Immunobiology 217 (2012) 307–315

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

# Polymyxin B increases the depletion of T regulatory cell induced by purinergic agonist

Claudio Cappelli[a,1], Ximena López[a,1], Yohana Labra[a], Margarita Montoya[a], Ricardo Fernández[b], Mónica Imarai[a], Juan Luis Rojas[c], Dante Miranda[d], Alejandro Escobar[e], Claudio Acuña-Castillo[a,c,*]

[a] Centro de Biotecnología Acuícola and Departamento de Biología, Facultad de Química y Biología, Chile
[b] Departamento de Ciencias Biologicas, Facultad de Ciencias Biologicas y Facultad de Medicina, Universidad Andres Bello, 837 0134 Santiago, Chile
[c] Laboratorio Emory Black, Facultad de Ciencias Médicas, Universidad de Santiago de Chile (USACH), Alameda 3363, Santiago, Chile
[d] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas Universidad de Chile, Sergio Livingstone 1007, Independencia, Santiago, Chile
[e] Departamento de Ciencias Básicas Facultad de Odontología, Universidad de Chile, Sergio Livingstone 943, Independencia, Santiago, Chile

ARTICLE INFO

*Article history:*
Received 13 April 2011
Received in revised form 11 October 2011
Accepted 18 October 2011

*Keywords:*
P2X7 receptor
Polymyxin B
T regulatory cells
Immune response
Purinergic agonist

ABSTRACT

Regulatory T cells (Treg) are important in the development of immune tolerance under normal physiological conditions. However, in pathological situations such as cancer, Treg increases have been correlated with bad prognoses. Treg depletion can be achieved *in vitro* under several stimuli, including the activation of the purinergic P2X7 receptor. Our aim was to determine whether polymyxin B (PMB), which is a positive modulator of this receptor, could affect mice Treg depletion by ATP and related compounds. For that purpose, we evaluated by flow cytometry changes in Treg populations under several treatments with PMB and/or purinergic agonists and antagonists. We found that both ATP and NAD induce a dose-dependent decrease on the Treg CD4+ CD25+ population. PMB not only potentiated the effect of exogenous ATP and NAD, but also decreased the CD4+ CD25+ population when it was applied alone. While ATP mediated effects are related to the P2X7 receptor, PMB effects appear to be related to another mechanism. We conclude that PMB positively modulates the depletion of the CD4+ CD25+ population of Treg. Therefore PMB could constitute a non-canonical drug with potential use on Treg depletion and cancer treatment.



## Introduction

Regulatory T CD4+ cells (Treg) are essential for immune homeostasis acting as negative regulators which help prevent autoimmune diseases. Several subtypes of Treg cells have been described on the basis of their origin, generation, and mechanism of action. Currently, there are two main origins of Treg cells identified, either natural or thymically derived Foxp3+ Treg cells, or inducible Treg cells. Phenotipically, CD4+ Treg cells are characterized by expression of the interleukin 2 (IL-2) receptor α chain (CD25), the transcription factor Foxp3, cytotoxic T-lymphocyte antigen-4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR). This population is referred as Treg CD4+ CD25+ (reviewed in Gavin and Rudensky 2003; Gavin et al. 2007; Ohkura and Sakaguchi 2010; Wing and Sakaguchi 2010).

In addition to the physiological functions, several current lines of evidences support a role for Tregs in tolerance response that favors tumor development. Several solid tumors show an accumulation of Foxp3+ CD25+ CD4+ Treg cells in both humans and rodents. This infiltration apparently hinders immune response to tumor cells, and moreover appears to impede the antitumor immune responses in cancer patients (Needham et al. 2006; Nishikawa and Sakaguchi 2010). The important role of Treg cells in suppressing anti-tumor immune responses can be deduced by the fact that Treg cell levels are significantly increased in tumor bearing individuals (Curiel et al. 2004; Woo et al. 2002). Also, animal studies show that depletion of CD25+ T cells results in enhanced anti-tumor immunity (Jones et al. 2002; Sutmuller et al. 2001). Therefore, Treg cells create an immunosuppressive environment in tumor bearing hosts that may further impede successful immunotherapies. In order to minimize the effect on tumor establishment, several therapeutic approaches have been developed to modify the tumor microenvironment and target Treg cells. In animal models, varying strategies have been

