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

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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.

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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

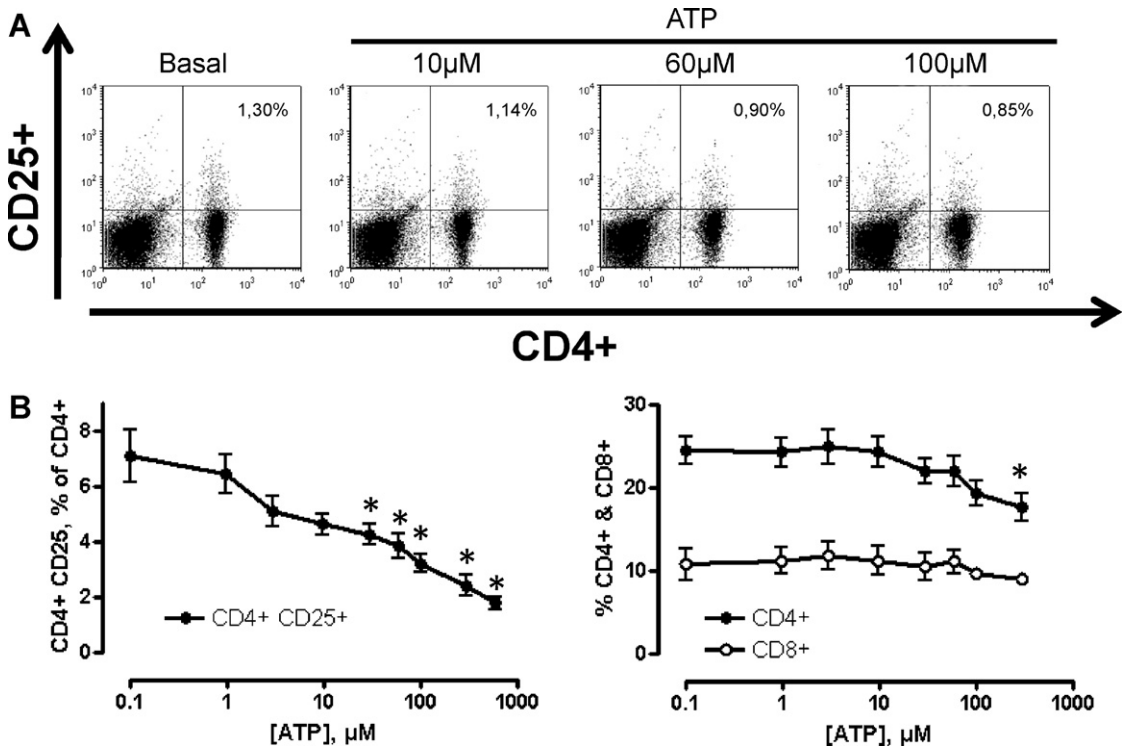

Fig. 1. CD4+ CD25+ cells are sensitive to ATP and NAD induced cell death. B6 spleen cells were incubated with increasing concentrations of ATP for 24 h, then stained for CD25, CD4, CD8, and analyzed by FACS as shown in representative dot plots. Representative dot plots are shown for CD4+ CD25+ population treated with 0, 10, 60 and 100 μM ATP are shown in A (from left to right). ATP (B, $n = 12$) induced effects were evaluated on independent experiments, and results were quantified and graphed for CD4+ CD25+ cell (B left panel, $p = 0.001$ Kruskal–Wallis test) and for CD4+ and CD8+ (closed and open circles on B right panel, respectively. CD4 $p = 0.001$, CD8 $p = 0.103$ by Kruskal–Wallis test). Data are expressed as mean ± SE, * represent a statistically significant effect for Dunn post test analysis ($p < 0.05$).

