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# Epitope-Specific Immunotherapy of Rheumatoid Arthritis

## Clinical Responsiveness Occurs With Immune Deviation and Relies on the Expression of a Cluster of Molecules Associated With T Cell Tolerance in a Double-Blind, Placebo-Controlled, Pilot Phase II Trial

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***Objective.*** **Induction of immune tolerance to maintain clinical control with a minimal drug regimen is a current research focus in rheumatoid arthritis (RA). Accordingly, we are developing a tolerization approach to dnaJP1, a peptide part of a pathogenic mechanism that contributes to autoimmune inflammation in RA. We undertook this study to test 2 hypotheses: 1) that mucosal induction of immune tolerance to dnaJP1 would lead to a qualitative change from a proinflammatory phenotype to a more tolerogenic functional phenotype, and 2) that immune deviation of responses to an inflammatory epitope might translate into clinical improvement.**

***Methods.*** **One hundred sixty patients with active RA and with immunologic reactivity to dnaJP1 were enrolled in a pilot phase II trial. They received oral doses of 25 mg of dnaJP1 or placebo daily for 6 months.**

***Results.*** **The dnaJP1 peptide was safe and well-tolerated. In response to treatment with dnaJP1, there was a significant reduction in the percentage of T cells producing tumor necrosis factor $\alpha$ and a corresponding trend toward an increased percentage of T cells producing interleukin-10. Coexpression of a cluster of molecules (programmed death 1 and its ligands) associated with T cell regulation was also found to be a prerequisite for successful tolerization in clinical responders. Analy-**

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**sis of the primary efficacy end point (meeting the American College of Rheumatology 20% improvement criteria at least once on day 112, 140, or 168) showed a difference between treatment groups that became significant in post hoc analysis using generalized estimating equations. Differences in clinical responses were also found between treatment groups on day 140 and at followup. Post hoc analysis showed that the combination of dnaJP1 and hydroxychloroquine (HCQ) was superior to the combination of HCQ and placebo.**

***Conclusion.*** **Tolerization to dnaJP1 leads to immune deviation and a trend toward clinical efficacy. Susceptibility to treatment relies on the coexpression of molecules that can down-regulate adaptive immunity.**

Rheumatoid arthritis (RA) is the most common systemic autoimmune condition worldwide and is more prevalent in women (1). RA is characterized by remittent systemic autoimmune inflammation and painful progressive joint deterioration. The rapid evolution of molecular immunology has changed the way we conceptualize the pathogenesis and treatment of this disease. With the advent of targeted therapies, specific biologic interference with pathogenic pathways, including cytokines and costimulatory molecules, is complementing, and in some cases replacing, generalized immune suppression with cytotoxic drugs or antimetabolites (2–8). Consequently, the focus in clinical research is veering toward early aggressive intervention to achieve remission or low disease activity and to prevent the development of joint erosions and functional limitations. Recent studies indicate that for some patients, disease-modifying antirheumatic drugs (DMARDs) can be withdrawn and low levels of disease activity maintained; however, most patients experience a relapse of their disease unless treatment is continued (9–11). Complementary approaches aimed at maintaining disease control are still needed (12,13), perhaps by shifting the focus from induction of immune suppression to maintenance of immune tolerance (14–19).

Several reports describing the effects of current therapies, particularly biologics, on the regulatory arm of the adaptive immune response, which controls immune tolerance, have been published. For instance, the different effects exerted by various anti–tumor necrosis factor $\alpha$ (anti-TNF$\alpha$) agents on Treg cells were recently reported (20–22). However, tolerization effects from current biologic agents appear to depend on continuous administration; in most cases, clinical control fades with withdrawal of therapy (2–8).

This project explored the ability of epitope-specific immune tolerization to induce immune deviation and clinical responsiveness in a double-blind, placebo-controlled, pilot phase II trial in RA patients. A complementary objective was the identification of the immune requisites that lead to susceptibility to tolerization. Our program targets the induction and maintenance of immune tolerance to dnaJP1, a dominant epitope from the heat-shock protein (HSP) dnaJ, as the tolerogen.

The 15-mer synthetic peptide dnaJP1 is derived from HSP dnaJ. The dnaJP1 peptide also shares homology with the "shared epitope" sequence conferring susceptibility to RA that is present in certain HLA class II alleles. We identified this peptide as a proinflammatory T cell epitope in patients with active RA (23–26). We proposed a pathogenic role for T cell inflammatory responses to HSP dnaJ based on loss of control of mechanisms that modulate cross-recognition of self-epitopes sharing homology with foreign immunogenic proteins. Recognition of the dnaJP1 epitope contributes to the amplification and perpetuation of autoimmune inflammation independently of its original triggers, which is a feature present in responses to various HSP-derived epitopes in several inflammatory autoimmune diseases (16,19,27–33). This reaction is maintained by overexpression of homologous HSP at sites of inflammation, and it is self-limiting under physiologic conditions or in naturally remitting diseases (25). Our intervention aims at restoring this self-regulating ability by inducing mucosal tolerance to a dominant proinflammatory epitope, such as dnaJP1. The independence of the HSP circuit from any primary trigger of autoimmune inflammation is an important conceptual difference from experimental animal models that use the inciting antigen as the tolerogen and from prior attempts in humans at tolerization to purported triggers of RA.

