Immune tolerance in multiple sclerosis and neuromyelitis optica with peptide-loaded tolerogenic dendritic cells in a phase 1b trial

Irat Zubizarreta,1–4 Georgina Flórez-Grau,3–5 Gemma Vila, Raquel Cabezón, Carolina España, Magi Androra, Albert Saiz,6–7 Sara Llufriu,6–7 Maria Sepulveda,6–7 Nuria Sola-Valls,6–7 Elena H. Martinez-Lapiscina, Irene Pulido-Valdeolivias, Bonaventura Casanovad, Marisa Martinez Gines, Nieves Tellezf, Celia Oreja-Guevarag, Marta España, Esteve Triash, Joan Cid, Manel Juan, Miquel Lozano, Yolanda Blanco, Lawrence Steinman, Albert Saiza, Sara Llufriu, Maria Sepulveda, Nuria Sola-Valls, Elena H. Martinez-Lapiscina, Irene Pulido-Valdeolivas, Bonaventura Casanovad, Marisa Martinez Gines, Nieves Tellezf, Celia Oreja-Guevara, Marta España, Esteve Trias, Joan Cid, Manel Juan, Miquel Lozano, Yolanda Blanco, Lawrence Steinman.

There are adaptive T-cell and antibody autoimmune responses to myelin-derived peptides in multiple sclerosis (MS) and to aquaporin-4 (AQP4) in neuromyelitis optica spectrum disorders (NMOSDs). Strategies aimed at antigen-specific tolerance to these autoantigens are thus indicated for these diseases. One approach involves induction of tolerance with engineered dendritic cells (tolDCs) loaded with specific antigens. We conducted an in-human phase 1b clinical trial testing increasing concentrations of autologous tolDCs loaded with peptides from various myelin proteins and from AQP4. We tested this approach in 12 patients, 8 with MS and 4 with NMOSD. The primary end point was the safety and tolerability, while secondary end points were clinical outcomes (relapses and disability), imaging (MRI and optical coherence tomography), and immunological responses. Therapy with tolDCs was well tolerated, without serious adverse events and with no therapy-related reactions. Patients remained stable clinically in terms of relapse, disability, and in various measurements using imaging. We observed a significant increase in the production of IL-10 levels in PBMCs stimulated with the peptides as well as an increase in the frequency of a regulatory T cell, known as Tr1, by week 12 of follow-up. In this phase 1b trial, we concluded that the i.v. administration of peptide-loaded dendritic cells is safe and feasible. Elicitation of specific IL-10 production by peptide-specific T cells in MS and NMOSD patients indicates that a key element in antigen specific tolerance is activated with this approach. The results warrant further clinical testing in larger trials.

Immune tolerance | dendritic cells | neuromyelitis optica | multiple sclerosis | Tr1 cells

Antigen-specific immunotherapies for autoimmune diseases aim to restore peripheral immune tolerance for pathogenic adaptive T- and B-cell responses without dampening the overall immune surveillance against microbes and cancer (1). Therefore, by using a highly selective approach, the safety and efficacy of immunotherapies should be enhanced. Several approaches have been tested aimed to restore immune tolerance, including peptide-based tolerance vaccines (either oral, i.v., intradermal, or i.m.), altered peptide ligands, DNA vaccination, or cell-based tolerance [T-cell vaccines, leukocytes coupled with peptides, mesenchymal stem cells, regulatory macrophages, T or B cells, or tolerogenic dendritic cells (tolDCs) (1–3)]. A probabilistic model of the immune system states that immune responses (including auto-immune responses) require a minimum number of MHC–peptide–T cell receptor complexes to be formed (4), providing support for testing antigen-specific tolerance based on increasing the exposure of self-antigens within an antiinflammatory environment, which can be provided by tolDCs (5). The induction of tolDCs is an approach tested for antigen-specific tolerance in autoimmune diseases such as rheumatoid arthritis, type 1 diabetes, and Crohn’s disease. These trials with tolDCs have shown satisfactory safety and some evidence of immune tolerance (6–10).

Genetic and immunological studies support the involvement of myelin-specific immune responses in the pathogenesis of multiple sclerosis (MS) (1). Even if the target antigen remains unknown, by promoting immune tolerogenic responses in adaptive immune cells that migrate to the inflamed CNS, an amelioration of the inflammatory process can be achieved (2). Previous trials of tolerance with myelin peptides in MS have been shown to be safe and that the peptides are capable of induction of immunomodulatory responses (11).