*Abbreviations:* PMB, polymyxin B; Treg, regulatory T cells; IL-2, interleukin 2; CD25, interleukin 2 receptor α chain; BBG, brilliant blue G; oATP, oxidized ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate; NAD, nicotinamide adenine dinucleotide; BzATP, benzoyl-*ATP*; ATP, adenosine-5′-triphosphate.

* Corresponding author at: Universidad de Santiago de Chile, USACH, Alameda 3363, Estación Central, Santiago, Chile. Tel.: +56 2 7181109.
*E-mail address:* claudio.acuna@usach.cl (C. Acuña-Castillo).

[1] Equally contributed.

0171-2985/$ – see front matter. 
doi:10.1016/j.imbio.2011.10.006

developed including, for example, the application of low doses of cyclophosphamide (Salem et al. 2008). These and other therapies, including administration of several drugs used in clinical trials have been reviewed extensively elsewhere (Needham et al. 2006; Nishikawa and Sakaguchi 2010; O'Day et al. 2007; Schabowsky et al. 2007).

Dennert's group works determined that, in contrast to other lymphocytes, CD4+ CD25+ Treg cells highly express functional purinergic P2X7 receptors (Aswad et al. 2005; Kawamura et al. 2005). The P2X7 receptor is a nonselective cationic channel member of the ATP-gated P2X receptor family. This receptor is the most divergent member of the P2X family; high ATP concentrations induce not only the opening of the cationic channel, but also the uptake of molecules up to 900 Da due to a "macropore formation", which is associated with apoptotic cell death (North 2002). P2X7 receptors are mainly expressed in the immune system, and their activation seems to be important for the release of mature IL-1β and other pro-inflammatory cytokines, such as IL-18 and TNF-α from macrophages and dendritic cells (Di Virgilio 2007). P2X7 receptors are sensitive to specific modulation by several compounds, such as divalent cations (Acuña-Castillo et al. 2007). Interestingly, Ferrari and coworkers reported that the antibiotic polymyxin B (PMB) also modulates the responses elicited by P2X7 receptor activation in cells expressing either the native or recombinant receptor.

In the present work we tested whether PMB could sensitize Treg cells to ATP-induced effects. We corroborated that ATP reduced CD4+ CD25+ and CD4+ CD25+ Foxp3 cell populations cultured *in vitro*, in a concentration-dependent manner, and showed that PMB by itself is able to reduce Treg cell populations, in a P2X7 unrelated fashion. As expected, PMB augments ATP-induced Treg cell depletion, while the conventional CD4+ cell population remains unchanged after the treatments. Altogether, our results show that PMB used in therapeutic doses is able to reduce the number of Treg cells by modulating the P2X7 receptor, with a potential therapeutic use in the antitumoral response.

## Materials and methods

### *Animals, cells and chemicals*

C57BL/6 mice were used in this study after institutional review and board approval. Animal care was in compliance with recommendations of the Guide for Care and Use of Laboratory Animals, National Research Council. Male or female 6- to 8-week-old C57BL/6 mice were obtained from the USACH Research Facility. Animals were euthanized by cervical dislocation, spleens were removed under sterile conditions, and splenocytes were obtained free of erythrocytes by treatment with ACK lysis buffer. Splenocytes were cultured in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% FBS (Biological Industries Ltd., Kibbutz Haemek 25115, Israel), 50 U/mL penicillin–streptomycin, and 2.5 μg/mL amphotericin B (Sigma–Aldrich, St. Louis, Mo, USA). All salts used were of analytical degree. Antibodies to CD4 and CD25 were purchased from Santa Cruz Biotechnology (clones RM-4 and PC61, respectively). Antibody to CD8 was obtained from BD Biosciences Pharmingen (San Diego, CA). Regulatory T cell kit detection assay was obtained from eBioscience (San Diego, CA). Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences Pharmingen. ATP determination kit was purchased from Promega. A740003 was purchased from Tocris (*Tocris* Bioscience, Ellis-ville, MO, USA), polymyxin B (PMB), brilliant blue G (BBG), oxidized ATP (oATP), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonate (PPADS), BzATP and ATP were purchased from Sigma Aldrich (St. Louis, MO, USA), and all salt used were of analytical grade.

