The substantiation of adoptive transfer of tolerogenic dendritic cells for treatment of rheumatoid arthritis in mice

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ABSTRACT

The tolerogenic properties of dendritic cells are of particular interest in the search for new approaches to the correction of Treg immunity in the treatment of autoimmune diseases.

*The **PURPOSE** is to study the effect of tolerogenic dendritic cells on the Treg immunity in mice with adjuvant arthritis at different stages.*

MATERIALS AND METHODS. Adjuvant arthritis (AA) was induced in CBA/H mice by sub-plantar injection of the Freund's complete adjuvant. Dendritic cells were obtained ex vivo from bone marrow mononuclear cells. On the 7th, 14th or 21st days of adjuvant arthritis, animals were injected with tolerogenic dendritic cells. Their arthritis index and T-immune status (CD4⁺CD25⁺ and FOXP3⁺ cells) were assessed.

RESULTS. Dysfunction of Treg immunity and clinical diagnostic parameters in animals at different stages of adjuvant arthritis have been demonstrated. On the 14th and 21st days of adjuvant arthritis, immunotherapy by obtained ex vivo dendritic cells, contributed to the increase of CD4⁺CD25⁺ and FOXP3⁺ cells content in the spleen and normalization of clinical diagnostic parameters in animals. In the acute stage of arthritis (7 days), adoptive therapy with tolerogenic dendritic cells was not effective.

CONCLUSION. Adoptive application of tolerogenic dendritic cells has contributed to the reduction of clinical manifestation of the arthritis. The greatest therapeutic efficacy was demonstrated by dendritic cells transferred on the 14th day of the pathology development, which contributed to the normalization of the clinical status of recipients with adjuvant arthritis.

KEY WORDS: adjuvant arthritis; adoptive immunotherapy; tolerogenic dendritic cells; Treg cells

An important component of the rheumatoid arthritis (RA) pathogenesis is dysregulation of the immune system, which induces impaired tolerance to auto-antigens and the development of autoimmune reactions. An important role in the inhibition of the autoimmune response belongs to regulatory T-cells (Treg), whose level of suppressor activity is largely determined by the expression of the transcription factor gene forkhead box protein 3 (Foxp3), required for the normal functioning and development of Treg [1]. Foxp3 acts as a transcriptional repressor of proinflammatory cytokines, being an antagonist of NFAT (nuclear factor of activated T-cells) function, which is central in the formation of the immune response in inflammation [2]. There is a decrease in the number of suppressor cells and Foxp3 gene expression in patients with RA [3, 4]. One of the mechanisms of this process might be a violation of the pro- and anti-inflammatory cytokines balance. For example, the level of anti-inflammatory cytokines TNF α , IL-6, IL-1 β , IL-17 increases and IL-10, IL-4 decreases in the blood

plasma at RA [5, 6]. Therefore, the decrease in the level of proinflammatory cytokines may contribute to the restoration of Treg suppressor function.

To date, the most common method of RA treatment is immunosuppressive therapy, which can significantly reduce the degree of the disease symptoms, but has severe side effects, in particular, a general reduction of infection resistance, suppression of antitumor immunity, mutagenic and cytotoxic effect on cells. Important factors limiting the immunosuppressive therapy of RA include the resistance to previously effective drugs, which often occurs at their long-term use [7, 8]. Thus, there is a need to find alternative treatments for RA. One of the promising therapeutic approaches is the application of autologous tolerogenic dendritic cells (tolDCs) [9, 10, 11, 12]. Dendritic cells represent a unique population of antigen-presenting cells that are capable of both stimulating and inhibiting the immune response. Tolerogenic DCs, unlike immunogenic

cells, are characterized by low expression of co-stimulatory molecules, high expression of immune regulatory cytokines and the ability to induce T-cell anergy and apoptosis [11, 12]. A new approach to the RA treatment is primarily aimed at the formation of tolerance to arthritic auto-antigens. However, the impact of tolDCs on natural antigen-specific Treg cells, which play a key role in tolerance to auto-antigens through inhibition of auto-reactive T-cells, remains unresolved. There are also no data on the efficacy of tolDCs therapy, depending on the time of cell transfer during the pathology development.

The **PURPOSE** of our study was to investigate the effect of tolDCs on the Treg immunity in mice with adjuvant arthritis (AA) at different stages of its development.