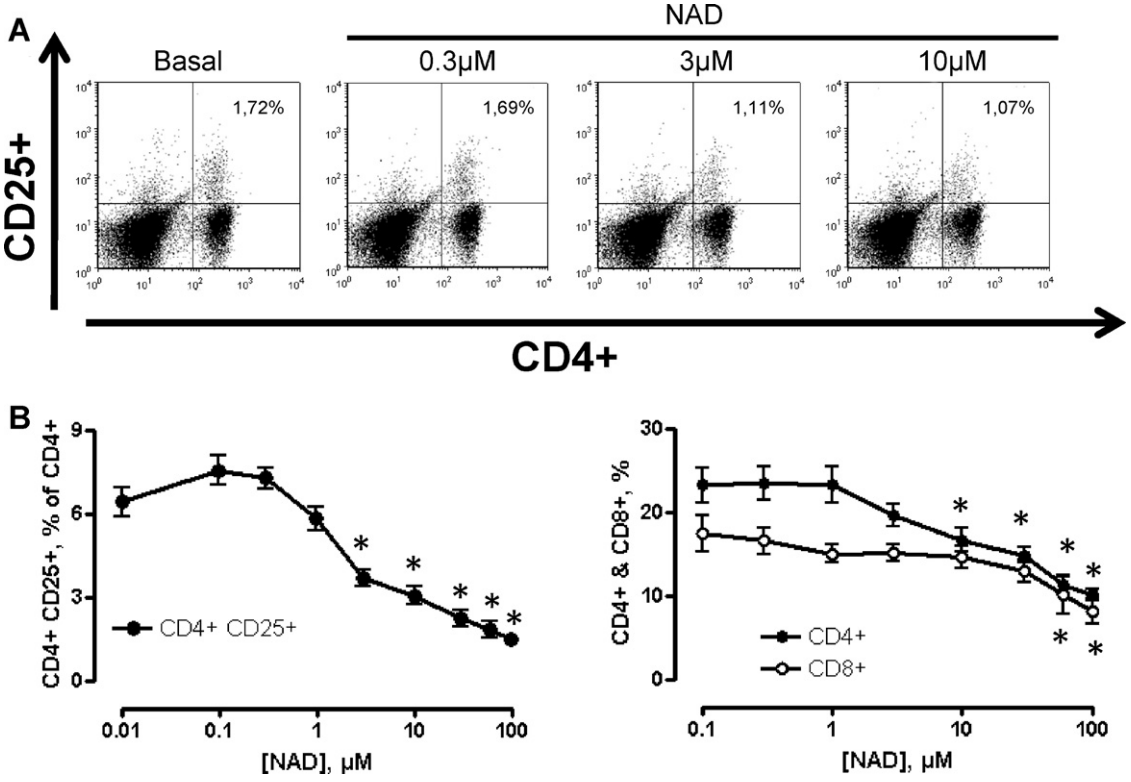

Fig. 2. CD4+ CD25+ cells are sensitive to NAD induced cell death. B6 spleen cells were incubated with increasing concentrations of NAD for 24 h, then stained for CD25, CD4, CD8, and analyzed by FACS as shown in representative dot plots. Representatives dot plots are shown for CD4+ CD25+ population challenged with 0, 0.3, 3 and 10 μM NAD are shown in A (from left to right). NAD (B, $n = 6$) induced effects were evaluated on independent experiments, and results were quantified and graphed for CD4+ CD25+ cell (left graph, $p = 0.001$ by Kruskal–Wallis test) and for CD4+ and CD8+ (closed and open circles on B right panel, respectively. CD4 = 0.001, CD8 $p = 0.012$ by Kruskal–Wallis test). Data are expressed as mean ± SE, * represent a statistically significant effect for Dunn post test analysis ($p < 0.05$).

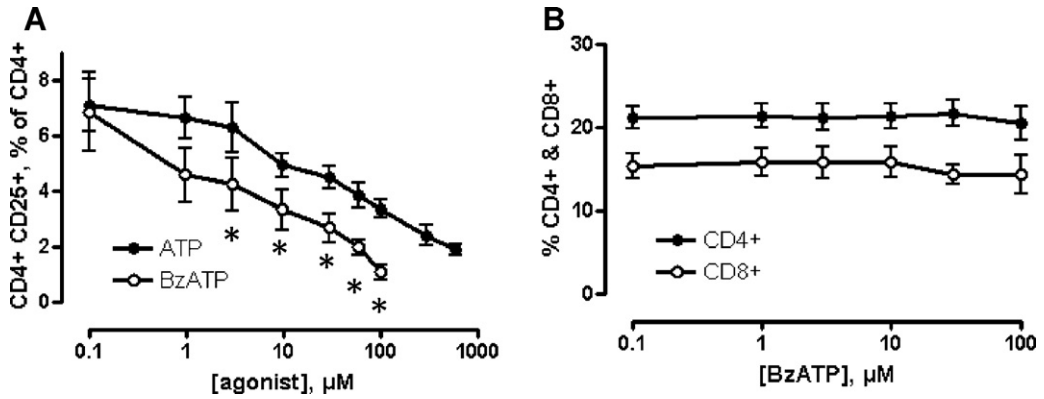

Fig. 3. CD4+ CD25+ cells are more sensitive to BzATP than ATP. B6 spleen cells were incubated with increasing concentrations of BzATP or ATP for 24 h, then stained for CD25, CD4, CD8, and analyzed by FACS as shown in representative dot plots. BzATP (open circles) and ATP (closed circles) induced effects were evaluated on independent experiments, and results were quantified and graphed for CD4+ CD25+ cell (A, $p > 0.0001$ by Kruskal–Wallis test). Similarly the BzATP induced effect was evaluated on CD4+ and CD8+ cell populations (closed and open circles on B right panel, respectively. CD4 $p > 0.1$, CD8 $p > 0.1$ by Kruskal–Wallis test). Data are expressed as mean ± SE, * represent a statistically significant effect for Mann–Whitney point-to-point analysis ($p < 0.05$).