Reviewer statements: E.F., University of Texas at Austin; and O.S., University of Texas Southwestern Medical Center.


I.Z. and G.F.-G. contributed equally to this work.

1To whom correspondence may be addressed. Email: steinman@stanford.edu or pvilloslada@clinic.ub.es.

2I.P.-V. and P.V. contributed equally to this work.

3www.pnas.org/cgi/doi/10.1073/pnas.1820039116

PNAS | April 23, 2019 | vol. 116 | no. 17 | 8463–8470

Published online April 8, 2019.
The neurological examination included the Expanded Disability Status Scale (EDSS) and the Multiple Sclerosis Functional Composite (MSFC). Blood tests for analyses and immunological assessment were done every 2 wk. OCT were performed at baseline and by week 12, as previously described (14). signed the informed consent. Brain MRI and an optical coherence tomography (OCT) were performed at baseline and by week 12, and a key tolerogenic cytokine, were detected after treatment. These results might lead to more extensive trials with this approach in autoimmune conditions where the antigenic target has been identified, including MS, NMO, myasthenia gravis, and Graves disease.

Neuromyelitis optica spectrum disorders (NMOSDs) represent an excellent opportunity for testing immune tolerance protocols. In NMOSD, the target autoantigen, is well identified, the aquaporin-4 (AQP4) (12, 13), and up to 80% of the patients have anti-AQP4 antibodies in the blood or the cerebrospinal fluid. All NMOSD patients in this trial were required to have anti-AQP4 antibodies.

In this study, we tested the safety of CNS peptide-loaded tolDC therapy and searched for signs of efficacy and markers of immune tolerance in patients with MS and NMOSD. We focused on tolDCs based on our previous experience in Crohn’s disease for which tolDCs showed excellent safety and signs of clinical efficacy (5, 9). Moreover, we combined in the same protocol patients with either MS or NMOSD to obtain evidence for the safety of tolerance induction with a diversity of CNS-related peptides. The use of a battery of tolDCs for different peptides would allow a potential approach for a broader swath of patients.

Materials and Methods

Ethical Statement. The protocol was reviewed and approved by the Ethics Committee of the Hospital Clinic of Barcelona and the Spanish Agency of Drugs and Sanitary Products (SI Appendix). All procedures were done following the rules of the Declaration of Helsinki Guidelines. The trial is registered under European Clinical Trials Database no. 2013-005165-39 and National Clinical Trial no. NCT02283671. All patients signed a written informed consent before inclusion in the study.

Study Design. The study was an open-label, single-center, multiple ascending-dose phase 1b clinical trial of 3 mo of follow-up (SI Appendix, Fig. S1). We included a total of 12 patients: 8 with MS and 4 with NMOSD. The trial was performed at the Hospital Clinic of the University of Barcelona, and patient recruitment was performed between September 2015 and July 2017.

Participants were assigned to four blocks of three patients each (two MS and one NMOSD per block), and they were consecutively included once the safety of the previous cohort of patients was confirmed based on the absence of serious adverse events in that previous dose after 3 mo of follow-up.

Intravenous administration of the cells was done slowly over 1 h, after resuspension of the product in physiological saline solution. The cell dose escalation was $5 \times 10^6$, $100 \times 10^6$, $150 \times 10^6$, and $300 \times 10^6$ tolDCs in total, separated in three independent doses administered every 2 wk. The last group did not receive the programmed doses (300 million tolDCs (100 million for each dose)) due to a technical limitation to obtain the required number of cells on the leukapheresis, and for this reason, they received the maximum yield of the cultures, always above 150 million cells in total.

We conducted 1-h monitoring after the administration for safety purposes. Cell viability, sterility, purity, and functionality of the final product were evaluated and registered for each patient and procedure, as previously described (9).

A baseline visit was conducted after patients fulfilled the inclusion criteria and signed the informed consent. Brain MRI and an optical coherence tomography (OCT) were performed at baseline and by week 12, as previously described (14). Blood tests for analyses and immunological assessment were done every 2 wk.

The neurological examination included the Expanded Disability Status Scale (EDSS) (15) and the Multiple Sclerosis Functional Composite (MSFC) (16), which were done at baseline and by week 12. Quality of life was assessed with the Short Form-36 questionnaire at baseline and end of follow-up (17).