The mechanistic hypothesis for therapeutic intervention was that mucosal induction of immune tolerance to dnaJP1 determines an immune deviation in effector T cells, leading to a qualitative change from a proinflammatory phenotype to a more tolerogenic functional phenotype. The clinical hypothesis was that immune deviation of responses to an inflammatory epitope might translate into clinical improvement.

## PATIENTS AND METHODS

**Patients.** Criteria for enrollment of patients required the concomitant presence of clinically active disease and in vitro immunologic reactivity of their peripheral blood mononuclear cells (PBMCs) to dnaJP1. Patients were ≥18 years old

and had active (6 tender and/or 9 swollen joints) RA of recent onset (≤5 years from onset). A stable dosage of nonsteroidal antiinflammatory drugs or of the DMARDs hydroxychloroquine (HCQ) or sulfasalazine was allowed, as was prednisone up to 10 mg/day. A washout period of 6 weeks was required for all other DMARDs, including methotrexate and biologic agents. Of note, none of the enrolled patients was previously exposed to DMARDs. Exclusion criteria comprised significant cardiac, pulmonary, hepatic, or renal disease as defined by grade ≥2 on the World Health Organization (WHO) toxicity scale (34), at serum creatinine level >1.5 times normal, an alanine aminotransferase level >2.5 times normal, a hematocrit value <30%, a platelet count <130,000/$\mu$l, or a history of lymphoma, any active malignancy, or cancer requiring treatment in the last 5 years, except for nonmelanoma skin cancers and carcinoma of the cervix in situ. Women of childbearing potential were tested for pregnancy before enrollment and had to maintain appropriate contraceptive procedures for the study duration. Patients were enrolled in outpatient clinics at 11 major medical centers in the US and Mexico.

Immunologic PBMC reactivity to dnaJP1 in vitro was defined as a 2% expression above background of interleukin-2 (IL-2), TNF$\alpha$, interferon-$\gamma$ (IFN$\gamma$), or CD69 by CD3+ cells as measured by fluorescence-activated cell sorting or proliferation of PBMCs upon stimulation with dnaJP1. This threshold was determined and validated in preclinical studies and in the phase I trial (35).

**Protocol.** The Data Safety and Monitoring Board (DSMB) and human research committee at each study site approved the protocol and the Manual of Operations and Procedures, which provided standard operating procedures for the trial to all of the sites. All patients gave written informed consent before undergoing a screening evaluation to determine eligibility. Clinical, laboratory, and immunologic assessments were repeated at monthly intervals for the duration of the study and 1 month after the last dosing (followup). Measurements of disease activity included a 68-joint examination for pain/tenderness and a 66-joint examination for swelling, a patient-generated modified Health Assessment Questionnaire (36) and 10-cm horizontal visual analog scales for pain and disease activity, an examiner's global assessment of disease severity, and measurement of erythrocyte sedimentation rate and C-reactive protein level. Adverse events were graded on the standard WHO scale.

**Treatment.** Enrolled patients were randomly assigned a study drug number, and 33 capsules of drug were dispensed monthly. Participants were assigned to take placebo (lactose) or dnaJP1 (25 mg of peptide) by mouth daily for 6 months. The dosage was determined by clinical and immunologic data obtained in phase I studies (35).

**Randomization.** Patients were randomly assigned to receive placebo or dnaJP1 with equal probability, using a permuted-blocks design with a block size of 10. Each center used separate blocks to stratify for that center. The investigational pharmacy at the University of California, San Diego, prepared the randomization table, labeled medication kits with codes, and kept kit codes, which were not made available until database lockup. The data coordinator at the coordinating center enrolled eligible patients in the study and assigned kits to the patients. An independent DSMB monitored safety issues and the general conduct of the study.

**Immunologic studies.** The sequence of peptides used was QKRAAYDQYGHAAFE for dnaJP1 and QYIKANSKFIGITE for tetanus toxoid (TT) 830–843. Flow cytometric studies were done with PBMCs isolated from blood collected on days 0 (T0) and 168 (Tend). Cells were cultured with media alone or with dnaJP1, phytohemagglutinin, or TT. After 24–96 hours (based on screening data), 1 $\mu M$ monensin (GolgiStop; BD PharMingen, San Diego, CA) was added. Four hours later, the cells were washed and first stained for CD3 and CD69 surface markers and then fixed and stained for the intracellular cytokines TNF$\alpha$, IFN$\gamma$, IL-2, IL-4, and IL-10 (antibodies were from BD PharMingen). The appropriate isotype controls were used and subtracted as background. For analysis, the response to media alone was subtracted from the response to the antigen to determine antigen-specific response.