BzATP (Fig. 3A). BzATP induced a dose-dependent Treg depletion with a maximal effect at 100 μM, closed to 10-fold more effective than those obtained with ATP (compare open and closed circles) Furthermore this effect was Treg specific at and did not affect conventional lymphocytes (Fig. 3B).

Polymyxin B depletes Treg cell subpopulation in vitro

Next we evaluated the effect of PMB, a P2X7 positive modulator (Ferrari et al. 2004). Strikingly, PMB alone induced Treg cell depletion in a dose-dependent fashion, becoming significant at 1 μg/mL, reaching a plateau of 50 ± 8% of depletion at 6 μg/mL (Fig. 4A and B, left panel). In contrast to ATP, the PMB effect was specific for the CD4+ CD25+ population, since none of the concentrations induced significant depletion of both CD4+ and CD8+ populations (Fig. 4B, right panel). Then we evaluated the specific effect of the treatments on CD4+ CD25+ Foxp3+ T cells. As shown in Fig. 5, CD4+ CD25+ Foxp3+ T cells were sensible to ATP, NAD and PMB similar to CD4+ CD25+ T cells as representative dot plots in upper panel, middle panel and lower panel, respectively. A summary of all experiments are showed in Fig 5B. In our experimental model, Foxp3+ cell population levels corresponded to 85 ± 5% of

