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A new approach to the role of IL-7 and TGF-ß in the in vitro generation of thymus-derived CD4 + CD25 + Foxp3 + regulatory T cells

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ABSTRACT

Thymus-derived regulatory T cells of CD4 + CD25 + Foxp3 + phenotype develop as a functional, mature population playing an essential role in self-tolerance and immune homeostasis, and exhibiting therapeutic potential to inhibit adverse immune response. Despite intensive research on thymus-derived Tregs, the knowledge about agents involved in their generation, survival, proliferation, and biological functions is still insufficient. In this research we have focused on the role of selected cytokines in previously developed *in vitro* model based on the application of anti-CD3 monoclonal antibodies. We have demonstrated an essential role of IL-7 and TGF-β in the generation of thymus-derived Tregs in the co-culture of thymocytes and JAWS II cells. In addition, *in vitro* generated Tregs exhibited their suppressive function similarly to Tregs sorted from freshly isolated thymus.

1. Introduction

The history of regulatory T cells (Tregs) of CD4 + CD25 + Foxp3 + phenotype has begun in the 90s of the XX century, when Sakaguchi S. and his group showed that activated CD4 + CD25 + T cells are able to maintain self-tolerance [1,2]. Despite the growing number of research confirming the role of Tregs in the maintenance of immune homeostasis [3] and their therapeutic potential in inhibiting autoimmune diseases, the knowledge about agents involved in their generation, survival, proliferation, and biological functions is still not satisfactory. There are two different types of Tregs of CD4 + CD25 + Foxp3 + phenotype: natural regulatory T cells developing in the thymus as a specialized population among the SP CD4 + subset, and induced regulatory T cells arising in peripheral lymphoid organs upon the activation of naive CD4 + CD25- T cells, in the presence of transforming growth factor β (TGF-β) [4,5]. Recently, the new terminology has been introduced in order to distinguish the origin of CD4 + CD25 + Foxp3 + regulatory T cell: thymus-derived Tregs (tTregs) formerly named natural Tregs (nTregs) and peripherally-derived Tregs (pTregs), previously known as induced Tregs (iTregs) [6,7]. The most commonly accepted hypothesis concerning tTreg development is based on typical stages of differentiation of conventional T cells, which are determined by the expression of surface markers CD4 and CD8: 1/double negative (DN: CD4-CD8-); 2/double positive (DP: CD4 + CD8 +); 3/single positive (SP CD4 +: CD4 + CD8-). What is interesting, tTregs do not undergo negative selection in condition of high-affinity TCR signal and CD28 costimulation in contrast to autoreactive thymocytes [8]. For the differentiation tTregs from immature SP CD4 + thymocytes a two-step model was proposed, which comprise: 1/ the encounter with self-antigen and 2/ CD28/B7 interaction and cytokines engagement. In physiological conditions, the conversion from self-reactive thymocytes to tTregs occurs during the positive selection involving cortical thymic epithelial cells, which present highly expressed MHC II/self-peptide complexes. In the second step, mainly dendritic cells are engaged by delivering costimulatory signals in an environment containing IL-2, other γ-chain cytokines, and possibly other unknown cofactors. Immature tTregs are Foxp3-negative CD25-positive and are converted to Foxp3-positive CD25high mature Tregs.

Thymus-derived regulatory T cell development takes place in an environment containing cytokines and other, less known factors, which are available in the thymus under physiological conditions. In order to study the contribution of various cytokines in the generation of thymus-derived Tregs, we used the *in vitro* model already described in our previous paper, based on the two-step model for Treg differentiation in the thymus [9].

The aim of this study was to examine the role of the following cytokines, considered to constitute important factors in the generation of CD4 + CD25 + Foxp3 + regulatory T cells:

– common γ-chain cytokines (IL-2, IL-4, IL-7, IL-15, and IL-21) considered as important ones in the development, maintenance and homeostasis of Tregs;

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– TGF-β as the crucial cytokine inducing Tregs;
– thymic stromal lymphopoetin (TSLP), an IL-7-like cytokine regulating early T cell development, but so far not associated with Treg development in the thymus.

2. Materials and methods

2.1. Animals

In order to examine the effect of cytokines on *in vitro* thymus-derived Treg differentiation, female, 6 to 8-week-old B6. Cg-Foxp3^{tm2Tch} mice were used, as it is considered that females are more susceptible than males to most autoimmune diseases, in which Tregs are particularly important.

In order to evaluate the suppressive activity of tTregs generated *in vitro* and co-cultured with JAWS II cells, C57BL/6 female mice were used due to higher endogenous suppressive activity of Tregs directly isolated from the thymus, when compared to B6. Cg-Foxp3^{tm2Tch} strain.

Mice were bred in the Animal Facility at the Department of Biology, University of Warsaw, in the Individually Ventilated Cages system, in the photoperiod of Light/Dark 12/12, with standard food and water *ad libitum*. All the procedures involving animal studies were approved by the Local Ethic Commission complying with the ARRIVE guidelines EU Directive 2010/63/EU for animal experiments.

2.2. Isolation of thymocytes

Mice were sacrificed in carbon dioxide. Freshly isolated thymi were homogenized in cold PBS. To remove tissue debris, the cells were filtered through 100 μm cell strainer.