MATERIALS AND METHODS

The experiments were performed on female CBA/H mice, 2.5-3 months old. The studies were conducted in accordance with the «General Principles of Animal Experiments», approved by the V National Congress on Bioethics (Kyiv, 2013) and agreed with the «European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes» (Strasbourg, 1986). The animals were euthanized under ether anaesthesia by cervical dislocation.

Isolation of murine bone marrow cells. The bone marrow was flushed out from the femur of mice ($n = 10$) with complete RPMI-1640 medium (*Sigma-Aldrich*, UK) supplemented with 3 % fetal calf serum (*BioLot*, Russia) and 2 % sodium citrate (*Sigma-Aldrich*, USA). The suspension of bone marrow cells was passed through a nylon filter with a pore diameter of 100 μm (*Falcon*, USA), centrifuged at 300 $\times g$ for 10 min and resuspended in the complete medium.

Isolation of bone marrow mononuclear cells. Mononuclear cells (MNCs) were isolated by centrifugation of bone marrow suspension in density gradient Trazograph 1.077 g/cm^3 (*Unic Pharmaceutical Laboratories*, India) [12].

Obtaining of tolerogenic DCs. MNCs were cultured in 35 mm plastic Petri dishes in RPMI-1640 medium supplemented with 10 % fetal calf serum and 1 % antibiotic solution (100 U/ml penicillin, 0.1 mg/ml streptomycin), at a concentration of $3\text{--}5 \cdot 10^6$ cells/ml or $9 \cdot 10^6$ cells per dish. The cells were cultured at 37 °C in 5 % CO_2 atmosphere. On 0 day, 20 ng/ml murine recombinant granulocyte-macrophage colony-stimulating factor – GM-CSF (*Sigma-Aldrich*, UK), 5 ng/ml IL-4 (*Sigma-Aldrich*, UK) [13] and 0.4 $\mu\text{g}/\text{ml}$ dexamethasone – Dex (*Sigma-Aldrich*, UK) [14] were added to the culture medium. After 3 days, a fresh culture medium with the factors and Dex was added. On the 6-7th days, non-adherent and weakly adherent cells were collected for further FACS analysis and injection to animals with adjuvant arthritis (AA). On 6-7th days of culture the relative content of tolDCs was 90 % of the seeded cells or $8 \cdot 10^6$ cells/dish.

Induction of adjuvant arthritis. Adjuvant arthritis (AA) was induced in CBA/H mice ($n = 15$) by subplantar injection with 0.2 ml/mouse Freund's Complete Adjuvant (*Santa Cruz*, USA). The development of AA was assessed using the arthritis index (AI), which is the ratio of the circumference of the test joint to the circumference of the control one. In healthy animals ($n = 5$) treated with saline at the same dose as Freund's Complete Adjuvant, IA was taken as 1 unit. The administration of saline did not cause a local inflammation.

Determination of inflammatory mediators at AA. The level of seromucoids in the serum of animals with AA was determined by the turbidimetric method using a kit of reagents (*Felicit-Diagnostics*, Ukraine) according to the manufacturer's instructions and was evaluated in turbidity units (S-H) by Shank and Hoagland. The level of sialic acids was determined by the Hess method [15].

Transfer of tolDCs to animals with AA. Syngeneic *ex vivo* grown DCs were administered intravenously at a dose of $5 \cdot 10^5$ cells/mouse [10] in 0.2 ml complete medium on the 7th ($n = 5$), 14th ($n = 5$) or 21st ($n = 5$) days of AA development. In each group, 7 days after the injection of DCs, on

14th, 21st and 28th days respectively, we determined the content of Treg in the spleen and AI of all experimental group animals. Five experiments were performed; each group consisted of five animals. Controls animals ($n = 5$) were injected with bone marrow mononuclear cells at the same concentration as tolDCs ($5 \cdot 10^5$ cells/mouse). The comparison group was animals ($n = 5$) administered intravenously with dexamethasone – Dex (*KRKA*, Slovenia) 0.022 mg/mouse in 0.2 ml saline [16].

Flow cytometric analysis of the cells. Monoclonal anti-mouse antibodies CD14-FITC, CD11b-FITC, CD83-FITC, CD80-FITC, CD86 and CD86-FITC (*BD Biosciences*, USA) were used to identification *ex vivo* grown tolerogenic DCs on a FACS Calibur flow cytometer (*Becton Dickinson*, USA) according to the manufacturer's instructions. The Treg content in the spleen of animals at various stages of AA development and after the introduction of tolDCs was evaluated using monoclonal anti-mouse antibodies CD4-FITC, CD25-PE, FOXP3-PE (*BD Biosciences*, USA) according to the manufacturer's instructions. Isotype-Ig antibodies (*BD Biosciences*, USA) were used as a negative control. Statistical analysis of the data obtained by flow cytometry was performed using WinMDi 2.8 software (*Joseph Trotter, Scripps Institute*, USA).