**Immunologic end point.** The immunologic end point was a ≥20% decrease from baseline in the percentage of CD3+ cells producing any TNF$\alpha$, IFN$\gamma$, or IL-2 or an increase of >20% from baseline in either of the tolerogenic cytokines IL-4 or IL-10. These thresholds were identified in preclinical studies and validated in the previous phase I clinical trial (35).

**Clinical end points.** Clinical responses were measured using the American College of Rheumatology (ACR) definition of response in RA (37). A clinical responder was defined as a patient meeting the ACR 20% improvement criteria (achieving an ACR20 response) at least once on day 112, 140, or 168 (or at followup). The primary end point was predetermined as the area under the curve (AUC) obtained by adding the ACR20 response codes (0 or 1) for visits on days 112, 140, and 168 (AUC 112–168) by Cochran-Mantel-Haenszel test. The secondary end point consisted of the percentage of patients with an ACR20 response on day 112.

**Clinical and immunologic safety.** Patients were evaluated monthly for clinical and laboratory signs of toxicity. Adverse events were scored according to the standard WHO scale. To determine whether dnaJP1 therapy affected the general immune response, we analyzed the in vitro reaction of patient PBMCs to the recall antigen TT.

**Statistical analysis.** Randomization was stratified by clinical center, and end point analyses used an intent-to-treat approach, adjusting for intercenter variability. The primary results assume that dropouts or missed visits have no ACR-N (ACR20, ACR50, ACR70) response (38), and we therefore score them as zero. All analyses shown are based on the total randomized cohort of 160 patients. Descriptive statistics provided summary information for the selection and characteristics of the enrolled patients. Differences in baseline data were analyzed with Mann-Whitney U tests and Fisher's exact tests. The primary analyses were conducted with Cochran-Mantel-Haenszel tests as planned in the original protocol. Due to sizable differences in clinical effect between centers, additional analyses were carried out by using logistic regression and generalized estimating equation (GEE) methods with adjustments for center. GEEs enable the use of repeated observations on each subject at each time point taking into account the correlation within subject, as opposed to the AUC approach, which counts successes over the entire interval. The GEEs are similar to the nonparametric approach provided by the Cochran-Mantel-Haenszel tests, but they often yield smaller $P$ values due to the model assumptions (39,40). Hence, adjustments to the $P$ values were applied to account for this potential

**Table 1.** Baseline demographic and clinical characteristics of the 160 randomized patients*

| | Placebo-treated group (n = 79)† | dnaJP1-treated group (n = 81)‡ |
|---|---|---|
| Age, % | | |
| <40 years | 22.8 | 25.9 |
| 40–60 years | 58.2 | 51.9 |
| >60 years | 19.0 | 22.2 |
| Weight, kg | 77.7 ± 21.4 | 78.2 ± 18.5 |
| Female, % | 83.5 | 74.1 |
| Race/ethnicity | | |
| Black | 1.3 | 3.7 |
| White | 70.9 | 71.6 |
| American Indian | 2.5 | 0 |
| Asian | 1.3 | 2.5 |
| Hawaiian/other Pacific | 0 | 0 |
| Hispanic/Latino | 21.5 | 22.2 |
| Other | 2.5 | 0 |
| Disease duration, years | 1.66 ± 1.43 | 1.63 ± 1.43 |
| RF titer, IU/ml | 126.3 ± 182.4 | 136.9 ± 239.8 |
| HLA–DRB1*04 positive, % | 51.9 | 41.9 |
| No. of tender joints, range 0–68 | 19.9 ± 14.1 | 21.5 ± 15.2 |
| No. of swollen joints, range 0–66 | 13.0 ± 7.5 | 13.2 ± 7.1 |
| Patient's assessment of pain, 0–100-cm scale | 39.5 ± 24.1 | 43.7 ± 24.2 |
| Patient's global assessment of disease activity, 0–100-cm scale | 41.6 ± 28.7 | 42.0 ± 24.9 |
| Physician's global assessment of disease activity, 0–11-cm scale | 35.2 ± 18.3 | 34.7 ± 17.1 |
| M-HAQ disability index, 0–100-cm scale | 0.42 ± 0.42 | 0.45 ± 0.48 |
| ESR, mm/hour | 24.3 ± 19.8 | 23.3 ± 20.0 |
| CRP, $\mu$g/ml | 3.5 ± 8.5 | 1.6 ± 2.3 |
| Morning stiffness, minutes | 99.2 ± 240.5 | 103.5 ± 227.9 |

