Immune responses induced by T-cell vaccination in patients with rheumatoid arthritis

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Abbreviations: APC, allophycocyanin; EULAR, European League Against Rheumatism; FITC, fluorescein isothiocyanate; MAb, monoclonal antibodies; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PHA, phytohaemagglutinin; RA, rheumatoid arthritis; TCR, T-cell receptor; Treg, regulatory T-cells

Introduction

It is well established that T-helper type 1-dependent autoimmunity is the core factor in the pathogenesis of rheumatoid arthritis (RA). Specific recognition of antigens expressed on the synovial articular surfaces by T lymphocytes triggers a series of events that lead to inflammation and joint destruction.¹,²

Autoreactive T-cells that recognize autologous antigens constitute part of the immune system in a healthy organism. This means that post-thymic T-cell development mechanisms control autoreactive T-cells and ensure protection against them. This regulation could include peripheral clonal deletion, modulation by cytokine antagonists, as well as inhibition of idiotypic T-cell receptor (TCR) interactions. Regulatory network impairments result in defective suppression of activated autoimmune cells leading to the development of autoimmune diseases.²⁻⁴

Conceivably, immunotherapy-based RA treatment strategies should be aimed at inactivation of autoreactive T- and B-lymphocytes, upregulation of natural and induced regulatory T-cell activity, and suppression of pro-inflammatory mediator production.

T-cell vaccination approach consists in administration of attenuated autoreactive T-cells, which activates many regulatory mechanisms of the immune response. Thus, T-cell vaccination induces anti-clonotypic T-cells. Anti-clonotypic T-cells regulate pathogenic T-cells via recognition of clonotypic determinants (idiotypes), thus ensuing anti-idiotypic response. In general, pathogenic T-cells belong to CD8⁺ T-cell lineage and are restricted to major histocompatibility complex (MHC) class I molecules; to lesser extent pathogenic T-cells belong to CD4⁺ T-cell lineage. Anti-clonotypic CD4⁺ T-cells lack cytotoxic properties with regard to autoreactive cells. However they are capable of inhibiting proliferation of autoreactive cells by recognition of idiotypic T-cell determinants in the context of MHC class II molecules. Anti-clonotypic CD4⁺ T-cells belong to different T-lymphocyte subpopulations, and namely IL-4-producing TH2 cells, and TGFβ-producing TH3 cells.⁴⁻⁷

T-cell vaccination is also affecting function of natural (pre-existing) CD4⁺CD25⁺ regulatory T-cells (Treg). There is

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evidence to suggest that some Treg are TCR-specific, and thus these cells are intrinsically involved into idiotype-anti-idiotype immunoregulation cascade. This means that T-cell vaccination could establish appropriate beneficial conditions leading to expansion of CD4^+CD25^+ Treg cells directed to vaccine-specific TCR.8

T-cell vaccination also influences anti-ergotypic regulation of T-cell reactivity that is not associated with recognition of idiotypic determinants. Independently of its antigenic specificity, anti-ergotypic T-cells are reactive only against activated, but not resting T-cells.4,9

T-cell vaccination induces not only T- but also B-cell reactivity, and namely production of anti-idiotypic antibodies capable of binding and inhibiting autoreactive T-cell clones. Anti-idiotypic antibodies belong to IgG isotype recognizing TCR determinants irrespective of MHC molecules. Mechanism of action of these antibodies could be related to shielding and functional TCR blockade.10

In conclusion, available experimental and clinical evidence suggest that T-cell vaccination could become an effective treatment strategy of autoimmune diseases with great development potential.11,12

The aim of the present study was to investigate immunological and clinical effects caused by T-cell vaccination in patients with RA.