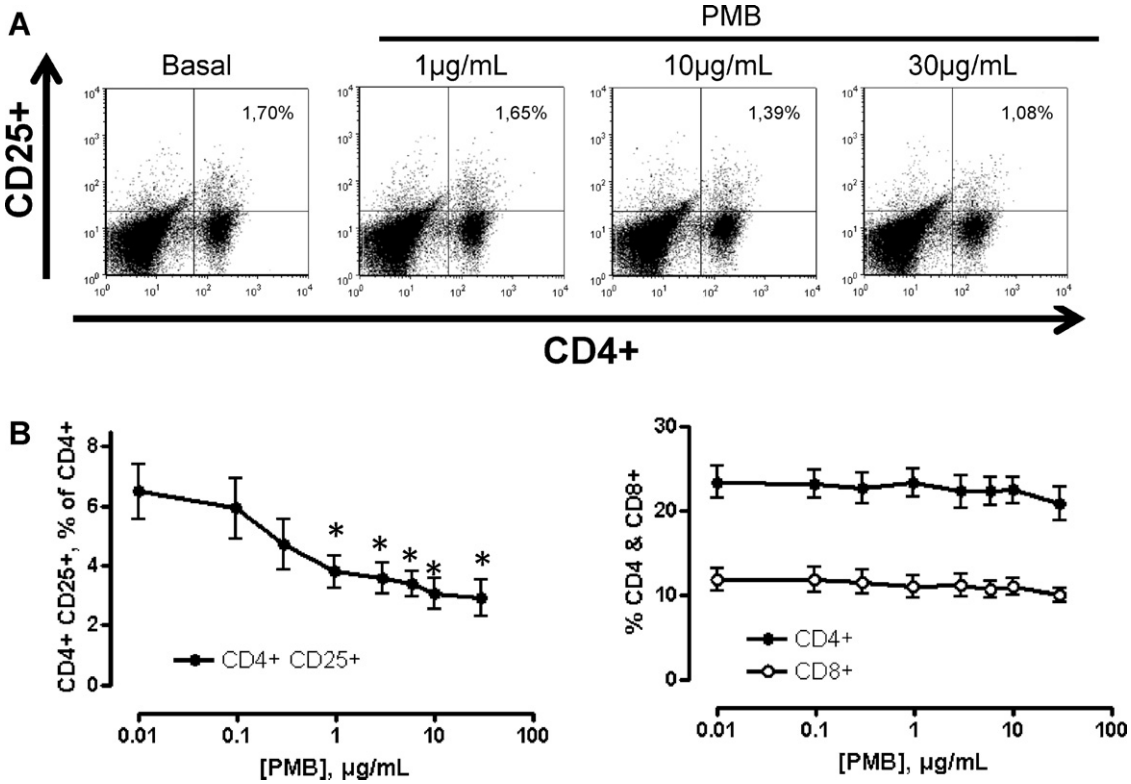

Fig. 4. CD4+ CD25+ cells are sensitive to PMB-induced cell death. B6 spleen cells were incubated with increasing concentrations of PMB for 24 h, then stained for CD25, CD4, CD8, and analyzed by FACS. (A) Dot plots showing representative effects for CD4+ CD25+ population treated with 1, 10, 30 mg/mL PMB. Quantification of 8–10 independent experiments are shown for CD4+ CD25+ population in the left panel on B ($n = 4$–8, $p = 0.002$ by Kruskal–Wallis test), and in the right panel CD4+ (open circles, $n = 12$, $p = 0.215$ by Kruskal–Wallis test) and CD8+ (closed circles, $n = 8$, $p = 0.422$ by Kruskal–Wallis test) are shown. Data are expressed as mean ± SE, * represent a statistically significant effect for Dunn post test analysis ($p < 0.05$).

