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Role of thymic B cells in the development of thymus-derived regulatory T cell *in vitro*

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ABSTRACT

The thymus contains a low frequency of B cells, about 0.1–0.5% of total population of thymocytes that were shown to contribute in thymic negative selection. The fact that B cells in the periphery have been contributed in the generation of the T regulatory cell compartment emerged the idea that this process may indeed be initiated firstly within the thymus. The results of this study revealed that activated thymic B cells maintained the high percentage of nTreg generation or counteracted the decrease of this percentage observed in non-activated culture, and both activators (LPS and IMQ) have the same effect in the process of nTreg generation by B cells. In addition, the activated cultures showed increase of the level of expression of Foxp3 transcription factor. In this study we confirmed that thymic B cells in the condition of our experiments did not influence the generation of nTregs, but rather maintain their viability when activated by both activators.

1. Introduction

B cells can play a role as antigen-presenting cell (APC) because of their constitutive high expression of MHC II and the ability to uptake, process, and present antigens to CD4+ T cells via B cell-mediated mechanism. Additionally B cells constitutively express costimulatory molecules such as CD80 and CD86 as well as CD40, and their expression increase upon activation [1,2]. These molecules, as it was described for dendritic cells, have an important role in the generation of nTregs [3–9]. The thymus contains a low frequency of B cells (about 0.1–0.5% of total population of thymocytes) that were shown to contribute in thymic negative selection [10,11]. Evidence that B cells can play a role in T cell selection process in the thymus has emerged from rodent studies examining minor lymphocyte-stimulating (Mls) Ags [12]. Results have indicated that clones reactive to specific Mls Ags, such as T cells bearing the Vβ6 TCR element reactive for Mls-1a, are deleted by thymic B cells [13–15]. Other studies have extended this concept to other model of Ags [16,10] demonstrating that B cells can contribute to the shaping the thymic T cell repertoire through negative selection.

Using an *in vitro* culture model, Lu et al. [9] revealed that thymic B cells have an important role in regulating the size of the naturally-occurring regulatory T cell (nTreg) compartment in the thymus. It has demonstrated that thymic B cells localized in the thymic medulla with nTreg precursors, particularly around Hassall's corpuscles, but also in the perivascular and intralobular spaces [17,18,8,9]. In addition, a deficiency of thymic B cells results in a significant decrease in both the frequency and number of thymic nTreg cells. Further, it has demonstrated that thymic B cells contribute to thymic nTreg cell number via cell–cell contact involving two independent pathways. In the first step, thymic B cells promote the generation of thymic precursor for nTreg cells from CD4+ SP subsets. Then thymic B cells directly promote the proliferation of thymic nTreg cells both *in vivo* and *in vitro* that is MHC II contact dependent and an intact BCR repertoire [8] with a fewer contribution of the costimulatory molecules CD40\CD80\CD86. Whereas the development of thymic nTreg cells was MHC II dependent but CD40\CD80\CD86 independent process. Thus signals required for thymic nTreg development may be distinct from those required for thymic nTreg proliferation [9].

In addition to mentioned functions of thymic B cells, these cells are directly implicated in the induction tolerance in the thymus [19]. In this regard, studying the mechanisms and cells responsible for the development of tolerance increases the treatment of allergic diseases, autoimmune diseases, preventing transplant rejection, and prevents the development of an immune response during gene therapy. It was suggested that B cells play a role in negative selection of CD4+CD8– SP but not CD8+CD4– SP thymocytes [10].

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Though activation of T cells by B cells as APC requires costimulation, resting B cells with low expression of B7 induce tolerance T cells (anergy). *In vivo*, dendritic cells can prevent such induced anergy by providing additional costimulatory signals (OX40L, cytokines), thus, in the absence of these signals showed the status of the T-cell tolerance [20,21].

Several studies have indicated to different molecules that implicate to achieve T cell tolerance. However, the role of each molecule in achieving tolerance is still to be needed further elucidation. MHC II and B7 (CD80\CD86) molecules were suggested to have an important role in the tolerance. The mechanistic studies have shown that transduced B cells must express MHC II and B7.2 (CD86) co-stimulatory molecules for successful tolerance induction [22,23]. It has well known that CD80 and CD86 are co-stimulatory pairs with CD28 and CTLA-4 through interaction between APC and T cells to transduce co-stimulatory signals [24,25]. Prolonged allograft survival in rodent models is observed after transient blockade of B7\CD28 co-stimulation by monoclonal antibodies [26]. Other results have revealed that exposure to intra-nasal prototypic protein antigens was associated with rapid partial activation of the antigen-specific B cells, characterized by increased expression of MHC II and co-stimulatory molecules, whereas in the absence of B cells, respiratory tolerance could not be induced [27]. In the present study we will explain the role of thymic B cells in the generation of nTreg in *in vitro* model.