Results

Proliferative responses and pro-inflammatory cytokine production in PBMC from RA patients

We observed statistically significant decline in proliferative PBMC responses to the protein cartilage antigen in RA patients in the course of T-cell vaccine treatment (Fig. 1). This observation could denote reduction in autoantigen-reactive T-cells in RA patients. Furthermore, 6 mo after the onset of T-cell vaccination significant and sustained reduction in plasma IFNγ and increased IL-4 were documented (Fig. 2). Compared with the baseline levels (before the onset of T-cell vaccination), PBMC from vaccinated RA patients displayed increased IL-4 production and increased IL-4 production pattern in the presence of cartilage antigen (Fig. 3). Consistent with these findings, intracellular staining and flow cytometry analysis revealed significant 1.6–1.8-fold reduction in IFNγ-producing CD4^+ and CD8^+ T-cells in RA patients after the induction course with the T-cell vaccine; this tendency was paralleled by the 1.7-fold increase in IL-4-producing CD4^+ lymphocytes (Fig. 4).
Study of memory T-cell cells

The results of this study suggest that there were no significant differences in "naïve" CD4⁺ CD45RO⁻CD62L⁺ T-cells and CD8⁺CD45RO⁻CD62L⁺ T-cells in healthy subjects and RA patients (Table 1). However, as compared with healthy donors, 5-fold increase in CD8⁺CD45RO⁻CD62L⁺ effector memory T-cells was observed in RA patients, whereas CD45RO⁺CD62L⁺ central memory T-cells increase was characteristic for both CD4⁺ T-cells and CD8⁺ T-cell populations. Our data denote that in RA clinical settings an accelerated differentiation of naïve T-cells into central CD4⁺ T-cells, CD8⁺ T-cells, and cytotoxic effector CD8⁺ T-cells could occur, which reflects antigen-specific T-cell expansion in response to repeated systemic antigen encounter¹³,¹⁴.

We observed that in the course of T-cell vaccination the number of CD4⁺ and CD8⁺ central memory T-cells was reliably and significantly down-regulated (5–6-fold reduction); effector memory CD8⁺ T-cell number was reduced by 2-fold (Table 1). The observed effect was maintained for 9 mo from the onset of T-cell vaccination, with the subsequent return of the qualitative and quantitative parameters nearly to the background values.

Study of regulatory T-cells

Data shown in Figure 5 suggest that as compared with healthy donors number of peripheral blood "naïve" regulatory CD4⁺CD25⁺FoxP3⁺ T-cells in RA patients was not considerably different; this number increased significantly (approximately 6-fold) as soon as 1 mo after the onset of T-cell vaccination. However, after 6 mo there is a significant weakening effect and has no significant difference compared with baseline were observed. This effect was maintained for 6 mo from the onset of immunotherapy, with the subsequent gradual reduction. Number of induced regulatory CD4⁺CD25⁺FoxP3⁺ T-cells did not significantly change in the course of the immunotherapy.
The onset of immunotherapy (of morning stiffness and fatigue, which were observed 3 mo after syndrome intensity, number of tender and swollen joints, duration DAS 28 index reduction, as well as by the decrease in pain syn-
thesis in RA patients suggested good/moderate clinical effect (according to the EULAR criteria) of the therapeutic protocol. 

Discussion

Conventional RA treatment is based on longitudinal unspe-
cific immunosuppressive therapy, which down-regulates immune system in general and carries high risk of serious side effects. There is obvious need for novel therapeutic strategies that would selectively inactivate pathogenic lymphocytes as an approach to treat RA and other autoimmune diseases. One such approach is based on vaccination of patients with autoimmune lymphocytes, as it is well known that there is no immune tolerance to lymphocyte antigen receptors formed in the postnatal period, and lymphocyte-lymphocyte idiotype-anti-idiotype interactions play an important role in regulation of the immune system.