* Except where indicated otherwise, values are the mean ± SD. No significant differences were found between the groups. ESR = erythrocyte sedimentation rate.
† Seventy-seven patients were tested for rheumatoid factor (RF), 74 were tested for HLA–DRB1*04 positivity, 78 provided an assessment of pain, and 65 provided a global assessment of disease activity.
‡ Seventy-eight patients were tested for HLA–DRB1*04 positivity, 64 provided a global assessment of disease activity, 80 were administered the disability index of the modified Health Assessment Questionnaire (M-HAQ), 80 were tested for C-reactive protein (CRP) level, and 79 were asked for their duration of morning stiffness.

error. Clinical results here are presented with both Cochran-Mantel-Haenszel and adjusted GEE values.

Differences in cytokine levels at each time point as well as differences between groups based on changes from baseline were assessed by *t*-tests. Analyses were conducted using software from SAS Institute (Cary, NC), GraphPad Software (San Diego, CA), and iStudy (San Diego, CA).

## RESULTS

**Participant flow and recruitment and baseline data.** We screened 257 patients from August 10, 2000 to February 22, 2005. PBMCs from 194 of those patients were responsive in vitro to dnaJP1 (75.49%), and 160 of those 194 patients were enrolled (further information is available online at http://albanilab.arthritis.arizona.edu/figure.html). Baseline demographic and clinical characteristics and concomitant medications were similar in both the placebo- and dnaJP1-treated groups (Table 1). Eighty-one patients received 25 mg of the peptide by mouth every day for 6 months, and 79 patients received a corresponding dose of inert placebo (i.e., lactose) daily for 6 months. The study was completed by 114 patients, 61 (75%) in the dnaJP1-treated group and 53 (67%) in the placebo-treated group (further information is available online at http://albanilab.arthritis.arizona.edu/figure.html).

**End points.** The objective of detecting both immunologic and clinical effects of the treatment inspired the design of the trial. Hence, patients were enrolled based on the presence of both clinical disease activity and proinflammatory T cell responsiveness in vitro to dnaJP1 at the screening visit.

*Immunologic.* The percentage of CD3+ cells producing TNF$\alpha$ in response to dnaJP1 in vitro decreased significantly at the end of treatment in the dnaJP1-treated group compared with the value at trial initiation (Figure 1). None of the other cytokines studied showed a significant change from baseline in the dnaJP1-treated group (data not shown). Of note, the

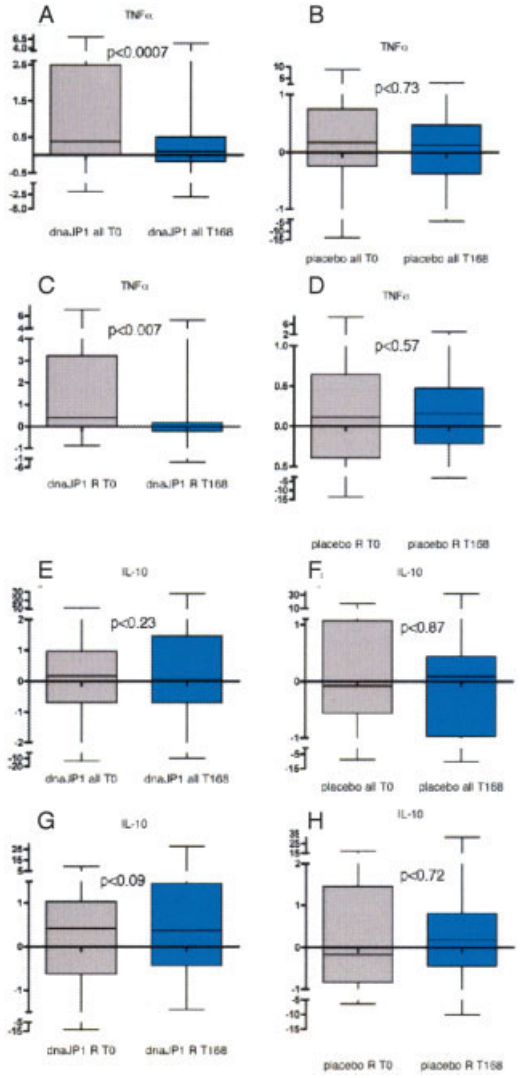


**Figure 1.** Percentage of CD3+ T cells producing tumor necrosis factor $\alpha$ (TNF$\alpha$) **(A–D)** or interleukin-10 (IL-10) **(E–H)** in response to stimulation in vitro with dnaJP1. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the error bars represent the minimum and maximum values. An intracellular staining assay was used, and the readout was performed using fluorescence-activated cell sorting analysis. The appropriate isotype controls were used and subtracted as background. For analysis, the response to media alone was subtracted from the response to the antigen to determine the level of antigen-specific responses. A clinical responder (R) is defined (per the trial Manual of Operations and Procedures protocol) as a patient meeting the American College of Rheumatology 20% improvement criteria at least once on day 112, 140, or 168 (or at followup). *P* values are for comparisons within the same group between day 0 (T0) and day 168 (T168). all = all patients.