2. Materials and methods

2.1. Mice

8–12 weeks old B6.Cg-Foxp3^{tm2Tch} male mice (expressing Foxp3 protein in co-expression of GFP protein) were used in experiments. Mice were bred and maintained in the Animal Facility at the Department of Biology, University of Warsaw, in the individually ventilated cages system (IVC), LD 12/12, with free access to standard food and water. All the procedures involving animal studies have been approved by the Local Ethic Commission.

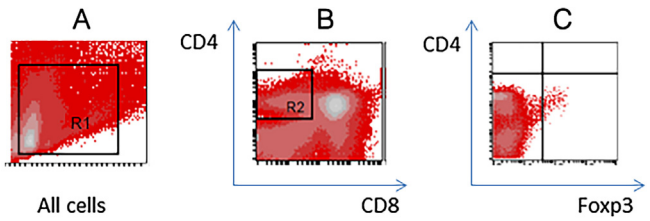

Fig. 1. Gating strategy for the analysis of CD4+Foxp3+ nTregs.
All cells in *in vitro* culture have been gated in R1 (A). To perform further analysis on nTregs, SP CD4+ cells have been gated (R2) for further analysis (B). Gated CD4+ thymocytes have been analyzed for Foxp3 expression as CD4+Foxp3+ (C).

2.2. B cell activation

Two activators for B cells were used in the experiments of current study dependent on the results of other authors. Lipopolysuccharide (LPS) from *Escherichia coli* (0111:B4) (1 μg\ml as high dose, and 0.01 μg\ml as low dose) [2], and Imiquimod (IMQ) (5 μg\ml as high dose, and 1 μg\ml as low dose) [28] were used for B cells activation *in vitro*. High dose of IMQ excluded from next experiments due to similarities between the results with low dose.

2.3. Cell culture

Cell cultures were conducted in 24 well flat-bottom plates at the density of 2×10^6 cells/ml in RPMI-1640 + Glutamax supplemented with antibiotics (Penicillin 50 mU/ml and Streptomycin 50 ng/ml),10% fetal bovine serum, 1 mM sodium pyruvate and 0.2 mM 2-mercaptoethanol for 24, 48, and 72 h, at 37 °C in a 5% CO_2 humidified atmosphere. 48 h cultures were excluded from next experiments because the expression of studied molecules was approximately similar to 24 or 72 h.

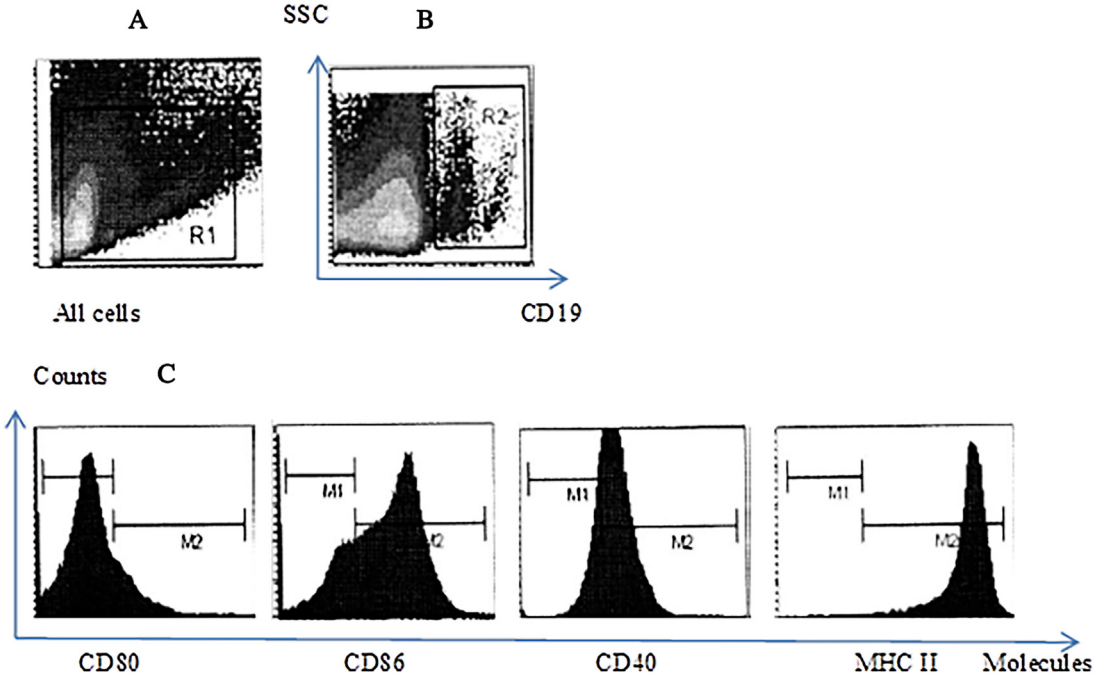

Fig. 2. Gating strategy for the analysis of MHC II as well as costimulatory molecules on thymic B cells.
Thymic B cells in *in vitro* culture of thymocytes have been gated in R1 (A). To perform further analysis on B cells only, CD19+ B cells have been gated (R2) (B). MHC II and costimulatory molecules have been investigated (C), all histograms were derived from control (non-activated) samples.