In comparison with other treatment strategies, a clear advan-
tage of the T-cell vaccination approach consists in its selectivity that aims specifically at inactivation of those lymphocytes that account for the development of autoimmunity. It is of particu-
lar importance that the effect of T-cell vaccination is dependent on several mechanisms and components, such as stimulation of anti-idiotype immune reactions, induction and stimulation of regulatory T-cell functional activity, induction of anti-idiotype antibody synthesis etc. Admittedly, there is not yet enough evi-
dence with respect to clinical application of the T-cell vaccination approach. Previously, most studies used cloned T lymphocytes for immunization purposes. However, propagation of human T-cell clones is quite an expensive procedure that requires a lot of time, and is not always successful. Moreover, inactivation of one or several autoimmune clones might not always render a beneficial effect onto disease progression, which could involve polyclonal immune responses directed to multiple antigenic determinants; in such a scenario the importance of a single clone in the autoim-
mune process could prove to be a minor one. The above consid-
erations sideline the effective clinical application of cloned T-cells in RA treatment, as well as other autoimmune diseases.

We have developed a method that yields sufficient quantity of autoantigen-reactive T-cells within a relatively short period of time. Under these conditions, the vaccine composition is deter-
mined by the initial individual T-cell reactivity representing most autoaggressive cells that received selective growth preferences owing to the presence of joint tissue antigens in cell culture.

One of the major aims of the present pilot clinical study was to assess immunological and clinical effectiveness of the proposed treatment protocol. Significant reductions in PBMC proliferative activity to the joint tissue antigens in RA patients indicate immun-
ological effectiveness of T-cell vaccination; this may reflect reduction in antigen-reactive T-cell quantity in those patients. An important role is also played by the alterations in IFNγ and IL-4 concentrations in plasma and in cell culture supernatants of PBMC from vaccinated patients. Down-regulation of the former
cytokine (IFNγ) and up-regulation of the latter cytokine (IL-4) are suggestive of functional rearrangements in the immune system, as well as indicate activation of non-specific immune mechanisms that inhibit immunopathological process. In RA settings T-helper type 1-produced IFNγ is considered to play a pivotal role in recruitment of macrophages and other immunocompetent T-cells to pathological inflammatory sites, whereas T-helper type 2-produced IL-4 is capable of down-regulating the IFNγ-mediated activity, thus causing an anti-inflammatory effect.

Immunotherapy-mediated reduction in central memory T-cells, and in particular effector memory CD8+ T-cells is also a beneficial factor with respect to disease progression due to the fact that CD8+ T-cells producing IFNγ, TNFα, and perforin participate in maintaining chronic inflammation and destruction of joint tissues.

On the other side, down-regulation of the immune reactivity could be achieved via an increase in quantity or up-regulation of functional activity of regulatory CD4+CD25+FoxP3+ T-cells, which are capable of causing a adjusting effect both on memory cell generation and effector cell function.15

The results obtained in the present study also suggest significant clinical efficacy of T-cell vaccination approach, as good and moderate clinical effects were achieved in 87% patients.

Hence, T-cell vaccination opens new possibilities in treatment of RA and other autoimmune diseases. T-cell vaccination is well tolerated, does not cause significant side effects that could restrict its clinical application, and allows for induction of long-lasting changes in the immune system that prevent disease progression. Further progress in the development of this particular technology is highly desirable, and new data will further define modality of its clinical application.

Patients and Methods

Technology of T-cell vaccine preparation

We have developed a two-step technology of T-cell vaccine preparation. The technology was technically straightforward, and did not include cell cloning procedures making it easily-reproducible under the GLP conditions.

The initial step involved antigen-specific selection of cells, which were expanded to the desirable quantity during the subsequent cultivation step.16,17 Specifically, peripheral blood mononuclear cells (PBMC) from patients were incubated at a concentration of 2 × 10⁶/ml in RPMI 1640 medium (N R7130), supplemented with 10% inactivated autologous plasma, 5 mM HEPES (N H8651), 2 mM L-glutamine (N G7029), 5 × 10⁻⁵ M mercaptoethanol (N M6250) (all reagents were purchased from Sigma-Aldrich) in the presence of a protein cartilage antigen (1 µg/mL) for 7 d in a humidified incubator with 5% CO₂ (Step one). Protein cartilage antigens were obtained from pig cartilage. Protein cartilage antigen was purified according to Strom S.C.18 Protein concentration was determined according to Lowry.19 Optimal concentrations of all reagents were determined in separate experiments.