percentage of CD3+ cells producing IL-10 in response to dnaJP1 in vitro in the dnaJP1-treated group showed a trend toward an increase at the end of treatment compared with the value at trial initiation (Figure 1). The increase in IL-10 production was more pronounced, although not significantly, when treated patients were stratified according to clinical response, thus indicating that the decrease in TNF$\alpha$ and the concomitant increase in IL-10 in clinical responders may be related. These changes were not present to the same degree when placebo-treated patients were stratified according to clinical responses (Figure 1). The concept that the changes in TNF$\alpha$ and IL-10 production are linked and are associated with clinical responsiveness was further corroborated when an analysis of the correlation between the changes in TNF$\alpha$ and IL-10 production showed a statistically significant ($P = 0.04$) inverse correlation in the dnaJP1-treated group ($r = -0.29$) but not in the placebo-treated group ($r = 0.01$).

The immune deviation observed was not only treatment specific but was also peptide specific. No significant differences in the production of TNF$\alpha$ by CD3+ T cells were found between the groups in response to TT 830–843, a main epitope of TT, thus indicating that the ability to react to recall antigens is not negatively affected by epitope-specific tolerization (data not shown).

A group of dnaJP1-treated patients did not respond clinically to the treatment but showed an immune deviation measured as a reduction in TNF$\alpha$ production at Tend similar to that observed in the clinical responders. However, production of IL-10 in all dnaJP1-treated patients did not increase to levels comparable with those in the dnaJP1-treated responders (Figure 1). This led to the hypothesis that mechanisms associated with T cell regulation and anergy may be necessary at baseline to induce clinically effective tolerization. We focused on a panel of regulatory molecules whose expression contributes to regulating the intensity and quality of T cell responses. PBMCs at the time of enrollment were obtained from comparable numbers of dnaJP1-treated clinical responders and nonresponders. PBMCs were stimulated in vitro with dnaJP1 for 48 hours. Complementary DNA was synthesized and preamplified for the genes of interest with the TaqMan Pre-Amp Master Mix (Applied Biosystems, Foster City, CA), and expression of genes was measured by TaqMan. When PBMCs were stimulated with dnaJP1, expression of programmed death 1 (PD-1), programmed death ligand 1 (PDL-1; or B7-H1), PDL-2 (or B7-DC), CTLA-4, and FoxP3 was significantly more elevated at enrollment in clinical responders than in nonresponders (Figure 2), thus indicating that clinical responsiveness to tolerization re-

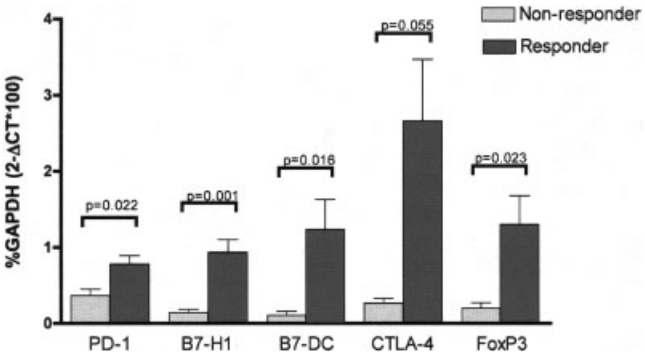


**Figure 2.** Gene expression of regulatory molecules in clinical responders and nonresponders. Peripheral blood mononuclear cells (PBMCs) had been obtained from patients at the time of enrollment. Of these patients, those who had been treated with dnaJP1 were determined on day 168 (or at followup) to be clinical responders (n = 13) or nonresponders (n = 10). PBMCs were incubated in vitro with dnaJP1 for 48 hours, and the cell pellets were lysed for mRNA isolation and cDNA synthesis. Complementary DNA was synthesized and preamplified, and expression of genes was measured by TaqMan. The results were analyzed as a percentage of GAPDH. Values are the mean and SD. *P* values were obtained by unpaired *t*-test. PD-1 = programmed death 1; B7-H1 = programmed death ligand 1 (PDL-1); B7-DC = PDL-2.

quires not only immune recognition of the antigen, but also a higher representation of immune pathways that directly relate to T cell anergy and tolerance even before therapy initiation.

