Journal of Autoimmunity 59 (2015) 53–60

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

B-Cells induce regulatory T cells through TGF-β/IDO production in A CTLA-4 dependent manner☆

A. Nouël [a], P. Pochard [a, 1], Q. Simon [a, 1], I. Ségalen [a, b], Y. Le Meur [a, b], J.O. Pers [a, *], S. Hillion [a]

[a] EA2216, INSERM ESPRI, ERI 29, Université de Brest and LabEx IGO, Brest, France
[b] Department of Nephrology, CHRU Cavale Blanche, Brest, France

ARTICLE INFO

Article history:
Received 17 October 2014
Received in revised form
11 February 2015
Accepted 16 February 2015
Available online 7 March 2015

Keywords:
Regulatory B cells
TGF-beta
Indoleamine 2.3-dioxygenase
CTLA-4

ABSTRACT

A number of studies have suggested that B cell mediated-regulation contributes to the establishment of immunological tolerance. However, the precise mechanisms by which regulatory B cells establish and maintain tolerance in humans remain to be determined. The objective of the current study is to understand the cellular and molecular bases of B-cell regulatory functions in humans. To describe the mechanisms regulating the functional plasticity of regulatory B cells, we used an *in vitro* co-culture model based on autologous mixed lymphocyte cultures involving freshly isolated B and T cells. The results show that activated B cells regulate T cell proliferation through producing transforming growth factor (TGF)-β and indoleamine 2,3-dioxygenase (IDO). The production of TGF-β and IDO leads to the induction of not only "natural" regulatory T cells but also of TGF-β-producing $CD4^+$ T cells and IL-10-producing regulatory T cells. Furthermore, we evidenced for the first time that CTLA-4 induces B-cells to produce IDO and to become effective induced regulatory B cells (iBregs). This study emphasizes a novel regulatory axis and open news insights in how to manage regulatory B cell functions in autoimmunity.

1. Introduction

The bio-pathological processes that underpin susceptibility to auto-immune diseases have been extensively explored by many investigators but remain largely undefined. The vast majority of these studies focused on mechanisms that trigger effector T lymphocytes since these cells were considered as the initiators of harmful immunological responses, including the production of pathogenic antibodies by B lymphocytes. However, targeting T cells as therapeutic approaches for treating such diseases have proved to be largely ineffective. In recent years, B lymphocyte functions have been reconsidered, and it is now accepted that these cells play much more diverse functions than simply just producing antibodies [1]. The key role played by B-cells in autoimmune diseases is further supported by therapeutic efficacy of B cell-ablation strategies for treatment [2]. Interestingly, a number of studies identified potential adverse effects in total B cell ablation suggesting that some B cell subsets with regulatory functions may provide ameliorating effects in many pathological settings [3].

Regulatory B lymphocytes were discovered in the past decade, principally, in studies involving murine models of autoimmune diseases. The cells were termed Breg cells, based on their ability to dampen or even suppress effector immune processes [4]. The most widely-studied immunosuppressive mechanism through which Breg cells exert their immune regulatory functions is generally-believed to be interleukin (IL)-10 based [5]. IL-10 production is one of the most important anti-inflammatory cytokines capable of down-regulating inflammation [6]. Although stimulated B cells produce IL-10, the importance of regulatory B cell-derived IL-10 in immunosuppression was only recently revealed. In 2002, Mizoguchi et al. identified a subset of IL-10-producing $CD1d^+$ B cells in gut-associated lymphoid tissues during the course of chronic intestinal inflammation [7]. When these Breg cells were enriched or transferred into recipient mice with intestinal inflammation the cells mediated an IL-10-dependent suppression of the inflammation. In a murine model of autoimmune encephalomyelitis (EAE), a key role for IL-10-producing splenic B cells was highlighted as important for controlling the disease [8]. In humans, Blair and

☆ This study was supported by a grant from "Investissements d'Avenir" program ANR11-LABX-0013-01.