Primary and Secondary End Points. The primary end point of the study was to evaluate the safety and tolerability of the treatment. Safety and tolerability were determined by the number and severity of adverse effects (AEs) related to tolDC administration using the MedDRA dictionary (https://www.meddra.org). Secondary end points were (i) assessment of clinical efficacy in terms of relapse rate and changes in the disability scales (EDSS and MSFC); (a) remaining relapse-free by week 12; (b) change on EDSS from baseline to week 12; and (c) change of MSFC from baseline to week 12; (ii) assessment of imaging efficacy: (a) number of new gadolinium-enhancing lesions at week 12; (b) number of new/enlarging T2-weighted fluid-attenuated inversion recovery (T2-FLAIR) lesions at week 12; and (c) changes in peripapillary retinal nerve fiber layer and macular volume (MV) from baseline to week 12; and (iii) changes in the immune responses (cytokine production and cell proliferation) to peptides and differential immune cell counts at week 12.

Inclusion and Exclusion Criteria. Inclusion criteria were (i) patients diagnosed with MS based on the 2010 McDonald criteria (18) or NMOSD with anti-AQP4 antibodies based on Wingerchuk criteria (19); (ii) disease duration $\geq 1$ y; (iii) age between 18 and 65 y; and (iv) EDSS between 3 and 8.5. All NMOSD patients were required to have anti-AQP4 antibodies. Excluding criteria were (i) presence of a relapse or use of steroids during the month before the screening; (ii) use of immunomodulatory/immunosuppressive therapy in the case of MS in the last 12 mo, but NMOSD patients were allowed to maintain their immunomodulatory treatment stable for 6 mo for ethical requirements (20); (iii) impossibility to perform brain MRI with gadolinium contrast, severe systemic diseases, or history of personal cancer or hereditary familial cancer; (iv) impossibility to proceed to the leukapheresis (e.g., absence of peripheral vein access); (v) participation in other experimental studies within the previous 3 mo; and (vi) pregnant or breastfeeding women.

Generation of Tolerogenic Dendritic Cells. Autologous monocyte-derived dendritic cells (DCs) were obtained by leukapheresis (Amicus; Fresenius Kabli), and tolDCs were generated under good manufacturing practice (GMP) conditions by conditionizing monocyte-derived DCs, as previously described (9, 21), and reported following minimum tolerogenic information about tolerogenic antigen-presenting cells criteria (22). Briefly, monocytes were cultured in the presence of 500 IU (international units)/mL of IL-4 and 800 IU/mL of GM-CSF (Miltenyi Biotec) during 7 d in X-VIVO-15 (BioWhittaker; Lonza) supplemented with 2% autologous serum. At day 3, fresh medium and cytokines were added; furthermore, 10–8 M dexamethasone (Merck) was added to the cells to induce the tolerogenic phenotype. At day 6, to boost peptide-specific DC tolerogenic properties, a mixture of cytokines including IL-1β, IL-6, TNF-α, and prostaglandin E2 (Miltenyi Biotec), as well as the immunogenic peptides (SI Appendix, Table S1), were added for 24 h and washed before administration. DCs from MS patients were stimulated with the seven myelin peptides from myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (described in ref. 11), and DCs from NMOSD patients were stimulated with a rheumatoid arthritis (RA)-specific cell immunogenic peptide from AQP4, as described in ref. 23. The peptides MBP13-32 (MBP1), MBP33-99 (MBP2), MBP111-129 (MBP3), MBP146-170 (MBP4), MOG1-21 (MOG1), MOG25-55 (MOG2), PLP139-154 (PLP1), and AQP463-75 were produced in GMP conditions (Hemmo Pharmaceuticals) and added at 5 μM to the cultures. After sterility testing and phenotype and purity characterization, tolDCs were administered by i.v. injection. Progressively increasing total DCs doses were injected in all of the cohorts (5 $\times 10^4$, 100 $\times 10^4$, 150 $\times 10^4$, and 300 $\times 10^4$ DCs) divided into three consecutive doses at weeks 0, 2, and 4 (SI Appendix, Fig. S1).

Characterization of tolDC Phenotype and Cytokine Production. tolDCs from each patient were characterized assessing for their phenotype, cytokine production, and immunogenicity. As depicted in SI Appendix, Fig. S2A, clinical grade-produced tolDCs presented semimature phenotype (lower expression of maturation marker CD83 and MHC) compared with mature DCs and increased expression of the glucocorticoid-induced receptor MERTK, which was described as a marker for tolDCs (24). Moreover, clinical grade-generated tolDCs produced higher amounts of the immunosuppressive cytokine IL-10 at baseline and after LPS challenge compared with mature immunogenic DCs (SI Appendix, Fig. S2B). Neither IL-12 nor IL-23 was produced by tolDCs (data not shown).