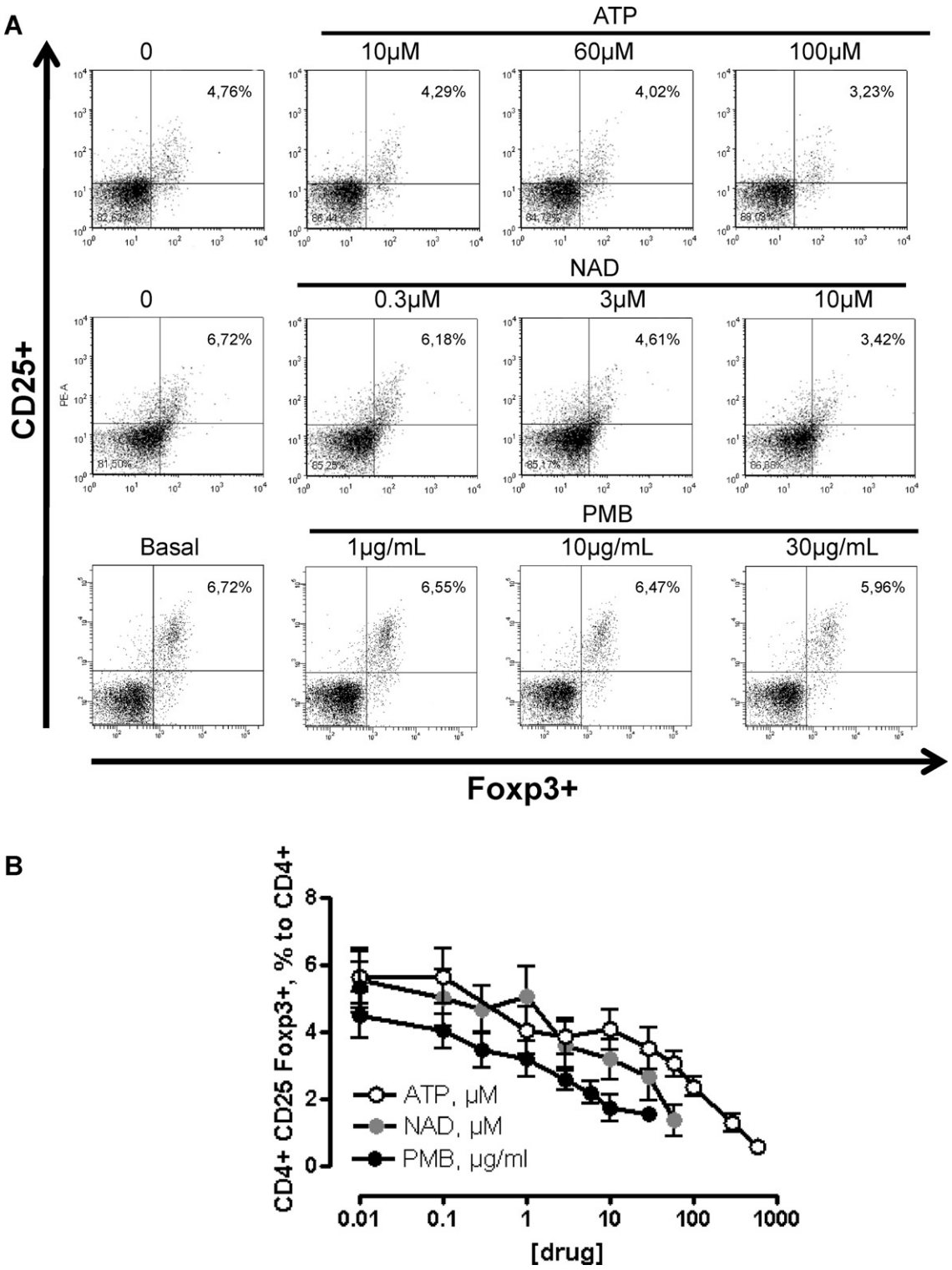

Fig. 5. Foxp3 CD4+ CD25+ cells are sensitive to ATP, NAD and PMB-induced cell death. B6 spleen cells were incubated with increasing concentrations of ATP, NAD and PMB for 24 h, then stained for CD25, CD4, CD8, and analyzed by FACS. The effect of some concentration of drugs: 0, 10, 60 and 100 μM ATP (A), 0, 0.3, 3 and 10 μM NAD (C) and 1, 10, 30 mg/mL PMB (D) on foxp3+ CD4+ CD25+ population are shown in dot plots (B). Quantifications of 8–10 independent experiments are shows. Representative dots plots are showed for CD25+ Foxp3+, from CD4+ cell gated. Data are expressed as mean ± SE, * represent a statistically significant effect ($p < 0.05$).

the total CD4+ CD25+ population, similar to previous reported values (Sakaguchi et al. 2008, 2009). Therefore, once we determined that in mice most CD4+ CD25+ cells are also Foxp3 positive, further analyses were done using only CD4+ CD25+ markers, unless others are specifically indicated.

In view that PMB itself is able to induce Treg depletion, it is reasonable to think that the treatment could induce ATP delivery resulting in a positive modulation of P2X7 by PMB. In order to determine this, we measured ATP levels after PMB treatment, but we did not detect any increase on extracellular ATP levels in

