DakoCytomation), anti-CD4 (PB, DakoCytomation), and anti-CD8 (PB, BioLegend) or with the corresponding isotype controls.

Antigen-specific T cell responses

The antigen-specific T cell responses toward the myelin peptides used in the study were measured in freshly isolated PBMCs before the tolerization procedure and after 3 months. Antigen-specific T cell responses were analyzed by proliferation assays with thymidine incorporation. Briefly, isolated PBMCs were seeded in 96-well plates at 1.5×10^5 PBMCs per well in X-VIVO 15 medium (Lonza) with 1 μ M of each peptide. Forty-eight wells were seeded per antigen, and six wells only with medium as negative control in each plate. TTx (5 μ g/ml) (Novartis Behring) was used as positive control. On day 7, plates were incubated for

15 hours with 1 μ Ci of [3 H]thymidine (Hartmann Analytic). [3 H]thymidine-pulsed plates were analyzed with a scintillation β counter (Wallac 1450, PerkinElmer). The scintillation counts (CPM) of each well were measured. Wells showing CPM higher than the mean + 3 SDs of the unstimulated wells were considered as positive.

Statistical analysis

Statistical analysis was done with GraphPad Prism 4 software (GraphPad Software Inc.). Descriptive statistics are reported as means $\pm$ SEM. The comparisons of clinical and immunological parameters were performed for two-group comparisons with a paired *t* test. Comparisons of three groups and more were assessed by one-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons or Kruskal-Wallis test with Dunn's post-test depending on the distribution of the data. *P* values <0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Study design

(A) The first six patients were followed before treatment with two clinical, MRI, and laboratory examinations at month -1 before treatment and day 0 and after treatment at weeks 2 and 6 and 3 months. Vital signs and AEs were recorded during infusion; 30 min, 1 hour, 2 hours, and 4 hours after infusion; and on day +1, day +3, week +2, week +3, week +6, and month +3. General physical examination was performed at month -1 and day +1. Neurological examination was done at month -1, day -1, week +2, week +6, and month +3. Brain MRI was assessed at month -1, day -1, week +2, week +6, and month +3. MSIS29 was assessed at month -1, day +1, week +6, and month +3. Patients were further followed for AEs, serious AEs (SAEs), and clinical and MRI disease activity at months +4, +5, and +6. The last three patients were followed at four time points (month -3 to day 0) before treatment, and further examinations were performed at week 2 and months 1, 2, 3, and 6 (scattered lines). (B) Patients were admitted to the phase 1 unit the day before treatment. At day 0, leukapheresis was performed. The autologous cell product manufactured was infused the same day, and patients were monitored for AEs for 24 hours. (C) Most important steps of the manufacturing process, which was performed in blood bags while maintaining a closed system. Erythrocytes are lysed with lysis buffer. The peptides are added, and the coupling procedure is initiated by addition of EDC. After quality control (QC), the ETIMS cell product is used for therapy. * includes washing steps and cell counting. RBCs, red blood cells.

Fig. 2. Peripheral blood cell counts after ETIMS treatment

Absolute peripheral blood counts (means $\pm$ SEM) of eosinophils, basophils, neutrophils, lymphocytes, monocytes, and platelets before and after treatment. Dotted lines indicate the time point of study drug administration.

Fig. 3. Clinical exacerbations after ETIMS treatment

Clinical exacerbations in the year before treatment (gray circles), during the first 3 months after treatment (black circles), and during the safety follow-up until month 6 for the nine patients included in the trial. Dose of ETIMS is shown on the right side. Dotted lines indicate the time point of study drug administration.

Fig. 4. Neurologic function after ETIMS treatment

EDSS (left graphs), SNRS (middle graphs), and MSIS29 (right graphs) in patients 1 to 6 (upper graphs; means $\pm$ SEM) and in patients 7, 8, and 10 (lower graphs) before and after treatment. Dotted lines indicate the time point of study drug administration.

Fig. 5. New T2 lesions and CELs after ETIMS treatment

New T2 lesions (left graphs) and CELs (right graphs) in patients 1 to 6 (upper graphs; means $\pm$ SEM) and in patients 7, 8, and 10 (lower graphs). T2 lesion graphs: y axis is number of T2 lesions; first column in gray indicates the number of total T2 lesions 3 months before study drug administration; columns in black indicate new T2 lesions before and after treatment. CEL graphs: y axis is number of new CELs (both brain and spinal cord CELs); new CELs before and after treatment are shown. Dotted lines indicate the time point of study drug administration.