Statistical data processing was performed using the SPSS Statistics 17.0 software (*IBM*, USA). The obtained experimental data are presented as the mean $\pm$ standard deviation. The reliability of differences between the groups was evaluated by Mann-Whitney U test. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The most manifesting sign of the initiation of the AA in rodents is the edema of the affected joint, which was determined in mice of the experimental group (**Fig. 1**).

According to literature [17, 18, 19], the edema of the joints in the term from 4 to 9 days after the induction of AA is a consequence of intraarticular changes of the synovium, followed by the formation of granulation tissue, which is characterized as an acute stage of the pathology. AI had the maximum value in animals on the 14th day of AA development (1.802 ± 0.058) (**Fig. 2 A**). The period from 10 to 20 days of AA development corresponds to the most intensive growth of connective tissue, which can cause more pronounced edema of the joints and is characterized as a phase of clinical manifestation of the disease. The following is a chronicity of the inflammation while maintaining a high level of AI throughout the observation period (up to 28 days).

RA is accompanied by the destruction of connective tissue, which main component is glycoproteins. Their level is determined by the content of sialic acids and seromucoids. In this regard, the determination of sialic acids and seromucoids as the markers of the inflammation in assessing the severity of the disease, monitoring its course and control of the treatment effectiveness is expedient [20, 21].

Thus, after AA induction, the animals had an increase in the level of seromucoids and sialic acids in the serum, which indicators reached a peak by the 14th day of disease development, which is consistent with the maximum arthritis index (**Fig. 2 A, B, C**). During the chronicity of the in-

Fig. 1. The image of local inflammation in the form of edema of the distal metatarsus of mouse hind paw with adjuvant arthritis model (A) compared to control mice (B).

Fig. 2. Dynamics of clinical indicators change at adjuvant arthritis: **A** – arthritis index (AI), **B** – level of sialic acids and **C** – level of seromucoids in the serum of blood; $n = 5$.

Note: FCA – Freund's Complete Adjuvant.

flammation, these index decreased, but until the 28th day remained above the control values.

One of the reasons for the tolerance disorder at RA may be a decrease in Treg content or their functional defects. Most researchers observed a decrease in the relative number of circulating Tregs [22, 23, 24], in some studies, there was its increase [25] or the absence of differences compared to healthy patients [26]. Our studies showed a decrease of Treg content in different periods of AA clinical course (Fig. 3).

In experimental murine models, it has been shown that Treg deficiency, namely CD4⁺CD25⁺ cells, although it is not a major factor in the arthritis pathogenesis, plays an important role in supporting the autoimmune process [27]. Reduced Treg activity in autoimmune diseases may be due to inadequate expression of cell surface molecules involved in the mechanism of contact-dependent suppression, such as CTLA-4, CD39, CD223, FAS ligand (CD95), granzyme A, as well as the loss of ability to secrete soluble factors TGF, IL-10, IL-35. The impact on immunosuppression mechanisms is primarily due to genetic factors. The location of immune cells, the involvement of antigen-presenting cells and cytokines (such as TNF- α), which can affect Treg function, are also of a great importance [5]. More commonly, the expression of the most specific marker, the Foxp3 transcription factor, which provides differentiation and suppressor function of FOXP3⁺ Treg, is associated with the Treg-cell population.

Given this, it was advisable to determine the content of FOXP3⁺ cells in the spleen cells of animals with AA, along with the number of CD4⁺CD25⁺ cells in the presence of intracellular protein scurf, which determines their suppressor function. It is known that FOXP3⁺ Treg along with CD4⁺CD25⁺ can be reckon to other cells with regulatory activity, such as Th3 and Tr1 producing anti-inflammatory mediators TGF- β and IL-10 [28].

Thus, we found that the relative number of CD4⁺CD25⁺ and FOXP3⁺ cells had no significant differences in intact mice of the control group. On the 7th day of AA development, the content of CD4⁺CD25⁺ i FOXP3⁺ cells decreased by 1.6 and 1.4 times, respectively, of the control (Fig. 3), which may contribute to the breakdown of tolerance in the development of the arthritis. This is consistent with the data of Fontenot J. et al., who discovered a special genetic mechanism of dominant tolerance mediated by the FOXP3 protein that protects the body against autoimmune diseases and chronic infections [29]. In our study, the increase of AI was the most important at immune disorders.