2.3. Cell cultures

Thymocytes were preactivated on anti-CD3 MoAbs coated 24-well flat-bottom culture plates. Anti-CD3-coated plates were obtained by overnight incubation at 4 °C with 5 μg/mL of MoAbs anti-CD3 (BD Biosciences) PBS solution. The preactivation of thymocytes (1x10^6 /well) was carried out in RPMI-1640 with Glutamax, fetal bovine serum (10%), Penicillin (50 mU/mL), Streptomycin (50 ng/mL), sodium pyruvate (1 mM), 2-mercaptoethanol (0.2 mM) for 24h, at 37°C, under a 5% CO_2 atmosphere. Preactivated thymocytes were co-cultured with JAWS II cells (ATCC, CRL-11904, U. S. Patent 5, 648, 219) according to previously developed model [9]. JAWS II cells are immature dendritic cells with characteristic features of antigen-presenting cells, found from bone marrow cells of a p53 KO C57BL/6 mouse. Co-cultures of thymocytes (1x10^6 /well) with JAWS II cells (1 × 10^5/well) were carried out in RPMI-1640 with Glutamax, fetal bovine serum (20%) Penicillin (50 mU/mL), Streptomycin (50 ng/mL), sodium pyruvate (1 mM) for 3 or 7 days. Cytokines were added to the medium once for 3 days co-culture. For 7-days co-culture, cytokines were supplemented twice with fresh medium containing equal cytokine concentrations: at time “0” and after 3 days. Cytokines: IL-7, IL-15, IL-21 (PeproTech), and IL-4 (Genzyme), TGF-β1 (PeproTech), TSLP (eBioscence) were used at the concentration of 10ng/mL IL-2 (Pepro-Tech) was administrated at 50U/ml. Concentrations of cytokines added to culture medium were based on the literature data, manufacturer's recommendations and preliminary studies.

2.4. Flow cytometry analysis of thymocyte viability, proliferation and CD4 + CD25 + Foxp3 + thymocytes generation

Thymocyte viability, proliferation and CD4 + CD25 + Foxp3 + Tregs generation were examined in co-cultures of thymocytes and JAWS II cells in cytokine-conditioned medium. To analyze the level of proliferation, thymocytes were stained with Violet Proliferation Dye 450 before anti-CD3 preactivation and co-culture with JAWS II cells. Analysis of thymocyte proliferation and CD4 + CD25 + Foxp3 + generation was performed in gated population of viable thymocytes (dead cells were gated out as 7-AAD positive). Phenotypic characterization of thymocytes was determined according to the gating strategy for *in vitro* generation of CD4 + CD25 + Foxp3 + regulatory T cells as previously described [9], basing on CD4-APC, CD8-PE, and CD25-PeCy7 staining. Cells were acquired on BD FACSVerse (BD Biosciences) and analyzed using FACSuite/FACSDiva Software. Monoclonal antibodies for surface markers staining, proliferation and viability dyes were purchased from BD Biosciences.

2.5. Sorting of thymus-derived and in vitro generated Tregs

Sorting of CD4 + CD8-CD25high thymocytes from freshly isolated thymi and *in vitro* co-cultures was performed on FACSAria (BD Biosciences) based on staining with fluorochrome-conjugated monoclonal antibodies: CD4-PE, CD8-PerCP, CD25-PeCy7. The purity of sorted population was > 97% upon reanalysis using the same gates as determined for sorting procedure. Sorted CD4 + CD8-CD25high thymocytes were used for the proliferation inhibition assay.

To determine whether Tregs generated *in vitro* are *bona fide* Tregs or induced Tregs the co-culture of JAWS II with thymocytes depleted of CD25 + GITR + (tTreg precursors) [10] has been performed. To deplete thymocyte population of CD25 + GITR + cells staining with CD25-PeCy7 and GITR-PE fluorochrome-conjugated monoclonal antibodies has been performed. CD25 + GITR + thymocytes were sorted out using FACSAria (BD Biosciences). The purity of sorted population was > 97% upon reanalysis using the same gates as determined for sorting procedure. The proliferation of CD25 + GITR + depleted thymocytes was evaluated basing on V-450 staining.

2.6. Proliferation inhibition assay

The suppressive activity of Tregs was determined by the inhibition of proliferation of lymph node CD4 + CD25- T cells (responder cells) according to CFSE assay [11]. In brief, 25 × 10^3 CFSE-stained responder cells were cultured in triplicates with 0.5 μg/ml anti-CD3 and 0.05 μg/ml anti-CD28 MoAbs (BD Biosciences), with or without 5 × 10^3 sorted Tregs. Negative control consisted of responder T cells cultured without activatory antibodies. Positive control consisted of activated responder T cells cultured alone. Responder T cell proliferation was measured after 3-days culture by flow cytometry using FACSCalibur (BD Biosciences). CellQuest software was used to analyze the percentage of proliferating cells. The suppressive activity of CD4 + CD25high Tregs on the responder cells proliferation was determined as the percentage of inhibition by the following formula [12]:

$$\%\text{inhibition} = 100 - \frac{\%\text{of prolife rated activated responder T cells cultured with Tregs}}{\%\text{of prolife ratedactivated responder T cells(positive control)}} \times 100$$

2.7. Cytokine detection

Culture supernatants from responder T cells co-cultured with sorted CD4 + CD25high thymocytes were collected and the concentration of IL-10 and TGF-β were assessed using Ready-Set-Go! ELISA assay from eBioscience following manufacturer’s instructions. The absorbance was measured at 450nm with a microplate autoreader μQuant, BioTek, and analyzed using Gen5 Data Analysis Software, BioTek.

2.8. Statistical analysis

All experiments were performed independently at least 3 times with 2–3 mice in each experiment. The results are expressed as mean values +/- standard deviation. ANOVA one-way test have been applied to test the statistical significance of the results. The following numbers of asterisks indicate each statistical significance: $^{*}p < 0.05$; $^{**}p < 0.01$;