Fig. 6. Immune cell subsets after ETIMS treatment

(A to C) Percentage of (A) B cells (CD45⁺CD19⁺), monocytes (CD45⁺CD14⁺), T cells (CD45⁺CD3⁺CD56⁻), NK cells (CD45⁺CD3⁻CD56⁺), and NK T cells (CD45⁺CD3⁺CD56⁺); (B) CD4⁺ T cells (CD3⁺CD4⁺CD8⁻), including T_H1 (CD4⁺IFN- γ ⁺), T_H17 (CD4⁺IL-17⁺), T_H2 (CD4⁺IL-4⁺), T_{regs} (CD4⁺FoxP3⁺), and Tr1 (CD4⁺IL-10⁺); and (C) CD8⁺ T cells (CD3⁺CD4⁺CD8⁺), including regulatory CD8⁺ (CD8⁺CD57⁺ILT2⁺) and proinflammatory CD8⁺ (CD8⁺CD161^{high}) measured by flow-cytometry in patients 6, 7, 8, and 10 before (month -1) and after (month 3) ETIMS treatment. Means $\pm$ SEM is shown. Dotted lines indicate the time point of study drug administration.

Fig. 7. Myelin-specific T cell response after ETIMS treatment

MOG₁₋₂₀, MOG₃₅₋₅₅, MBP₁₃₋₃₂, MBP₈₃₋₉₉, MBP₁₁₁₋₁₂₉, MBP₁₄₆₋₁₇₀, PLP₁₃₉₋₁₅₄, tetanus toxoid (TTx)-specific T cell responses, and unstimulated wells before and 3 months after ETIMS treatment in patients treated with low (1×10^3 to 5×10^8 , patients 1 to 5; left panel) or high (1×10^9 to 3×10^9 , patients 6, 7, 8, and 10; right panel) dose of antigen-coupled cells. Proliferative responses were measured by [^3H]thymidine incorporation assay. Graphs (y axis) represent the scintillation counts per minute (CPM). The dotted lines represent the threshold set for the mean + 3 SDs of unstimulated wells. All wells above this threshold are shown in red.

Table 1

Adverse events during 3 months of trial.

Patient ID	Adverse drug reaction	Severity	Relation to study procedure	Time interval (days)*
1	Vaginal infection	Mild	Not related	−1
	Diarrhea	Mild	Not related	2
	Headache	Mild	Not related	11
	Diverticulitis of sigma	Severe	Not related	42
2	Back pain	Mild	Not related	−28
	Metallic flavor	Mild	Reasonable possible	1
	Irritation of punctured vein	Mild	Not related	1
	Migraine	Mild	Not related	22
3	Neck pain	Mild	Not related	26
	Vision disturbance	Mild	Not related	28
	Dysaesthesia/pain in one hand	Mild	Not related	35
4	Cold	Mild	Not related	85
5	Cold	Mild	Not related	24
	Gastric pain	Moderate	Not related	66
6	Syncope	Moderate	Not related	3
7	Sinusitis	Mild	Not related	−72
	Sinusitis/bronchitis	Mild	Not related	−7
	Paravertebral dysaesthesia	Mild	Not related	74
8	Diarrhea after oral antibiotics	Mild	Not related	−1
	Dental infection	Moderate	Not related	−8
10	Headache	Mild	Not related	−65
	Cold	Mild	Not related	−44
	Cold	Mild	Not related	−37
	Herpes labialis	Mild	Not related	−24

* Time interval to administration of study drug (days).

Table 2

Clinical and demographic data.

Patient	Dosage [*]	Age (years)	Sex	MS type	Disease duration (years)	Relapse 12/24 mo [†]	MRI [‡]		EDSS	HLA DR15
							T1 Gd ⁺	T2		
inims-01	1 × 10 ³	49	F	SP	5	0/1	0	10	3.5	Positive
inims-02	1 × 10 ⁵	39	F	RR	5	1/1	0	12	1.5	Negative
inims-03	1 × 10 ⁷	27	F	RR	2	1/2	1	2	1	Negative
inims-04	1 × 10 ⁸	42	F	RR	2	0/1	0	4	1.5	Negative
inims-05	5 × 10 ⁸	44	F	RR	16	0/0	0	8	4	Negative
inims-06	1 × 10 ⁹	43	F	SP	13	0/0	0	27	4.5	Negative
inims-07	1 × 10 ⁹	34	F	RR	1.5	4/na	0	36	1.5	Positive
inims-08	2.5 × 10 ⁹	42	F	RR	3.5	2/4	1	23	1.5	Positive
inims-10	3 × 10 ⁹	42	M	RR	2.5	1/1	1	40	2	Negative

^{*} Absolute number of antigen-coupled cells infused.

[†] Number of relapses in the previous 12/24 months.

[‡] Number of lesions on brain MRI.

SP, secondary progressive MS; RR, relapsing-remitting MS; na, not available.