*Clinical*. When we evaluated the clinical effects of the treatment, a progressive separation between the dnaJP1-treated and placebo-treated groups for the ACR20 and ACR50 responses was evident after day 112, consistent with a process of active immune tolerization; the separation widened at the followup point, a possible consequence of a combination of persistent effects of the drug (Figures 3A and B) and waning of the high placebo effect. Results are shown with missing data considered as failures. This often results in an underestimation of the therapy effect. No inputs or extrapolations such as the last observation carried forward were allowed. It also should be noted that, to explore the effects of dnaJP1 as a first-line agent in early RA, we excluded concomitant medications such as methotrexate, which are commonly used in clinical trials and often increase clinical responses significantly.

The treatment appeared to be safe and well-tolerated, with self-remitting leukopenia being noted in 6 patients (further information is available online at http://albanilab.arthritis.arizona.edu/figure.html). The a priori primary end point (AUC 112–140–168) found more patients benefiting, although not significantly, from dnaJP1 based on Cochran-Mantel-Haenszel analy-

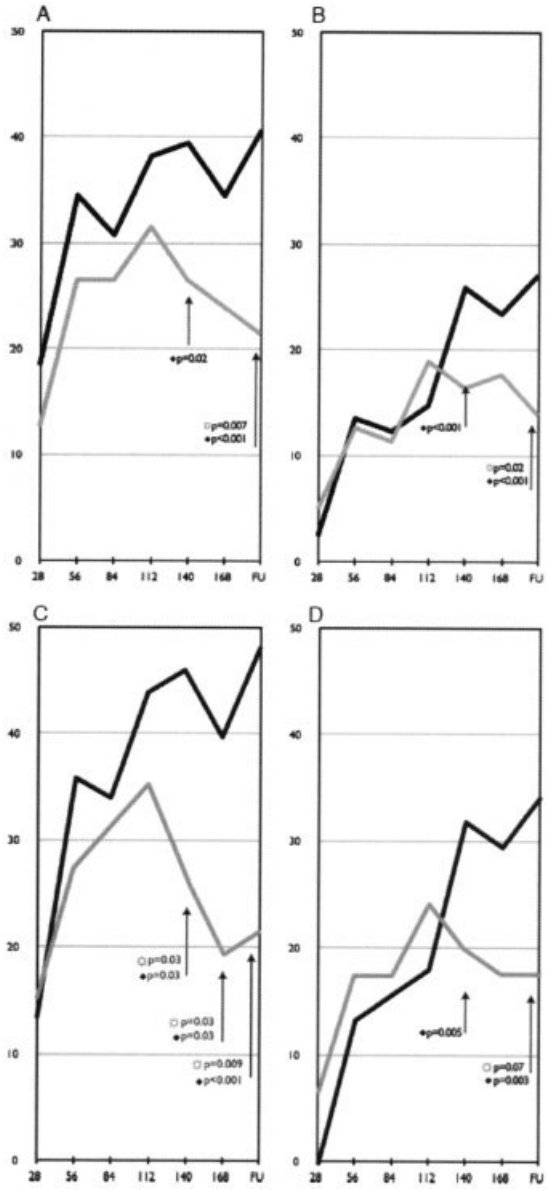


**Figure 3.** Percentage of patients meeting the American College of Rheumatology 20% improvement criteria (achieving an ACR20 response) (**A** and **C**) or achieving an ACR50 response (**B** and **D**) on different visit days (x-axes) throughout the study. Treatment lasted for 6 months. **A** and **B,** Percentage of ACR responders in the dnaJP1-treated group (n = 81; black lines) and in the placebo-treated group (n = 79; gray lines). **C** and **D,** Percentage of ACR responders within the hydroxychloroquine user subgroup in the dnaJP1-treated group (n = 45) and in the placebo-treated group (n = 46). All comparisons are of the dnaJP1-treated group with the placebo-treated group. *P* values next to open circles were obtained by the Cochran-Mantel-Haenszel test. *P* values next to solid diamonds were obtained by adjusted generalized estimating equation methods. FU = followup time point.

sis ($P = 0.09$). Since response rates were unequal across the centers, we used the adjusted GEE method to account for these differences. Since randomization was conducted within each center, this approach allowed the use of an individual patient's response over time within a center. Analysis of the primary end point by the adjusted GEE method rendered a $P$ value of 0.04. When considering whether a patient ever achieved an ACR20 response as adjusted for center, the Cochran-Mantel-Haenszel test showed an adjusted common odds ratio of 1.43 (95% confidence interval [95% CI] 0.97–2.12), with an adjusted $P$ value of 0.065 and with a $P$ value of 0.03 by the adjusted GEE method.