MRI. MRI examinations were performed on a 3T Siemens Trio MRI scanner at the Hospital Clinic of the University of Barcelona, as previously described (14). Scans were performed at baseline and at week 12 of follow-up. A 1.5T MRI scanner was used for patient MS01 because she has an implanted abdominal

Significance

Application of antigen-specific immune tolerance in autoimmune disease is a long-sought goal. We studied diseases with abundant information on the autoimmune target: in multiple sclerosis (MS), various myelin antigens are known targets of T cells and antibodies, whereas in neuromyelitis optica (NMO), the aquaporin-4 channel is attacked by T cells and antibodies. We tested whether engineered dendritic cells might induce a tolerogenic immune response in these two conditions. In this in-human clinical study, individual regulatory T cells, secreting IL-10, a key tolerogenic cytokine, were detected after treatment. These results might lead to more extensive trials with this approach in autoimmune conditions where the antigenic target has been identified, including MS, NMO, myasthenia gravis, and Graves disease.

Funding

This work was supported by Instituto de Salud Carlos III (ISCIII) Grant RD12/0036/0032 and the Spanish Ministry of Economy and Competitiveness. G.P.-P. was a recipient of the Spanish Ministry of Economy and Competitiveness Grant FPI. The authors declare no competing interests.
stimulator, which prevented the use of a high-field scanner. The sequences obtained were T1-magnetization prepared rapid gradient echo before and spin echo after gadolinium administration and T2-FLAIR sequences as previously described (14).

OCT. OCT was performed using a spectral domain device (Spectralis; Heidelberg Engineering), as previously described (14), and performed before therapy initiation and by week 12 follow-up. We calculated the thickness of the peripapillary retina nerve fiber layer thickness and MV from each eye at baseline and week 12 following criteria as previously described (25).

Flow Cytometry Analysis. Different immune cell subsets frequencies were analyzed from the patient’s peripheral blood mononuclear cells (PBMCs). The antibody panels for flow cytometry were (i) PBMC subtypes CD3, CD4, CD9, CD14, and CD56; (ii) T-lymphocyte subpopulations; (iii) Treg subsets: natural Tregs (CD4+CD25+Foxp3+) and Tr1 (CD4+IL-10+); (iv) T-helper subpopulations: Th1 (CD4+IFN-γ+), Th2 (CD4+IL-4+), and Th17 (CD4+IL-17+); (v) CD4 and CD8 GM-CSF “encephalitogenic” T cells; and (vi) V-cell subtypes by activation memory phenotype: CD4+CD161+ and CD8+CD161+.

The list of antibodies and panels is shown in SI Appendix, Table S2. Cells were analyzed in a Canto cytometer and analyzed using the FACSdiva software.

Antigen-Specific Proliferation. PBMCs 2 × 10^5 were cultured in a 96-well round plate in 200 μL of X-VIVO 15 medium (BioWhittaker; Lonza) supplemented with 2% AB human serum (Sigma-Aldrich). Cells were stimulated with each plate in 200 μL of supernatant from each condition was collected and frozen at −80 °C. Afterward, the plates were frozen until being read. Thymidine incorporation was measured using a Wallac Beta Liquid Scintillation counter (Perkin-Elmer). Measurements were obtained as cpm and calculated the stimulation index (SI) as cpm peptides/cpm unstimulated (26).

ELISA. Supernatant from PBMCs cultures was collected after 6 d of stimulation and analyzed by ELISA the production of IFN-γ, IL-17, and IL-4 (U-Cytech) were performed after 2-d stimulation of PBMCs with each individual peptide, according to the manufacturer’s guidelines. Results are reported as pg/mL.

Enzyme-Linked Immunospot Assay. Enzyme-linked immunospot (ELISPOT) assays for IFN-γ, IL-17, and IL-4 (U-CyTech) were performed after 2-d stimulation of PBMCs with each individual peptide, according to the manufacturer’s guidelines. Peptides were added as 1:10 dilution of the 5 mM peptide stimulus, and 2 μL of each peptide was added to each well. Results are reported as the number of cells with spots for a given cytokine, as described in refs. 27 and 28. Stimuli well spot number = (mean of three wells for each stimulus) – (mean of three wells of negative control) for 200,000 PBMCs per well.

HLA-DRB1* Typing. DNA HLA-DRB1* typing was conducted at the Department of Immunology of our center using high-resolution PCR.