### *Fluorescence-activated cell sorting (FACS) analysis and ATP analysis*

To monitor induction of cell death, splenocytes were incubated with various concentrations of P2X7 agonist ATP, BzATP and NAD (Sigma Aldrich, St. Louis, MO, USA). Sometimes cells were previously preincubated for 30 min with different antagonists and challenged with 60 or 100 μM ATP (Sigma–Aldrich) or, unless other conditions are specified, cultured in RPMI 1640 supplemented with 10% FBS, 50 U/mL penicillin–streptomycin, and 2.5 μg/mL amphotericin B. The effects of the treatment were evaluated after 24 h of culture. Total CD4+ CD25+ and foxp3+ populations were evaluated at 24 h after challenge as previously described by Leiva-Salcedo et al. (2011). In parallel, cells were recovered 3 h after-treatment and stained to identify CD4+ and CD4+ plus CD25+ cells with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). Stained cells were detected in a FACS Canto II (Becton Dickinson, NJ, USA).

To determine whether treatment induced an increase on ATP extracellular levels, culture media from 24 h treated cells were recovered, frozen and kept until use. Samples were unfrozen and ATP concentration was determined in a Luminuskan Ascent Thermo reader (Thermo Fisher Scientific, USA) using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA).

### *Data acquisition and analysis*

All protocols were performed at least five times in each animal. ATP-induced activation was evaluated in parallel with other protocols. Point-to-point analysis was performed using the Kruskal–Wallis test, with a Dunn post-test. All data were analyzed using GraphPad software (GraphPad software Inc., La Jolla, CA, USA) and are shown as average ± standard error (SE). Statistical differences were considered with $p < 0.05$.

## Results

### *Purinergic agonist depletes Treg cell subpopulation in vitro*

Previously, it was demonstrated that spleen CD4+ CD25+ Treg cells of mice are sensitive to depletion by NAD and ATP in a P2X7-dependent manner (Haag et al. 2007). In order to corroborate those results, splenocytes from C57BL/6 mice were isolated and incubated for 24 h in the presence of various ATP concentrations, and the effect on CD4+, CD8+, and CD4+ CD25+ T cells was quantified by flow cytometry; representative dot plots are shown in Fig. 1A. CD4+ and CD8+ populations are expressed as percentage of total splenocytes, whereas CD4+ CD25+ cells (and also Foxp3+) are shown as percentage of CD4+ total, unless otherwise specified. As shown in Fig. 1B left panel the CD4+ CD25+ T cell population appears sensitive to ATP treatment in a dose-dependent manner. Quantification of independent experiments showed a maximal Treg cell depletion of 75 ± 3% obtained at 600 μM ATP, with an apparent median effective concentration between 60 and 100 μM ATP. As shown in Fig. 1B right panel, CD8+ T cells are insensitive to ATP treatment, whereas CD4+ (those negative for CD25) appear to be sensitive to ATP concentrations higher than 100 μM. Next, the effects of NAD on CD4+ CD25+ T cell populations were evaluated (Fig. 1A, representative dot plots). In agreement with previous reports, NAD induced Treg depletion at lower concentrations than ATP with an apparent median effective concentration of 10–30 μM (Fig. 2A, left panel). NAD appears to be even less specific than ATP, because at concentrations above 30 μM it induced a significant depletion in both CD4+ and CD8+ cell populations (Fig. 2B, right panel). To confirm the involvement of P2X7 receptors on purinergic induced Treg depletion, we compared the depletion using the preferred P2X7 agonist