When individual time points were evaluated (using GEEs), significant differences between dnaJP1- and placebo-treated groups were found on day 140 for both ACR20 ($P = 0.02$) and ACR50 ($P < 0.001$) responses (Figures 3A and B). Of the dnaJP1-treated patients, 40.7% achieved an ACR20 response at followup (Tend) compared with 21.5% of placebo-treated patients (proportional differentiation 19.2%; $P = 0.007$ by Cochran-Mantel-Haenszel test and $P < 0.001$ by the adjusted GEE method) (Figure 3A). The AUC of the ACR20 response on days 112, 140, and 168 and at followup was significantly higher in the dnaJP1-treated group than in the placebo-treated group ($P = 0.03$ by Cochran-Mantel-Haenszel test and $P = 0.007$ by the adjusted GEE method).

**Potential synergy between epitope-specific therapy and concomitant HCQ use.** Significant differences were found between dnaJP1-treated patients (n = 45) and placebo-treated patients (n = 46) within the HCQ user subgroup (Figures 3C and D). These 2 groups were homogeneous in number, demographics, clinical characteristics at baseline, and concomitant therapies. The duration of HCQ therapy and the dosage of HCQ were comparable between the 2 groups. The AUC 112–168 for an ACR20 response reached a $P$ value of 0.04 by the Cochran-Mantel-Haenszel test and a $P$ value of 0.02 by the adjusted GEE method. Inclusion of the followup time point in the AUC yielded a $P$ value of 0.02 by the Cochran-Mantel-Haenszel test and a $P$ value of 0.002 by the adjusted GEE method for an ACR20 response. When other time points were assessed, day 140 showed significant differences between groups for both an ACR20 response ($P = 0.03$) and an ACR50 response ($P = 0.005$) by the adjusted GEE method. Analysis of data from day 168 also showed significant differences between groups for an ACR20 response ($P = 0.03$ by both the Cochran-Mantel-Haenszel test and the adjusted GEE method). In the group of dnaJP1-treated patients who were also taking HCQ, 48.9% achieved an ACR20 response at the followup time point ($P = 0.009$ by the Cochran-Mantel-Haenszel test and $P < 0.001$ by the adjusted GEE method), and 33.3% achieved an ACR50 response at the followup time point ($P = 0.07$ by the Cochran-Mantel-Haenszel test and $P = 0.003$ by the adjusted GEE method) (Figures 3C and D).

## DISCUSSION

This pilot phase II trial was designed to test whether immune tolerization to dnaJP1 could safely be translated into a signal of clinical improvement strong enough to encourage followup studies. The trial met 2 general objectives. The treatment was safe and well-tolerated, and it induced immune deviation, as measured by a decline in the production in vitro of the proinflammatory cytokine TNF$\alpha$ and a corresponding increase of the tolerogenic cytokine IL-10. These associations were not found in the placebo group, thus indicating that certain aspects of the immune tolerization process may attain the role of immunobiomarkers as a useful tool for additional studies. Significantly elevated expression of PD-1, CTLA-4, and their ligands was found before initiation of therapy in those patients who responded.

Despite the fact that the primary end point based on protocol-predetermined Cochran-Mantel-Haenszel analysis was narrowly missed, additional analyses taking into account intercenter variability provided evidence for clinical effect. A progressive separation between the dnaJP1-treated group and placebo-treated group ACR responses was present after an induction period, consistent with a process of active immune tolerization. The clinical effects remained evident at the followup point, suggesting a prolonged effect of active immune tolerization.

Post hoc analysis showed a potentially synergistic effect between epitope-specific therapy and HCQ. We are aware that this trial was not designed a priori to study the effects of the combination of dnaJP1 and HCQ and that post hoc subgroup analysis bears a risk of yielding false-positive results; however, there are objective reasons to support the hypothesis of a potential synergistic effect. First, due to the size and homogeneity of the groups treated with HCQ and either dnaJP1 or placebo, the 2 groups resemble the result of an intended randomization. Second, the effects seen are consistent in all clinical and immunologic parameters.

The potential clinical synergy observed may be the outcome of an efficient cooperation in the mechanisms of action. Our preliminary mechanistic data (results not shown) strongly suggest that treatment with HCQ induces the concomitant activation of a cluster of

immune pathways pertaining to immunologic tolerance and regulation. Hence, concomitant or preceding treatment with HCQ may induce the immune functional phenotype necessary for response to epitope-specific immunotherapy. This may be a prerequisite for a successful tolerogenic treatment regimen. As known by practitioners and shown by the results in the placebo-treated group, HCQ alone does not have much clinical effect but could be considered in a novel role as an adjuvant for successful immune intervention.