Table 1. Demographic and clinical variables of the cohort

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age, y</th>
<th>MS subtype</th>
<th>Duration, y</th>
<th>EDSS</th>
<th>Previous DMD</th>
<th>Concomitant DMD</th>
<th>HLA DRB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS01</td>
<td>F</td>
<td>48</td>
<td>SPMS</td>
<td>14.79</td>
<td>5.0</td>
<td>MP</td>
<td>NA</td>
<td><em>0101</em>0301</td>
</tr>
<tr>
<td>MS02</td>
<td>F</td>
<td>50</td>
<td>SPMS</td>
<td>9.67</td>
<td>6.0</td>
<td>IFN-γ1a s.c.</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MS03</td>
<td>M</td>
<td>27</td>
<td>PPMS</td>
<td>6.6</td>
<td>6.0</td>
<td>GA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MS04</td>
<td>F</td>
<td>57</td>
<td>PPMS</td>
<td>4.29</td>
<td>5.5</td>
<td>NA</td>
<td>NA</td>
<td><em>1501</em>1301</td>
</tr>
<tr>
<td>MS05</td>
<td>M</td>
<td>40</td>
<td>SPMS</td>
<td>8.18</td>
<td>6.5</td>
<td>GA, IFN-γ1a s.c.</td>
<td>NA</td>
<td><em>0701</em>1001</td>
</tr>
<tr>
<td>MS06</td>
<td>M</td>
<td>57</td>
<td>SPMS</td>
<td>29.29</td>
<td>6.0</td>
<td>IFN-γ1a i.m.</td>
<td>NA</td>
<td><em>1104</em>1301</td>
</tr>
<tr>
<td>MS07</td>
<td>F</td>
<td>56</td>
<td>PPMS</td>
<td>7.46</td>
<td>6.5</td>
<td>GA</td>
<td>NA</td>
<td><em>0102</em>0404</td>
</tr>
<tr>
<td>MS08</td>
<td>M</td>
<td>59</td>
<td>RRMS</td>
<td>1.81</td>
<td>6.0</td>
<td>IFN-γ1a s.c.</td>
<td>NA</td>
<td><em>0101</em>1101</td>
</tr>
<tr>
<td>NMO01</td>
<td>M</td>
<td>38</td>
<td>NA</td>
<td>3.37</td>
<td>3.5</td>
<td>AZA, RTX, IFN-γ1a s.c.</td>
<td>RTX</td>
<td><em>03</em>13</td>
</tr>
<tr>
<td>NMO02</td>
<td>F</td>
<td>39</td>
<td>NA</td>
<td>21.91</td>
<td>6.0</td>
<td>AZA, RTX, PDN, IVIG, MTX</td>
<td>MMF</td>
<td>NA</td>
</tr>
<tr>
<td>NMO03</td>
<td>F</td>
<td>40</td>
<td>NA</td>
<td>3.22</td>
<td>4.5</td>
<td>RTX, MP</td>
<td>RTX</td>
<td><em>0404</em>1001</td>
</tr>
<tr>
<td>NMO04</td>
<td>F</td>
<td>43</td>
<td>NA</td>
<td>8.57</td>
<td>4.0</td>
<td>RTX, CP, CyA, IVIG, MP</td>
<td>RTX</td>
<td><em>0301</em>1101</td>
</tr>
</tbody>
</table>

AZA, azathioprine; CP, cyclophosphamide; CyA, cyclosporine A; F, female; GA, glatiramer acetate; IVIG, i.v. immunoglobulins; M, male; MMF, mycophenolate mofetil; MP, methylprednisolone; MTX, methotrexate; NA, not available; PDN, prednisone; PPMS, primary-progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; RTX, rituximab; SPMS, secondary-progressive multiple sclerosis.
observe significant changes in retinal thicknesses measured by OCT (SI Appendix, Table S3).

**Immunological Responses After tolDC Therapy.** First, we evaluated changes in the antigen-specific cell proliferation induced by tolDCs by assessing [3H]thymidine incorporation in response to each peptide or the pool of peptides. We did not find significant differences on T-cell proliferation by week 12 after treatment for any of the peptides compared with baseline, although we found a trend for decreased proliferation in response to the AQP4 peptide by week 12 in NMOSD-treated patients ($P = 0.067$) (Fig. 1).