Step 2 consisted in the expansion of antigen-specific cells in the presence of phytohaemagglutinin (PHA, 5 µg/mL, N L9017, Sigma-Aldrich) and recombinant IL-2 (100 U/mL, Biotech) for 5 d. At the end of the cultivation step, cells were inactivated by irradiation at a dose of 2000 rad, and cryopreserved according to the standard method using plasma and 10% dimethyl sulfoxide. Cells were stored in liquid nitrogen until use. The total cell number obtained from one patient ranged between 18–27 × 10⁷.

Antigen-specific vaccine T-cells were validated for proliferative responses in the presence of cartilage and synovial antigens (1.8–2.0 fold increase compared with controls), as well as for IFNγ-producing CD8+ T-cells (3-fold increase), IFNγ-producing CD4+ T-cells (3–10-fold increase), CD3+CD45RO+ memory T-cells (2-fold increase), and CD3+CD45RO+ T cells (1.2–1.3-fold increase) upon antigen exposure.20

Proliferative PBMC responses

Antigen-induced proliferative responses of PBMC (2 × 10⁶) were tested in 96-well plates using RPMI 1640 medium, supplemented with 10% inactivated autologous plasma, 5 mM
Table 2. The dynamics of the articular syndrome and selected laboratory values in the course of T-cell vaccination (M ± m)

| Value                  | Pre-treatment, n = 42 | 3 mo n = 42 | 6 mo n = 42 | 12 mo n = 42 | 18 mo n = 32 | 24 mo n = 22 |
|------------------------|-----------------------|-------------|-------------|-------------|-------------|-------------|
| ESR (mm/h)             | 38.41 ± 2.20          | 31.12 ± 1.94** | 22.81 ± 1.26** | 21.75 ± 1.60** | 20.19 ± 1.74** | 21.14 ± 2.12** |
| Hemoglobin (g/L)       | 107.80 ± 1.45         | 113.70 ± 1.25** | 120.81 ± 1.01** | 125.20 ± 0.98** | 126.42 ± 1.17** | 126.46 ± 1.88** |
| Number of painful joints | 11.90 ± 0.63         | 7.26 ± 0.56** | 2.57 ± 0.47** | 2.09 ± 0.43** | 2.12 ± 0.53** | 2.41 ± 0.76** |
| Number of swollen joints | 6.97 ± 0.56         | 4.19 ± 0.55** | 1.33 ± 0.40** | 0.83 ± 0.33** | 1.01 ± 0.40** | 1.22 ± 0.52** |
| HAQ                    | 2.32 ± 0.06           | 1.78 ± 0.11** | 1.26 ± 0.12** | 0.87 ± 0.12** | 0.91 ± 0.14** | 1.11 ± 0.14** |
| Morning stiffness (min) | 78.10 ± 5.04          | 47.76 ± 5.16** | 20.01 ± 4.29** | 12.38 ± 3.52** | 12.38 ± 4.40** | 14.09 ± 5.53** |
| Pain (VAS, mm)         | 60.12 ± 2.65          | 39.40 ± 2.94** | 16.90 ± 2.79** | 11.60 ± 2.09** | 10.78 ± 2.46** | 11.14 ± 3.14** |
| Fatigue VAS (mm)       | 54.29 ± 2.46          | 34.83 ± 3.07** | 14.81 ± 2.81** | 9.92 ± 2.37** | 10.06 ± 2.55** | 10.59 ± 3.25** |
| DAS 28                 | 5.92 ± 0.13           | 4.93 ± 0.16** | 3.34 ± 0.18** | 2.99 ± 0.17** | 2.96 ± 0.21** | 3.12 ± 0.27** |

**P < 0.001, statistically significant differences in the groups studied before and after treatment (U criterion).

HEPES, 2 mM L-glutamine, 5 × 10^{-5} M mercaptoethanol (all from Sigma-Aldrich) in the presence or absence of the protein cartilage antigen (1 μg/mL) for 5 d in a humidified incubation with 5% CO₂. Cell proliferation was measured using standard [3H] thymidine incorporation method.