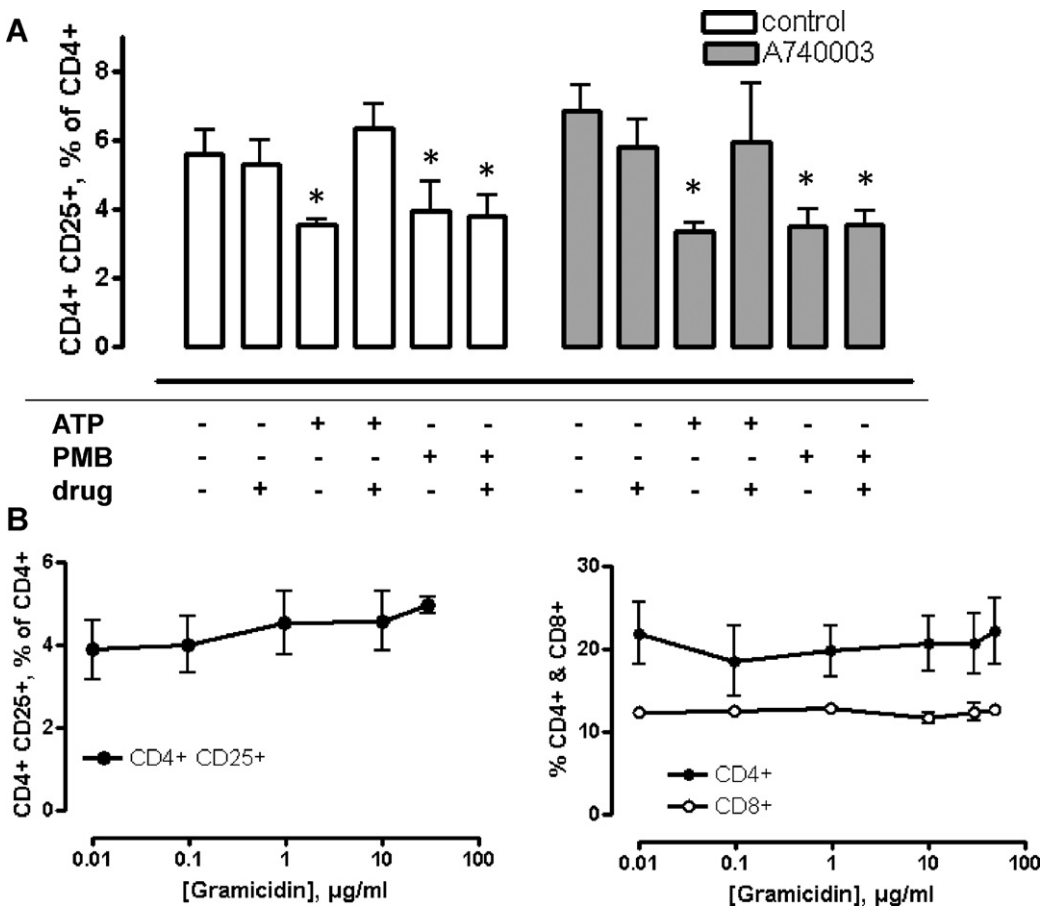

Fig. 6. PMB induced a specific effect independent of P2X7 receptor activation. (A) The effect of P2X7 unspecific antagonist BBG (white bars) and the specific antagonist A740003 (black bars) were evaluated on the effects induced by 1 μg/mL PMB, 60 μM ATP and ATP plus 1 μg/mL PMB. (B). The PMB effect was compared to gramicidin, another cyclic peptide with similar activity on CD4+ CD25+ population (B, left graph) and on conventional CD4+ and CD8+ cells (B, right graph closed and open circles, respectively). Data are expressed as mean ± SE, * represent a statistically significant effect for Wilcoxon paired test compared to untreated conditions ($p < 0.05$).

response to treatment compared to control ($p > 0.52$). In view that PMB mediated effects appear to be independent of endogenous extracellular ATP, our next approach was to pharmacologically evaluate the role of P2X7 receptor on this phenomenon. We evaluated the effect of the broad P2X antagonist, brilliant blue G (BBG), and the P2X7 specific antagonist A740003 on the PMB and ATP mediated depletions (Fig. 6A). While BBG and A740003, abolishing the effect induced by ATP, did not affect the PMB induced Treg depletion (Fig. 6A). However BBG and PPADS abolished the small Treg depletion induced by lower PMB concentrations (data not shown). As we expected, neither BBG nor A740003 were harmless for CD4+ conventional cells. PMB and other cyclic peptides such as gramicidin were able to induce IL1-beta maturation and secretion, and cell fusion. In order to determine the specificity of PMB activity on Treg depletion we evaluated the effect of gramicidin. As shown in the figure 6 gramicidin was unable to induce depletion in both Treg and conventional lymphocytes (Fig. 6B, left and right panel, respectively). Independent of those results, our next aim was to determine whether the PMB effect could be directly related to P2X7 activation.

PMB effect is additive to ATP-induced effect on CD4+ CD25+ T cells in vitro

In view that PMB and ATP mediated effects appear to be driven by different mechanisms, we evaluated whether is possible to induce an additive effect of PMB and ATP on Treg depletion. Fig. 7A, left panel shows the effect of 2 PMB concentrations on ATP treated population. PMB 1 μg/mL slightly modified the ATP-induced effect, and PMB at 10 μg/mL caused a significant downshift on Treg depletion. Similar additive effects were confirmed using the Foxp3+ population (Fig. 7B), while conventional CD4+ T cells were not affected (data not shown). Furthermore, PMB caused similar changes on NAD-induced Treg depletion, downshifting slightly and strongly the CD4+ CD25+ and foxp3+ NAD induced Treg depletion curves, respectively (Fig. 7B and D, respectively). When the variables were changed using a fixed ATP concentration and modifying PMB concentrations, we observed a dose dependent PMB-induced depletion with a maximal response close to 40 ± 10%, lower than ATP alone as shown in Fig. 8A. No significant effects were observed for CD4+ total population (Fig. 8B). Altogether these results suggest that the effect of ATP and PMB are additive.

Discussion

In this work, we corroborated previous reports showing that mouse Treg cells are killed by ATP and NAD in a mechanism involving P2X7 receptor activation (Aswad et al. 2005; Kawamura et al. 2005; Aswad and Dennert 2006; Seman et al. 2003). We determined that PMB, which is a P2X7R positive modulator (Ferrari et al. 2004), also induces Treg cell death *in vitro* and facilitates P2X7R-mediated cell death, with a mechanism that appears to be unrelated to the P2X7 receptor.