Second, we evaluated changes in cytokine secretion in response to specific peptide stimulation in PBMC culture supernatants by ELISA (INF-$
\gamma$, IL-17, IL-4, and IL-10). We found a significant increase of IL-10 production by week 12 compared with baseline for all myelin peptides (except MBP1) in patients with MS as well as for AQP4 peptides in patients with NMOSD (Fig. 2). IL-10 production has been previously described as associated with the induction of Tr1 cells without memory phenotype (CD8 $\text{CD161}^+$). We observed an upward trend in the frequency of Tr1 cells (CD4+$\text{CD161}^+$; $P = 0.14$) and a significant decrease in memory CD8 (CD8+$\text{CD161}^+$; $P = 0.021$), NK cells (CD56+CD161+ ($P = 0.012$) and CD14+CD56+ ($P = 0.041$)) in MS patients by week 12 compared with baseline, while the remaining subpopulations did not show significant changes (Fig. 4).

**Discussion**

In this trial, we have shown feasibility and safety of treating patients with MS and NMOSD with tolDCs loaded with either myelin antigens or AQP4 antigens at the highest dose of feasible by tolDCs (5, 29). Regarding changes in cytokine production by ELISPOT, we did not find significant differences for any of the cytokines analyzed at the group level (Fig. 3).

Finally, we assessed whether tolDC therapy induced changes in peripheral immune cell subset frequencies from baseline to week 12 after, including CD4 (Th1, Th2, Th17, naïve vs. memory cells, GM-CSF-expressing cells), Treg (natural Treg and Tr1), CD8, monocytes (CD14), B cells (CD19), and NK (CD56) cells, with or without memory phenotype (CD161). We observed an upward trend in the frequency of Tr1 cells (CD4+$\text{IL-10}^+$; $P = 0.14$) and a significant decrease in memory CD8 (CD8+$\text{CD161}^+$; $P = 0.021$), NK cells (CD56+CD161+ ($P = 0.012$) and CD14+CD56+ ($P = 0.041$)) in MS patients by week 12 compared with baseline, while the remaining subpopulations did not show significant changes (Fig. 4).

**Table 3. Disability assessed with the EDSS and MSFC from baseline to week 12**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>EDSS Baseline</th>
<th>T25FW Baseline</th>
<th>9HPT Baseline</th>
<th>Average Baseline</th>
<th>PASAT3 Baseline</th>
<th>MSFC Baseline</th>
<th>EDSS Week 12</th>
<th>T25FW Week 12</th>
<th>9HPT Week 12</th>
<th>Average Week 12</th>
<th>PASAT3 Week 12</th>
<th>MSFC Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>M502</td>
<td>6.0</td>
<td>31.065</td>
<td>NA</td>
<td>25.365</td>
<td>25.365</td>
<td>5</td>
<td>0.943</td>
<td>6.0</td>
<td>41.450</td>
<td>120.540</td>
<td>22.700</td>
<td>71.620</td>
</tr>
<tr>
<td>M503</td>
<td>6.0</td>
<td>12.035</td>
<td>NA</td>
<td>50.560</td>
<td>14</td>
<td>0.263</td>
<td>6.0</td>
<td>11.845</td>
<td>42.710</td>
<td>20.100</td>
<td>39.600</td>
<td>21.390</td>
</tr>
<tr>
<td>M504</td>
<td>5.5</td>
<td>7.400</td>
<td>25.030</td>
<td>24.855</td>
<td>24.355</td>
<td>48</td>
<td>-0.148</td>
<td>5.5</td>
<td>7.425</td>
<td>31.125</td>
<td>30.040</td>
<td>30.583</td>
</tr>
<tr>
<td>M505</td>
<td>6.5</td>
<td>11.940</td>
<td>27.230</td>
<td>21.760</td>
<td>24.495</td>
<td>39</td>
<td>-0.205</td>
<td>6.5</td>
<td>13.000</td>
<td>30.815</td>
<td>23.620</td>
<td>27.218</td>
</tr>
<tr>
<td>M507</td>
<td>6.5</td>
<td>32.100</td>
<td>22.100</td>
<td>22.750</td>
<td>22.575</td>
<td>37</td>
<td>0.398</td>
<td>6.5</td>
<td>43.350</td>
<td>20.415</td>
<td>23.370</td>
<td>21.893</td>
</tr>
<tr>
<td>NMO01</td>
<td>3.5</td>
<td>4.540</td>
<td>18.790</td>
<td>19.825</td>
<td>19.308</td>
<td>58</td>
<td>0.247</td>
<td>3.5</td>
<td>4.795</td>
<td>18.705</td>
<td>20.535</td>
<td>19.620</td>
</tr>
<tr>
<td>NMO02</td>
<td>6.0</td>
<td>8.725</td>
<td>22.565</td>
<td>23.180</td>
<td>22.873</td>
<td>39</td>
<td>-0.384</td>
<td>6.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NMO03</td>
<td>4.5</td>
<td>7.050</td>
<td>35.205</td>
<td>34.685</td>
<td>34.954</td>
<td>58</td>
<td>0.459</td>
<td>4.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NMO04</td>
<td>5.0</td>
<td>4.835</td>
<td>22.925</td>
<td>22.935</td>
<td>22.930</td>
<td>45</td>
<td>-0.387</td>
<td>5.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