On the 14th day, the number of CD4⁺CD25⁺ cells increased slightly compared to the 7th day (remaining below the control value by 25 %), while the content of FOXP3⁺ cells increased by 1.6 times. This may indicate the presence of FOXP3 protein in other cells with regulatory activity. It is known that overexpression of the FOXP3 protein may cause dysfunction of Treg and CD4⁺CD25⁺ cells, which leads to a decrease in their proliferation and production of anti-inflammatory cytokines and in some cases to the development of severe immunodeficiency [28].

On the 21st day of the arthritis development, on the contrary, there was an active proliferation of CD4⁺CD25⁺ cells at a low level of FOXP3⁺ cells in the general population of spleen cells, which testifies to their reduced functional activity. This expansion of Treg in the spleens of animals with AA during this period was noted by other authors [28, 30], which may be the result of increased expression of CD25 in response to an increase in proinflammatory cytokines IL2, prostaglandin E2, IFN γ , which are mediators of inflammation. There is every reason to suppose that at the stage of active proliferation of CD4⁺CD25⁺ cells, their suppressor activity is reduced, as evidenced by the low content of FOXP3 protein and high rate of arthritis index up to 1.686 ± 0.073 a.u. (Fig. 3).

On the 28th day of AA, the content of both CD4⁺CD25⁺ and FOXP3⁺ cells decreased compared to the 21st day of the arthritis development. In this period, the functional activity of CD4⁺CD25⁺ cells was reduced, resulting in high arthritis index (1.578 ± 0.099 a.u.). Thus, at all stages of AA, there was a marked dysfunction of the Treg immunity and violation of clinical and diagnostic parameters of animals that need correction of immune system.

Given the ability of tolDCs to induce naïve T-cells into Treg which secrete suppressive tolerogenic factors such as IL-10, TGF- β , retinoic acid and programmed death ligands [5], we investigated the possibility of using immature syngeneic DCs to induce Treg in the recipients with AA.

Fig. 3. The content of Treg in the spleen of animals and arthritis index in the dynamics of adjuvant arthritis development.

To this purpose, we have obtained DCs *in vitro* and confirmed they appropriate tolerogenic phenotype.

The analysis of the literature allowed us to select a number of monoclonal antibodies: CD14, CD83, CD11b, CD80, CD86, which are negative or positive markers that characterize the obtained DCs as tolerogenic (Table 1) [12, 13, 14].

Monocyte differentiation into immature DCs was accompanied by the loss of CD14 antigen, low expression of CD83 marker and co-stimulatory molecules CD80/86, as well as high level of CD11b. The resulting immature tolDCs had CD14⁺CD11b⁺CD83^{low}CD80/86^{low} phenotype. On the 7th day of cultivation, their relative content was 90 % of the seeded cells at the start of cultivation.

Since the resistance to *in vivo* maturation in autoimmune diseases is probably the most important feature among all other characteristic features of tolDCs [31, 32, 33], the next stage of the study was the approbation of tolDCs obtained in culture on the AA model.

The analysis of the Treg immunity in animals after tolDCs adoptive treatment depending on the AA stage (7, 14 and 21 days) is presented in Fig. 4. With the introduction of tolDCs into the animals on the 7th day of AA development (AA-7) (Fig. 4 A), there was an 1.6-fold increase of CD4⁺CD25⁺ cells content and a 1.5-fold decrease in FOXP3⁺ cells compared to untreated animals (AA-14). This may indicate the ability of tolDCs to stimulate CD4⁺CD25⁺ cell proliferation during this time with reduced production of FOXP3 protein in Treg, which is responsible for their suppressor function. In their turn, Dex and MNCs used as comparison groups contributed less to the normalization of these indicators. In addition, according to the arthritis index, the transfer of tolDCs at this time was not effective, since the AI after a week (14 days of effect evaluation) did not differ from that in untreated animals. Our data is consistent with that of van Duivenvoorde et al., who also did not obtain a positive effect with the introduction of tolDCs during the acute stage of arthritis [34]. It should be noted that the injection of Dex or MNCs to animals with AA-7 also did not cause a decrease in AI (1.736 ± 0.165 and 1.730 ± 0.093 a.u., respectively).