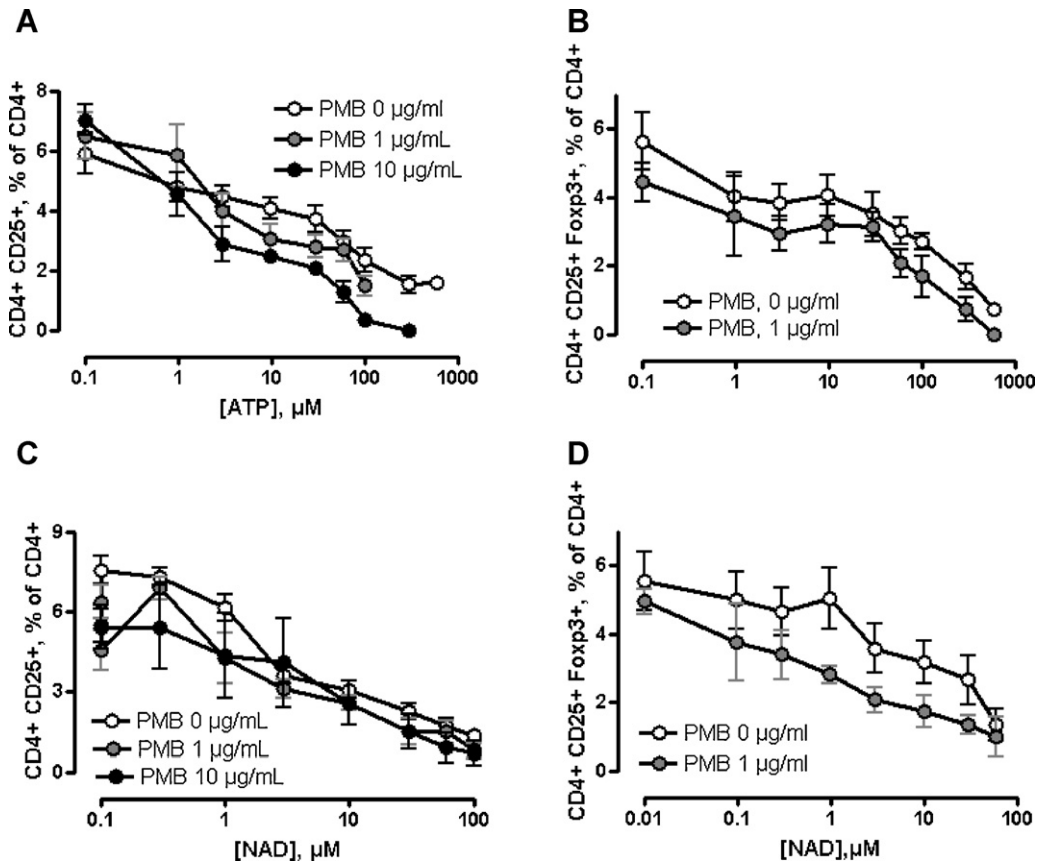

Fig. 7. PMB potentiates the ATP and NAD induced foxp3 Treg cell depletion *in vitro*. The effects of PMB 0 (open circles), 1 (gray circles) and 10 µg/mL (black circles) were evaluated on ATP (A, B) and NAD (C, D) dose–response mediated effect on CD4+ CD25 total population (left graph A, C) and on foxp3+ cell (right graph B, D) population. Quantification of 10–12 independent experiments for CD4+ CD25+ cells and 6–7 for foxp3+ cell populations are shown. Data are expressed as mean ± SE, * represent a statistically significant effect ($p < 0.05$).

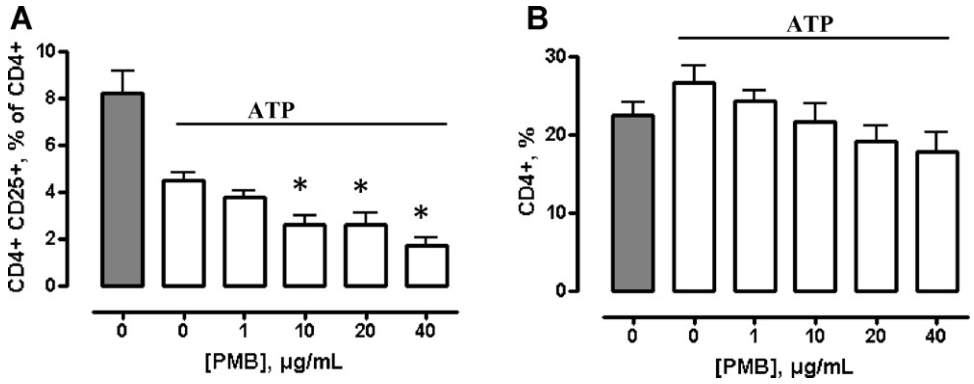

Fig. 8. PMB improves the ATP induced Treg cell depletion *in vitro*. The effects of PMB on ATP 60 µM treatment was evaluated on CD4+ CD25+ T cells (A) and CD4+ (B). Open bars correspond to ATP plus MBD treated cells, gray bars correspond to Treg and CD4+ untreated or basal levels. Quantifications of 5–10 independent experiments are shown. Data are expressed as mean ± SE, * represent a statistically significant effect for Mann–Whitney paired test compared to untreated conditions ($p < 0.05$).