9HPT, nine hole-peg test; NA, not available; PASAT3, paced auditory serial addition test 3 seconds; T25FW, timed 25 feet walking.
cells. Considering previous findings reporting reactivation of MS activity after altered ligand peptide therapy (30), it is important to highlight that therapy using tolDCs loaded with myelin peptide was safe. The design of the study combined patients with MS and NMOSD, treating both groups for tolerance induction with seven myelin peptides, while adding for patients with NMOSD, the combination of the AQP4 peptide plus the seven myelin peptides in the tolerizing regimen. This design allowed leveraging treatments for both conditions, with a constellation of different myelin antigens, and supplementing the regimen for patients with NMOSD by adding a peptide from AQP4. This allowed us to obtain safety data as a proof of concept for future antigen-tolerance induction clinical trials for both diseases. The trial was not powered for probing efficacy at the clinical level or at the imaging level, and for this reason, no conclusions in terms of efficacy should be raised. Finally, the immunological assessment revealed the induction of Tr1 cells producing high levels of IL-10, as it has been described in previous tolDCs trials (5, 29).

Therefore, we obtained evidence that tolDC therapy induces peptide-specific tolerance by promoting the adaptive regulatory T-cell function. Considering that Tr1 function is impaired in MS (31), augmenting Tr1 function by tolDCs may contribute to restoring peripheral immune tolerance in patients with autoimmune diseases (32–34). Future trials powered to show clinical efficacy would clarify whether the augmentation of the Tr1 response could modify the disease course in MS or NMOSD.

Fig. 1. Peptide-specific T-cell proliferation assays at baseline and week 12. MBP13-32, MBP83-99, MBP111-129, MBP146-170, MOG1-20, MOG35-55, PLP139-156, and AQP463-76-specific T-cell responses, at baseline (before treatment) and 12 wk after tolDC treatment in patients with MS (A) or NMO (B), are shown. Proliferative responses were measured by [3H]thymidine incorporation assay. Graphs (y axis) represent the SI.
Indeed, NMOSD is becoming a promising candidate as a disease that is optimal for testing immune tolerization, considering that the target antigen is well known (AQP4) for this condition. The target antigen AQP4 is actually part of the criteria for making a diagnosis of this disease, making it both a “biomarker” and a target for therapy. In addition, the clinical severity of NMOSD makes a case for development of new therapies that are effective and safe. Targeting the major pathologic immune response seems a compelling strategy.

The improved knowledge and fine mapping of AQP4 epitopes and associated HLA types should allow the design of specific tolDC protocols as a path for personalized medicine. Indeed, the fact that in our trial, the combination of peptides from different antigens was well tolerated and induced Tr1 cells, suggests that a pool of AQP4 peptides in different HLA class II contexts might achieve even better results in NMOSD, than those so far seen with one AQP4 epitope.

Regulatory DCs or tolDCs have shown the ability to dampen the immune response and restore cell tolerance in animal models of autoimmune diseases as well as in humans (3, 5, 35–37). TolDCs can be generated in vitro by promoting its differentiation with GM-CSF and IL-4 and inducing the regulatory phenotype with several stimuli such as IL-10, TGF-β, vitamin D3, prostaglandin E2 or dexamethasone among others (35). tolDCs generated with dexamethasone
and other stimuli have a stable phenotype, high expression of HLA class II molecules, intermediate expression of the costimulatory molecules CD80 and CD86, low expression of CD40, express high levels of IL-10 and TGF-β, and low levels of IL-12, IL-23, and TNF-α, overall conferring a low capacity to stimulate T cells, with the capacity to tolerize them (3, 35). Specifically, tolDCs induced with dexamethasone have a low expression of CD1a and CD14, and they express CD209 (34), which has been associated with the increase of Treg cells in patients with Crohn’s disease (9).