The target of our intervention is an immune circuit, based on recognition of HSP-derived epitopes. Immune recognition of HSP is perceived as a signal of "danger"; this circuit is part of a primary line of immune defense toward pathogens, which leads to a violent proinflammatory default response aimed at clearing the perceived bacterial infection (27,28,33). Overexpression of homologous HSP at the site of inflammation maintains this reaction, and under physiologic conditions or in naturally remitting diseases, it is self limiting (31,32).

In addition to its role as an HSP-derived epitope, dnaJP1 is peculiar because it mimics a "shared epitope" sequence common to HLA alleles associated with RA. In preclinical work, we suggested that the balance between recognition of and reactivity to epitope sequences containing the "shared epitope" might be altered by the presence of chronic inflammation (23–25,35). This would lead to a contribution to the inflammatory process itself by self (i.e., HLA) and non-self (i.e., dnaJP1) cross-reactive immune responses in the presence of impaired immune regulation. Our intervention aims at restoring this self-regulating ability by using epitope-specific tolerization.

The independence of these mechanisms from a putative initiator of autoimmune inflammation is an important conceptual difference when considering previous therapeutic experiences based on tolerization in animal models or humans (29,30,32,33). The initial effect is epitope specific and localized at the immune hubs within the gastrointestinal mucosa, where specialized antigen-presenting cells bind the peptide at high affinity via their HLA class II receptors and present it to transiting T cells in a tolerogenic environment. The primary effect is therefore on various types of epitope-specific Treg cells that induce an active modulation of the immune response. This process secondarily involves effector T cells and may also affect innate immunity. As we have shown, the total number of dnaJP1-specific T cells does not vary significantly between the beginning and end of treatment (35). Molecular analysis of these dnaJP1-specific T cells shows an active process of immune tolerization, very likely involving both adaptive and innate Treg cells, as shown by the treatment-dependent induction of IL-10, IFN$\gamma$, and FoxP3 genes. Ultimately, the tolerogenic effect involves effector T cells with a significant, treatment-specific reduction of their ability to produce proinflammatory cytokines, notably, TNF$\alpha$. The process is active rather than suppressive, as demonstrated by the concomitant increase in IL-10 production (35), and it is antigen specific, as shown by the intact ability of treated patients to respond to TT.

Considering the natural complexity of HSP-driven responses, which rely on innate and adaptive immunity, we see immune tolerization not merely as a change in the quality of response to an individual antigen, but rather as a complex and diverse pool of mechanisms that overlap in controlling, through active regulation, the inflammatory process (30–33, 35, 41). This concept is specifically supported by our data showing that both recognition of the antigen and expression of functional pathways associated with T cell tolerance and anergy are prerequisites for successful tolerization. PD-1 and CTLA-4 are associated with reversible T cell hyporesponsiveness to antigenic stimuli, as shown both in animal models and in progressive human immunodeficiency virus infection (42). Their increased expression in patients prone to tolerization as compared with their expression in those who were not, associated with the increased expression of the PD-1 ligands B7-H1 and B7-DC, underscores the role played by mechanisms of immune cross-talk in determining the susceptibility to induction of tolerance in human autoimmunity. These mechanisms are obviously complex and interlace with some T cell regulatory pathways, as shown by the increased representation of FoxP3 in the population susceptible to tolerization (Figure 3). Additional studies may confirm our refined hypothesis that a population of T cells expressing PD-1 and CTLA-4 has the ability to regulate those epitope-specific effector T cells contributing to the pathogenic inflammatory process.

In terms of relevance to current clinical practice, the potential of synergy with HCQ underscores the possible flexibility of use of epitope-specific therapy as a "work with" approach. With an appropriate development plan, which will stem from the data currently achieved, epitope-specific immunotherapy may be positioned not only as an orally administered first-line agent, in combination with methotrexate and HCQ, but also as a valuable complement to current therapies used to maintain clinical control upon induction of tolerance. Our recently reported data support these concepts,

showing that epitope-specific tolerization combined with an induction/withdrawal anti-TNF$\alpha$ regimen in an animal model resulted in clinical and histologic disease control that was comparable with that obtained with full-dose etanercept (43).

These data address some questions in a controversial area of translational medicine by identifying prerequisites of susceptibility to antigenic tolerization in human autoimmunity. These prerequisites do not appear to be related to any of the parameters currently used for randomization in clinical trials in RA. This may explain the contentious and never fully satisfying results from attempts to induce antigen-specific tolerance for therapeutic purposes in human autoimmunity.

Our data also open some interesting novel avenues for research. The validation of these findings in different trial settings may lead to the identification of biomarkers that can be used to preselect potential responders to immune therapy. Induction of the immune phenotype described here prior to immune tolerization may also increase the proportion of patients who may respond to the treatment. A detailed functional and molecular analysis may lead to the identification of novel regulatory mechanisms that play a pivotal role in the induction and maintenance of immune tolerance in humans.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Albani had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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