The role of P2X7 receptors on immune response homeostasis has been well documented. In immune cells such as microglia and macrophages, P2X7 is involved in several physiological and pathophysiological conditions such as induction, maturation and delivery of IL-1β after *in vitro* lipopolysaccharide (LPS) exposure (Di Virgilio 2007; Ferrari et al. 2006). P2X7 knockout mice show lower mortality and pro-inflammatory cytokine production under systemic LPS challenge compared to normal animals, suggesting a role for the P2X7 receptor in inflammatory response *in vivo* (Labasi et al. 2002). Similarly this receptor has been implicated in Treg cell homeostasis, as the CD4+ CD25+ population expresses high levels of P2X7 compared with conventional T effector cells, and this expression correlates positively with purinergic agonist induced cell death (Aswad et al. 2005; Aswad and Dennert 2006). Supporting this idea P2X7R KO mice present a large amount of Foxp3-expressing CD4+ CD25+ cells compared with wild type animals, both in the circulation and lymphoid organs (Aswad et al. 2005).

We found that PMB by itself is able to induce a Treg cell depletion, and it potentiates an ATP-induced response on Treg cells. This is a highly favorable feature for a drug that might be used

to induce an antitumoral response by means of Treg cell depletion and suggests that P2X7 receptors are endogenously activated under our basal experimental conditions. Previously, Ferrari et al. (2004) demonstrated that PMB potentiates responses elicited by ATP-stimulated transfected cell lines, macrophages and B cells expressing P2X7 receptor. The mechanisms associated with induction of activity appear to be common for several cyclic peptides. Moreover, IL1beta production in macrophages has been associated with the presence and activity of other cationic peptides such as the derivate peptide LL37 and SSA (Niemi et al. 2011; Elssner et al. 2004). Furthermore, Allam and co-workers determined that polymyxin B and other cyclic antibiotics induce IL-1β secretion in bone marrow dendritic cells and macrophages in a K+ efflux dependent fashion (Allam et al. 2011). This activity is also induced in cells from P2X7 KO animals, suggesting that the mechanism could be independent of P2X7 receptor. Similarly a pannexin 1 has been suggested as responsible for several activities associated with the purinergic receptor such as caspase 1 activation, IL-1β maturation and delivery, and ethidium bromide uptake (Kanneganti et al. 2007; Pelegrin and Surprenant 2006), but not for others such as macropore formation (Yan et al. 2008). Recently Lemaire and coworkers showed that P2X7 and pannexin are both crucial to the process of cell-to-cell fusion into multinucleated macrophages (MA). They showed, in agreement with our results, that PMB itself is able to induce cell fusion, and while P2X7 appears to play a role, pannexin 1 inhibition completely abrogates this effect. Altogether those studies suggest that PMB related effects could be mediated by pannexin, however this specific observation will be addressed in a future study.

PMB related effects have been described for APC cells, but the effects on T cell population and particularly on Treg cells have not been previously tested. Interestingly, we also demonstrated that PMB at the concentrations used here had no effects on other T cell populations. On the other hand, apoptosis was detected in Treg cells after ATP but not after PMB treatment. PMB has been previously associated with induction of cell death (Mihich group in 1993), i.e., this drug is cytotoxic to certain tumor cells, with differential sensitivity. For instance, EL4 cells and EL4/ADM cells were very sensitive (lysed by ≥10 μg/mL PMB); C1498 cells and REH cells were moderately sensitive (lysed by ≥20 μg/mL PMB); and 6 others different cell lines were resistant (lysed only by 100 μg/mL) to PMB (Verstovsek et al. 1993).

Several efforts have been made in order to generate an adjuvant therapy against cancer by induction of Human Treg cell depletion. Until now, the most effective drugs in depleting Treg cells are driven specifically against Treg cell receptors, such as CTLA4 and CD25 and cyclophosphamide. Unfortunately, both receptors are also expressed in activated lymphocytes and conventional T cells that could be depleted by action of this drugs (Needham et al. 2006; Nishikawa and Sakaguchi 2010; O'Day et al. 2007; Schabowsky et al. 2007), moreover cyclophosphamide induces undesired effects at higher concentrations, inhibiting the immune response (Motoyoshi et al. 2006). We described here that modulation of P2X7 appears to be a plausible alternative to deplete Treg cells in a more specific way. Our results suggest that the use of the antibiotic PMB could be an important step towards developing an adjuvant immunotherapy against cancer, however several experiments including *in vivo* approaches are still necessary.

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