Regarding the migratory capacity of DCs, in vivo imaging of pulsed tolDCs in the experimental autoimmune encephalomyelitis model showed that these cells reached the liver and the spleen at 24 h after i.v. injection, and in smaller amounts the lymph nodes, thymus, and bone marrow, and remained stable for 7 d (38). Although it is unknown whether tolDCs are able to migrate to the inflamed CNS in MS and NMOSD, the migration to primary and secondary lymphoid organs suggest they can modulate the peripheral immune response in such lymphoid organs and alter the autoimmune attack to the brain. Regarding the mechanism by which tolDCs exert their tolerogenic effects, they may include contact-dependent mechanisms [e.g., PD-L1, Fas-ligand, ICOS-ligand, Ig-like transcript-2 (ILT-2), ILT-3, ILT4, HLA-G, and others] or contact-independent mechanisms such as immunomodulatory cytokines (e.g., IL-10 and TGF-β) or enzymes that generate immunomodulatory molecules or related to nutrient deprivation (e.g., IDO, heme-oxygenase-1, iNOS, and arginase 1), all mainly mediated by regulatory cells (3). In this study, we have obtained evidence for the induction of IL-10 and Tr1 responses in patients with MS or NMO treated with tolDCs.

Considering the critical role of DCs in the induction and maintenance of central and peripheral tolerance, clinical-grade human tolDCs have been tested in phase 1b clinical trials in type 1 diabetes, rheumatoid arthritis, and Crohn’s disease with evidence for feasibility and safety (6–9). Most of the trials have shown as the main immunological effect an increase in regulatory cells and their related cytokines (10), in agreement with our finding of increased IL-10 production and frequency of Tr1 cells.

The search for immune-tolerance therapies or antigen-specific vaccination to treat autoimmune diseases such as MS or NMOSD is based on the need of more selective immunotherapies designed to restore self-tolerance without causing general immune suppression (1). The probabilistic model of the immune system states that the outcome of the immune response is based on the probability of presenting a given antigen in the appropriate context (pro- or antiinflammatory), showing a remarkable robustness in the preference for pathogen targeted responses compared with autoimmune responses (4). Considering that the immune system exhibits the remarkable ability to detect sudden increases in the abundance of self-antigens [“the 100-fold increase in effective abundance” proposed in the model (4)] within an antiinflammatory environment provided by the tolDCs (5).
In the case of MS, the target antigen is not known yet, although proteins within the myelin sheath, such as MBP, MOG, and PLP, are important targets of the autoreactive immune response (30, 39–41). To develop antigen-specific tolerance for MS, several myelin immunodominant peptides have been identified ex vivo from patients with MS (41, 42) and tested in clinical trials using autologous leukocytes chemically coupled with such peptides (11). Tolerance induction with alpha B-crystallin (HSβB5) has been tested in patients with MS showing good safety as well (43).

In the case of NMO, almost 80% of patients have specific antibodies against AQP4, providing a good target for antigen-specific tolerance-induction strategies (12, 13). As such, the T-cell immunogenic peptide AQP453–76 described by Zamvil and co-workers (23) was used in this trial, because it was the only known human disease-related epitope when we started the trial. New AQP4 epitopes have been described (44, 45), which would be tested in future trials.


In summary, this study shows that therapy with CNS peptide-specific loaded tolDCs is safe in patients with MS and NMO/MS and is able to induce immune tolerance as indicated by the induction of regulatory T cell, Tr1, activity.

ACKNOWLEDGMENTS. We thank Mark Setfon for English revisions, Prof. Jerry Nepom (Besarroya Institute and the Immune Tolerance Network) for his advice in immunological biomarkers, and the Group Affects Esclerosis Multiple (GAEM) Foundation for logistic support for the study. This study was supported by the GAEM Foundation, La Caixa Foundation Grant LC/PR/ G11/2012530001, the Guthy Jackson Charity Foundation, the Instituto de Salud Carlos III (Fondo Europeo de Desarrollo Regional (FEDER) funds “Otra manera de hacer Europa,” Grant PI15/0601) (to P.V. and A.S.); and Red Española de Esclerosis multiple Grants RD16/0015/0002, /0003, and /0015, and the Centres Excellencia Recerca Catalunya Programa/Generalitat de Cataluña. I.Z. is a recipient of the Río Hortega research contracts from the Instituto de Salud Carlos III, Spain (Grant CX06/00113). N.S.-V. received funding from the Instituto de Salud Carlos III, Spain; and FEDER Grant F16/00251, Predoctoral Grant for Health Research.