* Corresponding author. Laboratory of Immunology, Brest University Medical School Hospital, BP824, F-29609, Brest, France. Tel.: +33 298 22 33 84; fax: +33 298 22 38 47.
E-mail address: pers@univ-brest.fr (J.O. Pers).

[1] P.P. and S.Q contributed equally to this work.

http://dx.doi.org/10.1016/j.jaut.2015.02.004
0896-8411/

colleagues subsequently demonstrated that CD24^{high} CD38^{high} Breg cells inhibited interferon (IFN)γ and tumor necrosis factor (TNF)α production by autologous co-cultured T-cells through the production of IL-10 and that Breg function is deficient in patients with systemic lupus erythematosus (SLE) [9]. The existence of Breg cells in humans was subsequently confirmed in various *in vitro* models [4,10,11]. However, beyond the IL-10-mediated suppressive effects, strong evidence exist that part of the immunosuppressive effects of Breg cells is dependent on interactions with other regulatory cell lineages. Thus, repeated nasal antigenic challenge induced a Breg population that suppressed lung inflammation and asthma in recipient OVA-sensitized mice following adoptive transfer. The effectiveness of these Breg cells was linked to the generation of CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cell (Treg cells) that produced transforming growth factor-β (TGF-β) [12]. Other studies revealed that human Breg cells can inhibit T cell proliferation through cell-to-cell contact, leading to anergy, or T cell apoptosis [13]. Our own studies revealed that activated human CD19$^+$ IgD$^+$ CD38^{high} CD5$^+$ B cells strongly suppressed autologous T cell proliferation independently of IL-10 production [14]. Although effectiveness of induced Breg cells (iBregs) has been clearly demonstrated, the precise mechanisms through which these cells control immune responses remain to be elucidated.

Indoleamine 2,3-dioxygenase (IDO) enzyme degrades the essential amino-acid tryptophan and promotes immune tolerance by inhibiting T cell activation [15]. Inhibition of IDO with a competitive inhibitor, 1-methyl-tryptophan (1 MT) resulted in rejection of pancreatic islet allograft and aggravated autoimmunity [16,17]. The production of IDO is transitory induced in dendritic cells (DC) by IFN-γ whereas TGF-β mediates durable IDO-dependent regulatory functions [18]. Cytotoxic T lymphocyte associated antigen-4 (CTLA-4), is a central inhibitory regulator of T-cell proliferation and expansion [19]. Mice genetically deficient in CTLA-4 develop a massive lymphoproliferative disorder and an early lethality [20,21]. CTLA-4 pathway through ligation to CD80 and CD86 on antigen presenting cells (APC) has been shown to upregulate Foxp3 expression induced by TGFβ leading to induction of adaptative CD4$^+$ CD25$^+$ regulatory T cells [22]. Moreover CTLA-4 engagement of B7 ligands on DCs can induce release of IDO involved in maintenance of peripheral tolerance [23].

These findings strengthen the need for better characterize the mechanisms involved in Breg cell functions. Such insights would be an essential step to therapeutically exploit the function of Breg cells in diseases. In this context, we have presently investigated, in *in vitro* models, the mechanisms by which human iBreg cells regulate T cell functions. We reveal a novel regulatory pathway in B cells, that is mediated by the TGF-β/IDO axis in a CTLA-4 dependent manner. This novel regulatory pathway in Breg cells provides news perspectives for future treatment strategies in autoimmune diseases.

2. Materials and methods

2.1. Cell isolation

Peripheral blood from the healthy controls (HC) was collected, layered onto Ficoll-Hypaque and fractionated by centrifugation. Mononuclear cells were incubated with neuraminidase-treated sheep red blood cells and T cells depleted by a second 30-min round of centrifugation. B cells were further enriched by negative selection with a B cell enrichment kit (CD19$^+$CD43$^+$ Stem Cell Technologies) according to the manufacturer's instructions. All preparations were >97% B cells (CD19$^+$CD11c$^-$HLADR$^+$) excluding dendritic cells or >98% CD19$^-$ CD5$^+$ T cells.