The introduction of tolDCs on the 14th day of arthritis model (AA-14) within a week caused significant changes of CD4⁺CD25⁺ and FOXP3⁺ cells content, which were not significantly different from the intact control group (Fig. 4 B). This may indicate a stimulation of Treg function after the introduction of tolDCs at this time. Moreover, only in this group, compared to other groups, the AI decreased significantly, as close as possible to the control group (1.154 ± 0.109 and 1.0 ± 0.01 a.u., respectively).

The introduction of tolDCs on the 21st day of arthritis (AA-21) largely than Dex and MNCs corrected the arthritis index and both Treg subpopulations. At the same time, the reduction of the joint edema was less than with the transfer of tolDCs on the 14th day (Fig. 4 C).

The use of tolDCs during the clinical manifestation of the arthritis (14th, 21st days) showed greater corrective activity of the Treg immunity, leading to a decrease in arthritis index, compared to Dex and MNCs groups.

When using Dex, we also observed an increase in the content of FOXP3⁺ cells, but they exhibited less suppressor activity than induced by tolDCs, according to the arthritis index. Moreover, as noted by Prado C. et al. [35], dexamethasone ability to potentiate FOXP3 protein expression in Treg *in vitro* did not correlate with their regulatory activity. However, in our studies, the positive dynamics of the animal's clinical status, namely arthritis index, were noted with the injection of Dex on the 14th and 21st days of AA. The fact is that the mechanism of Dex impact has certain features. First, corticosteroids are powerful immunosuppressive agents [36]. The transcription factor GILZ is crucially important in immunosuppression. It is activated by glucocorticoids, causing the inhibition of nuclear factor kappa-B (NFκB), which in its turn contributes to the reduction of proinflammatory cytokines production [37]. In addition, epigenetic regulation of genes, primarily DNA methylation, plays an essential role in controlling their function and is involved in the expression of Treg transcriptional regulator FoxP3 [38]. The mechanism underlying this unique epigenetic effect of glucocorticoids is associated with a decrease in DNA methyltransferase gene expression, which leads to a significant inhibition of DNA methylation [39]. This causes impaired gene transcriptional activity and, consequently, can lead to an increase in FoxP3 production.

Table 1. Phenotypic markers of tolerogenic DCs.

CD14	glycoprotein receptor expressed on monocytes, macrophages, Kupffer cells, leukocytes
CD83	immunoglobulin superfamily molecule, the most characteristic marker of fully differentiated dendritic cells; absent or poorly expressed on tolerogenic DCs
CD80/86	membrane proteins of immunoglobulins superfamily expressed on antigen-presenting cells, as well as mature DCs and serve as a co-stimulatory signal for T-lymphocytes activation and survival
CD11B	integrin Mac-1, a molecule expressed on immature tolerogenic and absent on mature immunogenic DCs

After the transfer of MNCs on the 14th and 21st days of arthritis, AI also decreases, but to a lesser extent comparing to Dex and even less toIDCs groups. MNCs are known to contain mesenchymal stem cells that produce anti-inflammatory mediators. Due to the secretion of IDO, TGF- β , they are able to induce Treg, and due to the secretion of PGE2 to inhibit DCs maturation [40]. It is this fact that was noted in our study. The injection of MNCs stimulated the proliferation of CD4⁺CD25⁺ cells, but did not affect the content of FOXP3⁺ Treg cells, which is characteristic of toIDCs. Therefore, the induction of FOXP3⁺ Treg is one of the main mechanisms by which DCs maintain immune tolerance [11, 28].

Fig. 4. The content of Treg cells and arthritis index before and after the injection of toIDCs, Dex, BM-MNCs to animals with adjuvant arthritis (AA). Injections: **A** – on the 7th day of AA, assessment on the 14th day; **B** – on the 14th day, assessment on the 21st day; **C** – on the 21st day, assessment on the 28th day; n = 5.

CONCLUSION

Summarizing the above data, we can conclude about the role of terms of cells injection in the manifestation of their therapeutic effect. The greatest therapeutic efficacy was shown by tolDCs introduced on the 14th day of the arthritis. It was during this period that they adjusted the Treg content in recipients, which promoted to normalize their clinical status.

Obviously, tolDCs implement their immune-correcting effect much earlier than glucocorticoids, which is characterized by a later than at the injection of tolDCs manifestation of the corrective effect of dexamethasone.

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