Cytokine measurements in plasma and cell culture supernatants

Quantitative IFNγ and IL-4 cytokine measurements were performed in blood plasma samples and 72 h cell culture supernatants using commercial ELISA diagnostic kits (Vector-Best Co.).

Intracellular staining for detection of IFNγ- and IL-4 – producing lymphocytes

Quantification of IFNγ- and IL-4-producing lymphocytes was performed by an intracellular cytokine staining method in 4 h PBMC cultures in the presence of 30 ng/mL phorbol ester (N 194804), 1 μg/mL ionomycin (N 155070) and 10 μg/mL brefeldin A (N 194802) (all reagents from ICN). Following cultivation, cells were incubated with mouse anti-human monoclonal antibodies (mAb) against T-cell surface markers CD4 (N 345770) and CD8 (N 345774) labeled with peridinin-chlorophyll-protein (PerCP) (BD Biosciences), fixed, permeabilized, and incubated with mouse anti-human mAb against IFNγ labeled with fluorescein isothiocyanate (FITC) and IL-4 labeled with phycoerythrin (PE) (N 340456, reagents were from BD Biosciences). Cells were washed and analyzed by flow cytometry on FACSCalibur™ (BD Biosciences).

Memory T-cell analysis

Relative quantification of the memory T-cells were performed by flow cytometry using CD4- and CD8-specific mouse anti-human MAbs labeled with PE (Sorbent) and mouse anti-human CD45RO-specific MAbs labeled with FITC (BD Biosciences), as well as mouse anti-human CD62L-specific MAbs labeled with allophycocyanin (APC) (N 17–0629–73, eBioscience). The following cell populations were detected depending on the expression pattern of the corresponding molecular cell surface markers: naive T-cells (CD45RO-CD62L-), central memory T-cells (CD45RO-CD62L+), and effector memory T-cells (CD45RO-CD62L-). Relevant data are shown as percentage of each cell population from the total number of lymphocytes.

Measurement of regulatory T-cells

Cellsurface markers of regulatory T-cells (CD4-CD25-FoxP3-) were determined using mouse anti-human CD4-specific FITC-labeled mAb (N 11-0048042), and mouse anti-human IL-2 receptor (CD25)-specific PE-labeled mAb (N 12-0259-42). FoxP3 expression was evaluated using mouse anti-human APC-labeled mAb (N 17-4776-42, all reagents from eBioscience). Percentage of positive cells from the total lymphoid cell population was determined by flow cytometry.

Clinical studies

Clinical and experimental studies were performed in compliance with the protocol approved by the Scientific council and Ethical committee of the Institute for Clinical Immunology, Siberian Branch of the Russian Academy of Medical Sciences, Russia. An informed consent statement was obtained from each patient participating in this study. Forty-two patients (age 28–58 y; 37 women, 5 men) with RA (disease history ≥ 2 y) were subjected to the T-cell vaccination treatment. Clinical studies were based at the Federal State-funded Health Care Institution “Clinical Hospital” No 85, Moscow, Russia. The patients enrolled in the study had no clinical evidence for any chronic gastrointestinal and renal diseases. Hepatitis A, B, and C viruses, a cytomegalovirus, type 1 and 2 herpesviruses, as well as chlamydia were not detected in their bloods by a PCR analysis. All patients received basic therapy with methotrexate (≤ 10 mg weekly). Adjunct therapeutic regimen with the T-cell vaccine included 4 weekly s.c. injections (induction course) followed by subsequent monthly vaccinations. Vaccine dose ranged between 2.0–4.0 × 10^7 cells. The effectiveness of the T-cell vaccination was assessed according to the criteria of European League Against Rheumatism (EULAR) based on the DAS 28 score evaluation. In addition, the following RA activity indicators were assessed: pain intensity, duration of the morning stiffness, number of painful joints and...
disclosure of potential conflicts of interest

no potential conflicts of interests were disclosed.

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