2.2. Cell culture

Purified cells were cultured in 96-well plates in complete medium (RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine (Invitrogen Life Technologies), 200 U/ml penicillin, and 100 μg/ml streptomycin). T cells were seeded at 4×10^4 cells/ml on AffiniPure F(ab')2 fragment of goat anti-mouse IgG (Jackson Immunoresearch) coated onto wells of culture plate. Cells were stimulated with anti-CD3 (OKT3; 0.2 μg/ml; BioLegend, CA) and anti-CD28 (CD28.2; 0.2 μg/ml; Beckman Coulter) monoclonal anti-bodies (mAbs). For co-culture experiments, B cells were seeded at 4×10^4 cells/ml with CpG (human ODN 2006; 0.25 μM; Cayla-InvivoGen) and co-cultured with the T cells for 5 days at a 1:1 ratio as already described [24]. Blocking experiments were carried out with polyclonal anti-TGF-β Ab (5 μg/ml; Abcam), anti-IL-10 Ab (JES3-9D7; 5 μg/ml; BD Biosciences), anti-CTLA-4 Ab (L3D10; 10 μg/mL Biolegend) or by treatment with 1 MT (1-Methyl-DL-tryptophan; 600 μM; Sigma–Aldrich), an inhibitor of IDO. Isotype control for each of the used specific antibodies was used at the same concentration as the specific Ab.

2.3. Proliferation assays

Freshly isolated T cells were labeled with CFSE (5 μM; Invitrogen-Molecular Probes) before stimulation. T cell proliferation was evaluated by flow cytometry (FC500, Beckman Coulter) using cell proliferation index of the CFSE-labeled T cells in the FlowJo® software. Inhibition of proliferation was the percentage of the proliferation index in T cells alone compared to T cells co-cultured with B-cells. In co-culture experiments, cells were stained with PE-linked to Cyanin 7 (PC7)-conjugated anti-CD19 mAb (J3-119; Beckman Coulter), and proliferation index analyzed for CD19-negative cells.

2.4. ELISA assays

Commercial ELISA kits with paired Abs were used to detect IFNγ and TNFα (Beckman Coulter) and IL-10 (BD Biosciences) in the culture supernatants. The different ELISAs were performed according to the manufacturers' instructions.

2.5. Flow cytometry and cell sorting

In co-culture experiments, intracellular staining for IL-10, TGF-β and IDO was carried out after cell permeabilization using cytofix/cytoperm permeabilization kit (BD Biosciences). FITC-conjugated anti-IL-10 (127107; R&D Systems), PE-conjugated anti-TGF-β (1D11; R&D Systems) and polyclonal sheep anti-human IDO Abs (Thermo Fisher Scientific) were used in combination with PE-linked to Cy5 (PC5) and anti-CD4 mAb or PC7 anti-CD19 mAb. The secondary Ab used to detect IDO was AffiniPure F(ab')2 fragments of FITC-conjugated donkey anti-sheep IgG Ab (Jackson Immunoresearch). Foxp3-positive T cells were measured using PE-conjugated anti-Foxp3 Ab (236A/E7; BD Biosciences) according to the manufacturer's instructions. Isotype-matched mAbs were used as negative controls for IL-10, TGF-β, IDO, CTLA-4 and Foxp3 staining to demonstrate specificity and to establish background-staining levels. CTLA-4 staining was performed using APC-conjugated anti-CTLA-4 Ab (L3D10 Biolegend) in combination with FITC-anti-CD4 mAb. In some experiments CD19$^+$ CD5$^-$ B cells and CD5$^+$ CD19$^-$ T cells were sorted after 3 days of coculture using Moflo™ XDP (Dako-Beckman Coulter). The purity of B and T cells was greater than 98%.